HUMAN PLATELET-DERIVED GROWTH FACTOR RECEPTORS

The present application is a continuation-in-part application of commonly assigned patent application U.S.S.N. 07/309,322, filed February 10, 1989, which is a C-I-P of U.S.S.N. 07/151,414, filed February 2, 1988, both of which are incorporated herein by reference.

The Government has rights in this invention pursuant to Grant No. R01 HL-32898 awarded by the National Institutes of Health.

Field of the Invention

The present invention relates to the development of diagnostic and therapeutic agents and, in particular, to compositions based on platelet-derived growth factor receptors.

BACKGROUND OF THE INVENTION

Platelet-derived growth factor (PDGF) is a major mitogen for cells of mesenchymal origin. The protein mitogen is usually a 32 kDa protein heterodimer usually composed of two polypeptide chains, A and B, linked by disulfide bonds. In addition to the PDGF AB heterodimer, two homodimeric forms of PDGF, denoted AA and BB, have been identified.

The first event in PDGF-mediated mitogenesis is the binding of PDGF to its receptor at the cell membrane. This interaction triggers a diversity of early cellular responses including activation of receptor tyrosine kinase, increased phosphatidylinositol turnover, enhanced expression of a group of genes, activation of phospholipase A2, changes in cell shape, an increase in cellular calcium concentration, changes in intracellular pH, and internalization and degradation of bound PDGF. These changes are followed by an increase in the rate of proliferation of the target receptor containing cells.

The ability of a polypeptide to stimulate growth of a particular cell type *in vitro* does not prove that it serves the same function *in vivo*, but the roles of many growth factors on cells are being studied to determine the roles that the factors play in the whole organism. *In vitro*, platelet-derived growth factor is a major polypeptide mitogen in serum for cells of

This Sheet Has Been Replaced

REPLACED SHEETS SG
☑ Description : paged ...1.... to paged ...107....
☑ Claim(s) : paged ...106... to paged ...112....
☑ Drawing(s) : paged ...17... to paged ...9/9....
☑ Abstract : paged ...13... to paged ...1....
DATE OF RECEIPT .....

This Sheet Has Been Replaced

mesenchymal origin such as fibroblasts, smooth muscle cells, and glial cells. In vivo, PDGF does not circulate freely in blood, but is stored in the α granules of circulating blood platelets. During blood clotting and platelet adhesion, the granules are released, often at sites of injured blood vessels, thus implicating PDGF in the repair of blood vessels. PDGF may stimulate migration of arterial smooth muscle cells from the medial to the intimal layer of the artery where the muscle cells may proliferate. This is likely to be an early response to injury.

PDGF is being studied to determine how cell proliferation is controlled in the body. The growth factor has been implicated in wound healing, in atherosclerosis, in myeloproliferative disease, and in stimulating genes associated with cancerous transformation of cells, particularly c-myc and c-fos. Therefore, PDGF agonists may be useful in promoting wound healing. PDGF antagonists may also be useful in preventing atherosclerosis, in retarding blood vessel narrowing that occurs after cardiovascular intervention and in controlling cancerous proliferation.

The interaction of PDGF with cells is mediated, in part, by a receptor for the mitogen. The PDGF receptor is therefore a very important component in mitogenic stimulation by the growth factor. However, the inability to characterize the direct interaction between the PDGF and its receptor and between the PDGF receptor and intracellular components has hampered the development of reagents needed in the diagnosis or treatment of physiological conditions or disorders characterized by abnormal or undesired PDGF responses. For these reasons, a dramatic need exists to characterize the structural and physiological properties of PDGF receptors.

SUMMARY OF THE INVENTION

In accordance with the present invention, DNA sequences encoding human platelet-derived growth factor receptor (hPDGF-R) polypeptides have been isolated and sequenced. In one embodiment, expression constructs are provided comprising one or more sequences that encode

This Sheet Has Been Replaced

PDGF-receptor proteins that can be secreted or associated with the membrane of a mammalian cell. The membrane associated receptor should be functionally similar to or equivalent to a wild-type receptor thereby conferring a PDGF sensitive mitogenic response on cells lacking the receptor. The construct can be used, inter alia, for producing large amounts of the PDGF-receptor or fragments, for enhancing PDGF response of cells, for determining the regions of the receptor polypeptides involved in transducing the mitogenic signal in response to PDGF binding, for providing mutated analogs of the receptor, for evaluating drugs for their physiologic activity, and for probing the integrity of sequences nearby the chromosomal loci of the receptor genes. In particular, various soluble fragments of the receptor are provided, many possessing various properties of cell associated hPDGF-R proteins. Novel methods utilizing these constructs are also provided.

The present invention provides a purified and isolated recombinant nucleic acid of less than about 50 Kbp comprising at least about 24 contiguous nucleotides which encode a human platelet-derived growth factor receptor (hPDGF-R) polypeptide segment. Preferably the segment is a soluble polypeptide. In particular embodiments, the segment consists essentially of a full length extracellular region of a B type or an A type hPDGF receptor, e.g., a sequence of a polypeptide in Table 2 or Table 3. In other embodiments, the nucleic acid encodes a segment with a phosphorylation site.

Usually, the encoded segment is less than about 300 amino acids, and will preferably be capable of binding to PDGF, be a substrate for phosphorylation, or be capable of binding to a PI3 kinase. In other embodiments, the encoded segment lacks a substantially complete intracellular region.

The invention also embraces a cell transformed with the described nucleic acids, typically where the cell is a mammalian cell. In particular embodiments, the cell further contains a glycosylation enzyme originating from a non-fungal species.

Expression vectors are also provided, and in certain embodiments, the nucleic acid nucleotides encoding the segment

This Sheet Has Been Replaced

are operably linked to a promoter. Recombinant nucleic acids are provided which further encode a heterologous polypeptide which is fused to the hPDGF-R segment.

As another aspect of the invention, methods are provided for evaluating the ability of a compound to function as a hPDGF-R agonist or antagonist, utilizing the step of comparing the amount of a PDGF-induced response from a control cell with that in a cell transformed with a hPDGF-R peptide fragment encoding nucleic acid. In various embodiments, the PDGF-induced response is compared by measuring synthesis of DNA in the cells. After contacting the cells with cell with PDGF.

Polypeptide embodiments include a substantially pure hPDGF-R polypeptide fragment of at least about twenty amino acids having platelet-derived growth factor (PDGF) binding activity or tyrosine kinase activity. Typically, the polypeptide fragment will be soluble.

In other embodiments, hPDGF-R fragments are provided having hPDGF-R binding activity consisting essentially of amino acids beginning at about 1 and ending at about 499 of a type B hPDGF-R, e.g., derived from Table 2, or consisting essentially of amino acids beginning about 1 and ending at about 501 of a type A hPDGF-R, e.g., derived from Table 3. The invention embraces compositions having an unglycosylated hPDGF-R fragment, preferably where the fragment is substantially pure. In other embodiments, the hPDGF-R fragment exhibits a glycosylation pattern which is non-fungal and non-human. Particularly useful compositions have a hPDGF-R polypeptide fragment which is essentially the extracellular region of a type B or a type A hPDGF-R, e.g., derived from sequences of Table 2 or Table 3. An additional embodiment is a composition comprising a combination of: (a) a recombinant nucleic acid encoding a human platelet-derived growth factor receptor polypeptide (hPDGF-R) fragment; and (b) a non-fungal glycosylation enzyme capable of glycosylating said fragment when expressed.

The present invention provides various methods for introducing a hPDGF-R activity to a cell, comprising the step of introducing a hPDGF-R protein fragment of at least about

five hundred daltons to a cell. A method for assaying the presence of a ligand for a PDGF receptor in a sample is also provided, comprising the steps of: (a) combining the sample with a hPDGF receptor ligand binding site; and (b) detecting binding between the ligand and the hPDGF receptor ligand binding site.

With respect to the intracellular region of the PDGF receptors, the present invention provides an isolated polypeptide of less than about 200 amino acids comprising a receptor kinase insert region. In various embodiments, the polypeptide has a phosphorylated amino acid residue, e.g., phosphotyrosine. Usually, the polypeptide has a sequence substantially homologous to a kinase insert segment of a PDGF receptor, e.g., a sequence from Table 2 or Table 3. The invention also provides a composition with the polypeptide and a pharmaceutically acceptable carrier.

Various methods are provided by the invention, including methods for modulating the biological activity of a first protein which binds to a phosphorylated region of a second protein, the method including a step of adding to the first protein a peptide analogue of the phosphorylated region, where the analogue is capable of inhibiting the binding of the first protein to the second protein. Other methods are provided for selecting a molecule capable of inhibiting binding of a protein which binds to a target phosphorylated polypeptide. This method has steps of contacting the protein with the target phosphorylated polypeptide in the presence of the molecule in a first analysis; contacting the protein with the target phosphorylated polypeptide in the absence of the molecule in a second analysis; and comparing these analyses to determine the effect of the molecule on the binding. In particular embodiments, these contacting steps are performed in succession.

Other methods for modulating a PI3 kinase activity as provided, comprising the step of adding a phosphorylated PDGF receptor kinase insert region polypeptide to the PI3 kinase, thereby allowing binding between the polypeptide and the PI3 kinase.

This Sheet Has Been Replaced



The present invention provides methods for purifying, from a sample, a protein capable of binding to a PDGF receptor kinase insert segment, comprising the step of contacting the sample with an analogue of a phosphorylated polypeptide substantially homologous to a PDGF receptor kinase insert region polypeptide, thereby allowing the protein to bind specifically to the phosphorylated polypeptide.

Methods of isolating a nucleic acid encoding a protein capable of binding to a PDGF receptor are provided, comprising the steps of combining a labeled and phosphorylated PDGF receptor kinase insert region polypeptide with cells expressing various proteins, thereby labeling those cells which express the nucleic acid to produce a protein which binds the phosphorylated polypeptide; and isolating those cells which have been labeled. This method is particularly useful to isolate nucleic acids encoding PI3 kinase or c-fms.

IThis Sheet Has Been Replaced



BRIEF DESCRIPTION OF THE FIGURES

Figure 1 illustrates the association of 85 kD/PI3 kinase with the type B PDGF receptor. The association of phosphoprotein with the PDGF receptor (a and b) and phosphatidylinositol 3' kinase (PI3 kinase) activity (c and d) were studied in intact cells (a and c) and in vitro (b and d). In the intact cell experiments PDGF-stimulated (+) or control cells (-) were solubilized and the receptor was immunoprecipitated using anti-receptor antibodies. The receptor-associated phosphoproteins were detected by incubating the receptor-associated protein complex with $MnCl_2$ and γ^{32}P-ATP followed by autoradiography of an SDS polyacrylamide gel. The PDGF-stimulated receptor associated with 85 kD and 110 kD phosphoproteins and PI3 kinase activity, but the unstimulated receptor was not. Equivalent amounts of receptor were immunoprecipitated from the lysates from stimulated and unstimulated cells. In vitro associations were performed by incubating the baculovirus-expressed receptor with lysates from PDGF-stimulated (+) or unstimulated (-) cells. Receptor-associated phosphoproteins (b) and PI3 kinase activity (d) were detected. The position of the 85 kD protein is indicated by the arrows.

Figure 2 illustrates the association of 85 kD protein and PI3 kinase activity with the wild type and kinase insert deletion mutant receptor in vitro. Lysates from unstimulated 3T3 cells were mixed with immunoprecipitated wild type PDGF type B receptor or kinase insert deletion mutant receptor that were prepared using a baculovirus expression system. The immunoprecipitates containing receptor-associated proteins were washed extensively. In vitro protein kinase assays (panel a) or PI3 kinase activity assays (panel b) were performed on the proteins. The first lane of panel (a) shows the in vitro protein kinase assay of the baculovirous-expressed receptor that was not mixed with cell lysate. The top two bands of the second and third lanes of panel (a) are from the baculovirus-expressed receptor (second lane) or the mutated receptor (third lane) and their respective precursors. The arrow indicates the 85 kD protein.

This Sheet Has Been Replaced

This Sheet Has Been Replaced

Figure 3 illustrates the need for requirement of receptor autophosphorylation for association with PI3 kinase activity and 85 kD protein. PDGF receptors were immunoprecipitated from Sf9 cells infected with the wild type PDGF receptor recombinant baculovirus. Immunoprecipitates containing phosphorylated receptor were incubated with solubilized 3T3 cell lysates. PI3 kinase assays (a and b) and in vitro kinase assays (c) were performed. (a) Association of PI3 kinase activity with phosphorylated PDGF receptors were incubated with potato acid phosphatase (PAP) or PAP plus orthovanadate.

Figure 4 illustrates the use of peptides to block the association of the 85 kD phosphoprotein and PI3 kinase activity with the receptor. The peptides listed in Table AJ were preincubated with unstimulated 3T3 cell lysates prior to incubation with immunoprecipitated receptor. The detection of the receptor-associated phosphoproteins (panel a) and the receptor-associated PI3 kinase activity (panel b) were performed as in Figs. 1 and 2. The 85 kD protein is indicated by the arrow.

Figure 5 illustrates the concentration-dependance of the blocking activity of peptide Y719P. A series of concentrations of peptide Y719P were tested for the ability to block the binding of proteins (panel a) or PI3 kinase activity (panel b) to baculovirus-expressed immunoprecipitated receptor. The arrow points to the 85 kD phosphoprotein. The assays were performed as described in Figs. 1, 2 and 3.

Figure 6 illustrates that the association of PDGF receptor with PLC-γ and GAP is not affected by preincubation of 3T3 unstimulated lysates with receptor phosphopeptide, Y719P. Unstimulated 3T3 lysates were preincubated in the presence and absence of a receptor phosphopeptide (Y719P) prior to the association with the baculovirus-expressed receptor. The peptide/lysate mixture was incubated with the immunoprecipitated baculovirus-expressed receptor as described in Figs. 1, 2 and 3. The presence of PLC-γ and GAP in the receptor complex was determined by immunoblot analysis using PLC-γ and GAP antibodies as probes. In the lane marked

"lysates", crude lysates of 3T3 cells were immunoblotted as controls to show the electrophoretic position of the PLC-γ and GAP.

Figure 7 illustrates the association of soluble radiolabeled receptor with immobilized 85 kD protein ("receptor blot"). Baculovirus-expressed PDGF receptors were immunoprecipitated using receptor antibodies and the receptor was ^{32}P-labeled in vitro by autophosphorylation. The solubilized radiolabeled receptor was used to probe nitrocellulose filters containing SDS-polyacrylamide gel-fractionated lysates from control (lanes 1, 3, 4 and 5) or PDGF stimulated (lane 2) cells. In the peptide competition experiments (lane 3, 4, and 5) the peptides were added to the nitrocellulose paper along with the radiolabeled receptor probe. The arrow indicates the position of the 85 kD protein.

Figure 8 illustrates a strategy for oligonucleotide directed in vitro production of a soluble hPDGF-R extracellular region. The abbreviations used are:

PR = PDGFR; intact
P = PDGFR; extracellular region
TM = transmembrane region
K = kinase
S = signal sequence

This Sheet Has Been Replaced

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

Human platelet derived growth factor receptor (hPDGF-R) compositions are provided by the present invention. These compositions will be full length natural forms, fragments of the natural forms, fusion proteins with those fragments, modified forms of each, and multi-protein complexes comprising them. In particular, soluble polypeptides exhibiting hPDGF-R functions are made available, both extracellular and intracellular region fragments.

Methods for producing protein compositions based upon hPDGF receptors are provided. Nucleic acid constructs which encode the various hPDGF-R protein compositions are also disclosed. The nucleic acid constructs will be useful to transfect cells, providing an efficient and economical means to produce commercially useful quantities of the protein compositions. These cells, and others containing hPDGF receptors, will also be useful in diagnosis or for study of mechanisms of PDGF mitogenic action. They will be used to evaluate new drugs which affect signal transduction of PDGF ligand binding, and in treatment of diseases associated with hPDGF receptors. The constructs can be used to transfect cells, providing membrane-bound receptors that are functionally equivalent to wild-type receptors, and conferring a PDGF-sensitive mitogenic response on cells lacking receptors. The transfected cells can be used as a model for studying the PDGF-induced response of cells, determining the regions involved in transducing the signal in response to PDGF ligand binding, and evaluating drugs for their physiologic activities. Encoded receptors or their binding regions also find use in evaluating PDGF analogues.

The receptor fragments and their analogues will find use in determining regions of the receptor polypeptides which are involved in signal transduction in response to PDGF binding. In particular, the ability to test various combinations of structural features, e.g., ligand binding determinants, will allow dissection of receptor features to determine the importance to ligand binding affinity, ligand specificity, and physiological responses.

This Sheet Has Been Replaced



This Sheet Has Been Replacec

The hPDGF-R proteins will also find use in producing antibody reagents. These reagents should be capable of interfering with, or simulating, particular molecular interactions of the receptor polypeptides.

Soluble proteins possessing PDGF binding capacity will be useful in blocking PDGF action, as reagents for the quantitation of PDGF in diagnostic samples, for soluble testing of binding interactions between modified determinants for ligand binding and natural, modified, or derivatized ligands, and for screening for monoclonal antibodies against hPDGF-R polypeptides. They will also find therapeutic uses.

The DNA sequences will also find use as probes to detect genetic abnormalities, e.g., deletions or rearrangements in the region of chromosome 5 where a number of growth-control related genes are clustered, in the region of chromosome 4 near the c-kit oncogenes, or to detect other genes encoding tyrosine kinases or homologous genes.

Certain regions of the cytoplasmic region of the PDGF receptor, among them the kinase insert (KI) region, allow the PDGF receptor to interact with, e.g., bind with, other cellular proteins. Peptides substantially homologous to these regions, as well as organic analogue molecules, will find use in inhibiting the interaction between the PDGF receptor and other proteins, in identifying, screening for, and purifying proteins with which the PDGF receptor interacts, and in cloning genes encoding these proteins. These homologous peptides will also find use in medical diagnosis and in drug therapies, e.g., affecting PDGF receptor activities and receptor interactions with other proteins.

Moreover, the understanding of the role of phosphorylated residues to protein-protein interactions is applicable to other receptors and phosphorylated proteins. The phosphorylation of specific amino acids in particular polypeptide segments or substantially homologous segments, e.g., phosphorylation sites, has special biological significance. Identification of the phosphorylation sites and regions of protein interactions are important in preparing reagents useful in inhibiting such interactions, leading to a

modulation of the function of such proteins. The peptide analogues will also be useful in identifying and purifying proteins which interact with phosphorylated residues and in isolating and cloning genes encoding these proteins.

OUTLINE

I. General Description
A. PDGF-R
1. structural features
a. extracellular region
i. signal sequence
ii. Ig domains
b. transmembrane segment
c. intracellular region
i. tyrosine kinase
ii. insert
2. function
a. bind PDGF
b. bind to PDGF-R peptide
c. tyrosine kinase activity
B. Physiological Functions
1. cellular
2. tissue differentiation
3. organismal
II. Polypeptides
A. Soluble Forms
B. Truncated Forms
C. Fusion Proteins
D. Genetic Variants (site-directed mutagenized)
E. Compositions Comprising Proteins
III. Nucleic Acids
A. Isolated Nucleic Acids
B. Recombinant Nucleic Acids
C. Compositions Comprising Nucleic Acids
IV. Methods for Making PDGF-R
A. Protein Purification
1. affinity with derivatized PDGF
2. various ligands, same receptor
B. Expression of Nucleic Acids
V. Antibodies
VI. Methods for Use
A. Diagnostic
B. Therapeutic

* * * * *

This Sheet Has Been Replaced



I. General Description

The isolated full-length human platelet-derived growth factor (hPDGF) receptor mRNA encodes a single hydrophobic polypeptide segment similar to a membrane-spanning segment (designated the transmembrane segment). The segments

This Sheet Has Been Replaced

of a PDGF-R amino proximal to the transmembrane segment make up the extracellular region, while the segments carboxy proximal to the transmembrane segment are designated the intracellular region. From the amino terminus, the extracellular region has an NH_2-terminal hydrophobic putative signal sequence, an immunoglobulin-like domain (designated IgI), and second, third, fourth, and fifth immunoglobulin-like domains (designated IgII, IgIII, IgIV, and IgV, respectively). Although various structural features are identified in the external region of the hPDGF-R, the most important functional property which defines the region is the binding to the receptor ligands, e.g., members of the PDGF family and analogues thereof.

The intracellular region is characterized, in part, by the presence of a split tyrosine kinase structural domain. In the human type B receptor polypeptide, this domain is about 244 residues long and has an insert of about 104 amino acids. See Table 2. In the human type A receptor polypeptide, the tyrosine kinase domain is also about 244 residues long with a kinase insert of about 103 amino acids. See Table 3. Functionally, this domain is defined, in part, by its tyrosine kinase activity, typically modulated by ligand binding to the extracellular region, and appears to function in a dimer state. The substrate for phosphorylation includes various tyrosine residues on the accompanying receptor polypeptide chain, and other proteins which associate with the receptor. The tyrosine kinase domain is also defined, in part, by its homology to similar domains in other tyrosine kinase activity containing proteins. See, e.g., Yarden et al. (1986) Nature 323: 226-232. As such, the actual boundaries, determined by homology to other similar domains, may vary by a few amino acid residues, perhaps as many as about 7 residues, but probably within about 4 residues, and more probably within about one or two residues. A protein substantially lacks a complete intracellular region when it lacks a prototypical intracellular region, particularly lacking a tyrosine kinase domain; a similar concept is provided with respect to lack of a complete transmembrane region.

A typical PDGF-R nucleic acid sequence encodes a transitory NH_2-terminal hydrophobic sequence, which is usually

cleaved during the membrane translocation process. The classical function of a signal sequence is to direct the nascent polypeptide chain to membrane bound ribosomes, thereby leading to membrane translocation and subsequent processing and targeting steps. Since the signal sequence is typically removed in the translocation process, the signal sequence is absent in a mature polypeptide. However, other features of the amino proximal sequence of the mature protein may also be important for expression or correct localization or targeting of receptor fragments.

The putative boundaries of the different regions of the receptors are indicated in Table 1, derived, in part, by homology to the mouse PDGF receptor. In particular, a hydrophobic segment has been identified and designated a transmembrane segment, due to its structural homology and physical properties characteristic of a membrane spanning segment. The segment divides the protein into an extracellular region and an intracellular region. The intracellular region has homology to various other receptors believed to exhibit tyrosine kinase activity, though this receptor has an insert region, as indicated, which is embedded within the region of high homology to other tyrosine kinase segments.

This Sheet Has Been Replaced

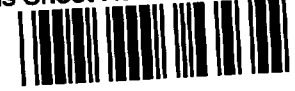


TABLE 1

approximate segment boundaries

	Type B	Type A
XR	leu (1) - lys (499)	gln (1) - glu (501)
TM	val (500) - trp (524)	leu (502) - trp (526)
TK1	arg (572) - his (662)	leu (572) - his (664)
KI	arg (663) - leu (766)	lys (665) - leu (767)
TK2	ser (767) - phe (919)	thr (768) - phe (920)

This Sheet Has Been Replaced



This Sheet Has Been Replaced

The intracellular region of the receptor contains a tyrosine kinase activity. PDGF receptors have a characteristic split tyrosine kinase domain, with an insert segment of about 103 or 104 amino acids. This insert segment has regulatory and other properties which are described in greater detail below.

The present invention provides isolated nucleic acids and polypeptides relating to PDGF receptors. An isolated molecule is one which is substantially separated from substances which naturally accompany the molecule when found in its natural form. For example, an isolated polypeptide is one which is substantially purified away from naturally accompanying human cell components, e.g., human nucleic acids, lipids, and other proteins. Of particular interest are contiguous segments of polypeptide which might be separated by genetic recombination or deletion techniques. Thus, an expression product of an isolated and manipulated genetic sequence is an isolated polypeptide, as used herein, even if expressed in a homologous cell type. Synthetically made forms or molecules expressed by heterologous cells are inherently isolated molecules.

Physiologically, the receptor is responsible for initiating a number of metabolic changes in, e.g., phosphatidylinositol levels, pH, calcium ion levels, cytoskeletal structure, gene expression, cAMP levels, and ligand receptor levels. Many of these metabolic changes are characteristic of a mitogenic, e.g., ligand induced, response.

The type A and type B PDGF receptor forms, when combined in the various dimer combinations into functional receptor complexes, also have characteristic binding affinities for the various forms of PDGF's, e.g., for the AA, AB and BB forms. For example, a type B isoform receptor binds the BB isoform PDGF at high affinity, the AB heteroform PDGF with lower affinity, and the AA isoform PDGF at low or no affinity. The type A isoform receptor binds to the AA isoform PDGF with high affinity, and with the AB heterodimer and BB isoform PDGF's with lower affinity.

The term "ligand" refers to molecules, usually members of the platelet-derived growth factor family, that bind

This Sheet Has Been Replaced

the segments involved in the growth factor binding. Also, a ligand is a molecule which serves either as a natural ligand to which the receptor, or an analogue of the receptor, binds, or a functional analogue of a natural ligand. The functional analogue may be a ligand with structural modifications, or may be a wholly unrelated molecule which has a molecular shape which interacts with the appropriate ligand binding determinants. The ligands may serve as agonists or antagonists, see, e.g., Goodman et al. (eds) (1990) Goodman & Gilman's: The Pharmacological Bases of Therapeutics (8th ed), Pergamon Press.

II. Polypeptides

The PDGF receptor is believed to be a dimer of similar polypeptides and the component polypeptides determine the dimerized receptor binding affinity for ligand.

The AA homodimer PDGF ligand is preferentially bound with high affinity by one form of the hPDGF receptor, and this form is correlated with a polypeptide chain referred to as the A receptor, the α receptor, and, as used herein, the type A receptor polypeptide (A-hPDGF-R). The receptor dimer is apparently a homodimer of the type A receptor polypeptides.

The BB homodimer PDGF ligand is preferentially bound with high affinity by two forms of the hPDGF-receptor. These forms are correlated with a second polypeptide chain referred to as the B receptor, the β receptor, and, as used herein, the type B receptor polypeptide (B-hPDGF-R). These two receptor dimer forms are apparently the homodimer of the B receptor polypeptides and the heterodimer type B/type A form.

The designations type A and type B polypeptides are each intended to also cover all alleles of each respective isoform. Thus, naturally occurring variants of each are embraced by the invention, as are other variants, analogues, and modified sequences.

The nucleotide sequence of a cDNA sequence encoding one B-hPDGF-R allele is set forth in Table 2 (See SEQ ID No.: 1) together with the deduced amino acid sequence of the receptor precursor. The following descriptions indicate

presumed gross structural and functional characterizations based upon analogy to the mouse and other growth factor receptors and proteins.

The sequence beginning at the amino acid numbered 1 corresponds to the putative amino terminus of a mature form of a human PDGF-R. The first 32 amino acids (designated -32 to -1) encode the putative signal peptide sequence. The putative transmembrane sequence corresponds to amino acid residues val(500) to trp(524). Potential N-glycosylation sites are at positions corresponding to amino acids asn(13) - ser(15); asn(57) - thr(50); asn(71) - ser(73); asn(183) - ser(185); asn(198) - thr(200); asn(260) - thr(262); asn(275) - thr(277); asn(322) - thr(324); asn(339) - ser(341); asn(436) - ser(438); and asn(447) - thr(449). The putative tyrosine kinase domain is interrupted by an amino acid insertion between about arg(663) and leu(766), see Table 1. The putative polyadenylation site is at the 3' end of the given cDNA sequence.

This Sheet Has Been Replaced



TABLE 2, panels A--?
sequence of the type B hPDGF-R from 12418-14 disk, file 01table
both nucleic acid and polypeptide sequence

This Sheet Has Been Replaced

The nucleotide sequence of a cDNA sequence encoding one allele of a type A hPDGF-R is set forth in Table 3 (See SEQ ID No. 5), together with the deduced amino acid sequence of the receptor (See SEQ ID No. 3). The structural features, as described, are again based upon analogy to the mouse PDGF receptors and other growth factor receptors and proteins.

The sequence beginning at the amino acid numbered 1 corresponds to the putative amino terminus of a mature form of a human PDGF-R. The first 23 amino acids (designated -23 to -1) encode the putative signal peptide sequence. The putative transmembrane sequence corresponds to amino acid residues leu(502) to trp(526). Potential N-glycosylation sites are at positions corresponding to amino acids asn(19) - ser(21); asn(53) - ser(55); asn(80) - thr(82); asn(156) - thr(158); asn(330) - thr(332); asn(336) - thr(338); asn(435) - thr(437); and asn(445) - ser(447). The putative tyrosine kinase domain is interrupted by an amino acid insertion between about lys(665) and leu(767), see Table 1.

This Sheet Has Been Replaced



TABLE 3, panel A

sequence of the type A hPDGF-R from 12418-14 disk; file 02table

This Sheet Has Been Replaced

This Sheet Has Been Replaced

As seen in Tables 2 and 3, the presumed intracellular tyrosine kinase domain of the type A and type B receptors have about 80% identical residues. The putative extracellular regions of the type A and B receptors have about 34-35% identical residues, an additional 14% of the remaining residues being conservative substitutions, i.e., substitutions with amino acids having similar chemical properties. The designated transmembrane regions of the hPDGF receptors have about 48% identical residues. Of the 52% of residues that differ, 70% are conservative substitutions. As seen in the tables, both receptor sequences have an amino acid insertion of about 103 or 104 amino acids interrupting the putative tyrosine kinase region. The overall homology between the two polypeptide sequences is about 44%.

The term platelet-derived growth factor receptor (PDGF-R) refers to a composition having properties characteristic of a PDGF-R. The native human PDGF receptors are examples of the class of PDGF-Rs, as are each of the polypeptides whose sequences are disclosed in Tables 2 and 3, and allelic variants. The natural receptor is typically a membrane glycoprotein having a molecular weight of about 180 kd. The receptor is normally found in vascular smooth muscle cell, fibroblasts, and glial cells, but not commonly observed in endothelial cells or on most hematopoietic cells.

As indicated above, the PDGF receptors have three major identifiable regions. The first is an extracellular region which contains the ligand binding determinants for the PDGF, i.e., the ligand binding segments. The extracellular region is also divided into Ig-like domains with the characteristics described above. The second major identifiable region is a transmembrane region and the third is an intracellular region. The intracellular region contains a characteristic type of tyrosine kinase domain, interrupted by a kinase insert (K1) segment.

The description "human" refers to the origin of the composition. In one use, it implies that the composition was found, at one point, within a human or human-derived cell or a minor variant from a naturally occurring human cell. With

respect to a protein or nucleic acid or sequence, it refers to a composition encoded by a natural human gene, or a closely related gene. Thus, the present invention includes proteins and nucleic acids encoding such proteins. The nucleic acids include RNA, cDNA, genomic DNA, synthetic forms, and mixed polymers, both sense and antisense strands. Thus, a protein derived from the sequence information disclosed, even if made synthetically, would be considered a human sequence. Furthermore, different alleles of each isoform are each also considered a human PDGF-R.

Thus, for the purposes used herein, the term human PDGF-R, when applied to a polypeptide, means a protein or polypeptide, which is substantially homologous to the amino acid sequences depicted in Table 2 or 3 and any alleles of them, or a fragment thereof. Ordinarily, hPDGF-R's will be at least about 80 percent homologous to the described PDGF-R sequences, preferably in excess of about 90 percent homology, and, more preferably, at least about 95 percent homology. The receptor will ordinarily also exhibit at least some biological activity in common with a hPDGF-R provided in the relevant sequences, e.g., PDGF binding, tyrosine kinase activity, phosphatidylinositol 3' kinase (PI3 kinase) binding, or immunologic properties. The hPDGF receptor embraces forms of the molecule which share the primary structural sequence, and is intended to encompass chemical and biochemical modifications, e.g., glycosylation, phosphorylation, ubiquinization, disulfide bonds, and other minor alterations in the basic primary sequence.

In particular, glycosylation alterations are included, made by modifying the glycosylation patterns of a polypeptide during its synthesis and processing, or in further processing steps. Particularly preferred means for accomplishing this are by exposing the polypeptide to glycosylating enzymes derived from cells which normally provide such processing, e.g., mammalian glycosylation enzymes. Also embraced are versions of the same primary amino acid sequence which have phosphorylated amino acid residues, e.g., phosphotyrosine, phosphoserine, or phosphothreonine.

This Sheet Has Been Repla



This Sheet Has Been Replaced

The type B hPDGF-R is an isotype of a hPDGF-R polypeptide whose properties have been described above, and includes all alleles of that sequence and minor modified sequences derived therefrom. Generally, the term also is intended to include fragments of the protein, particularly those which are characteristic of the human type B isoform. Corresponding meaning is intended in reference to the type A hPDGF-R.

In certain uses, the term applies to the functional receptor. As described, the functional form is believed to be a dimer complex of the type A receptor polypeptide, or of the type B receptor polypeptide, or the heterodimer form.

A heterologous fusion protein is a fusion of proteins or segments which are naturally not normally fused in the same manner. Thus, the fusion product of an immunoglobulin with a hPDGF-R polypeptide is a continuous protein molecule having sequences fused in a typical peptide linkage, typically made as a single translation product and exhibiting both immunoglobulin and hPDGF-R polypeptide dependent propertis. A similar concept applies to heterologous nucleic acid sequences.

A polypeptide fragment, or segment, is a stretch of amino acid residues of at least about 5 amino acids, often at least about 7 amino acids, usually at least about 9 amino acids, more usually at least about 11 amino acids, typically at least about 13 amino acids, more typically at least about 15 amino acids, and, in various embodiments, at least about 17 or more amino acids. In a nucleic acid, a fragment or segment is a stretch of at least about 6 nucleotides, often at least about 9 nucleotides, usually at least about 12 nucleotides, more usually at least about 15 nucleotides, typically at least about 18 nucleotides, more typically at least about 21 nucleotides, and in various preferred embodiments, at least about 24 or more nucleotides.

A polypeptide fragment is "physiologically active" when it substantially contributes to an activity characteristic of a natural polypeptide or fragment, e.g., a PDGF-R polypeptide. Typically, a hPDGF-R polypeptide, when associated into a functional receptor, has the set of activities performed

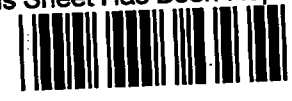
by the receptor in a biological context (e.g., in an organism or an in vitro biological context). In the case of the hPDGF-R, a functional fragment has these physiological activities, or functional activities characteristic of a hPDGF receptor, which include PDGF binding, tyrosine kinase activity and any of the mitogenic or other cellular responses mediated by the receptor. However, the protein fragment may exhibit other specific or inherent functions, such as vital conformational constraints, serving as a substrate site for glycosylation, contributing the signal sequence for cellular targeting, presenting characteristic epitopes absent in a mouse PDGF-R, or presently unrecognized functions possessed inherently.

Solubility of a polypeptide depends upon the environment and the polypeptide. Many parameters affect polypeptide solubility, including the temperature, the electrolyte environment, the size and molecular characteristics of the polypeptide, and the nature of the solvent. Typically, the temperature at which the polypeptide is used ranges from about 4°C to about 65°C. Usually the temperature at use is greater than about 18°C and more usually greater than about 22°C. For diagnostic purposes, the temperature will usually be about room temperature or warmer, but less than the denaturation temperature of components in the assay. For therapeutic purposes, the temperature will usually be body temperature, typically about 37°C for humans, though under certain situations the temperature may be raised or lowered in situ or in vitro.

The electrolytes will usually approximate in situ physiological conditions, but may be modified to higher or lower ionic strength where advantageous. The actual ions may be modified to conform to standard buffers used in physiological or analytical contexts.

The size and structure of the polypeptide should generally be in a substantially stable and globular state, and usually not in a denatured state. The polypeptide may be associated with other polypeptides in a quaternary structure, e.g., to confer solubility.

This Sheet Has Been Replaced



This Sheet Has Been Replaced

The solvent will usually be a biologically compatible buffer, of a type used for preservation of biological activities, and will usually approximate a physiological solvent. On some occasions, a detergent will be added, typically a mild non-denaturing one.

Solubility is usually measured in Svedberg units, which are a measure of the sedimentation velocity of a molecule under particular conditions. The determination of the sedimentation velocity was classically performed in an analytical ultracentrifuge, but is typically now performed in a standard ultracentrifuge. See, Freifelder (1982) Physical Biochemistry (2d ed.), W.H. Freeman, and Cantor and Schimmel (1980) Biophysical Chemistry, parts 1-3, W.H. Freeman & Co., San Francisco, each of which is hereby incorporated herein by reference. As a crude determination, a sample containing a putatively soluble polypeptide is spun in a standard full sized ultracentrifuge at about 50K rpm for about 10 minutes, and soluble molecules will remain in the supernatant. A soluble particle or polypeptide will typically be less than about 30S, more typically less than about 15S, usually less than about 10S, more usually less than about 6S, and, in particular embodiments, preferably less than about 4S, and more preferably less than about 3S.

The PDGF receptors or the specific extracellular regions of the receptors as provided herein may be used to affinity purify respective PDGFs. The full length, or entire, extracellular region, comprises the ligand-binding determinants of the type B receptor in Table 2, and extends from about leu(1) to lys(499). The full length, or entire, extracellular region comprising the ligand-binding determinants of the type A hPDGF-R shown in Table 3 extends from about gln(1) to glu(501). The ligand-binding determinants vary with different PDGF receptor alleles and may be anywhere from 5% to all of the extracellular region. The minimal amount of protein sequence essential for ligand binding may be determined by excising various segments of the extracellular region and measuring ligand binding. Studies of ligand-receptor interaction indicate that the ligand-binding region is located in the

This Sheet Has Been Replaced

extracellular region of the receptor. More sophisticated studies on the effects of various residues on the specificity of ligand binding are also made possible by the reagents of this invention. As used in this application, PDGF receptor or PDGF-R ligand-binding activity means having the ability to bind a PDGF, a PDGF agonist or antagonist, or other specific ligand for a hPDGF receptor. Usually these ligands will be members of the PDGF family, typically a human form. Therefore the external region has utility in establishing whether an analogue is a PDGF agonist or antagonist.

It is also likely that the PDGF-R, like many other growth factor receptors, is found naturally in a multimeric protein complex, most likely in dimer form. Thus, other important regions of a receptor will be those segments, either extracellular or otherwise, which are involved in dimerization or protein-protein interaction.

Besides substantially full-length polypeptides, the present invention provides for biologically active fragments of the polypeptides, or analogues thereof, including organic molecules which simulate the interactions of the peptides. Significant biological activities include ligand-binding, immunological properties, dimer association, serving as a kinase substrate, interacting with other mediators specific for binding to a PDGF-R protein, and other biological activities characteristic of human PDGF receptor polypeptides. Immunological activities include both immunogenic function in a target immune system, sharing of immunological epitopes for binding, and serving as either a competitor or substitute antigen for a human PDGF receptor epitope. Assays for these immunological activities are known in the art, see, e.g., below in the Experimental section; Harlow and Lane (1989) Antibodies: A Laboratory Manual, Cold Spring Harbor Press, New York; and Escobedo et al. (1988) J. Biol. Chem. 263:1482-1487, which are each hereby incorporated herein by reference. For example, ligand-binding or other segments may be "swapped" between different new fusion polypeptides or fragments. Thus, new chimeric polypeptides exhibiting new combinations of specificities will result from the functional linkage of

This Sheet Has Been Replaced

ligand-binding specificities and intracellular regions. For example, the Ig domains from other related polypeptides may be added or substituted for other Ig-like domains of these receptors. The resulting protein will often have hybrid function and properties.

For immunological purposes, immunogens may be produced which tandemly repeat polypeptide segments, thereby producing highly antigenic proteins. Alternatively, such polypeptides will serve as highly efficient competitors for specific binding. Production of antibodies to hPDGF receptor polypeptides is described below.

The present invention also provides for other polypeptides comprising fragments of PDGF receptors and polypeptides substantially homologous thereto. The receptor peptides of the present invention will generally exhibit at least about 80% homology with naturally occurring sequences of hPDGF receptor, typically at least about 85% homology with a natural receptor sequence, more typically at least about 90% homology, usually at least about 95% homology, and more usually at least about 97% homology. The length of comparison sequences will generally be at least about 16 amino acids, usually at least about 20 residues, more usually at least about 24 residues, typically at least about 28 residues, and preferably more than about 35 residues.

Homology, for polypeptides, is typically measured using sequence analysis software, see, e.g., Sequence Analysis Software Package of the Genetics Computer Group, University of Wisconsin Biotechnology Center, 1710 University Avenue, Madison, Wisconsin 53705. Protein analysis software matches similar sequences using measure of homology assigned to various substitutions, deletions, substitutions, and other modifications. Conservative substitutions typically include substitutions within the following groups: glycine, alanine; valine, isoleucine, leucine; aspartic acid, glutamic acid; asparagine, glutamine; serine, threonine; lysine, arginine; and phenylalanine, tyrosine.

Fusion polypeptides between the receptors and other homologous or heterologous proteins are also provided.

This Sheet Has Been Replaced

Homologous polypeptides may be fusions between different growth factor receptors, resulting in, for instance, a hybrid protein exhibiting ligand specificity of one receptor and the intracellular region of another, or a receptor which may have broadened or weakened specificity of binding. Likewise, heterologous fusions may be constructed which would exhibit a combination of properties or activities of the derivative proteins. Typical examples are fusions of a reporter polypeptide, e.g., luciferase, with a segment or domain of a receptor, e.g., a ligand-binding segment, so that the presence or location of a desired ligand may be easily determined. See, e.g., Dull et al., U.S. No. 4,859,609, which is hereby incorporated herein by reference. Other gene fusion partners include bacterial β-galactosidase, trpE, Protein A, β-lactamase, alpha amylase, alcohol dehydrogenase, and yeast alpha mating factor. See, e.g,., Godowski et al. (1988) Science 241:812-816.

Such polypeptides may also have amino acid residues which have been chemically modified by phosphorylation, sulfonation, biotinylation, or the addition of other moieties, particularly those which have molecular shapes similar to phosphate groups. In some embodiments, the modifications will be useful labeling reagents, or serve as purification targets, e.g., affinity ligands.

Fusion proteins will typically be made by either recombinant nucleic acid methods or by synthetic polypeptide methods. Techniques for nucleic acid manipulation are described generally, for example, in Sambrook et al. (1989), Molecular Cloning: A Laboratory Manual (2d ed.), Vols. 1-3, Cold Spring Harbor Laboratory, which are incorporated hereby by reference. Techniques for synthesis of polypeptides are described, for example, in Merrifield (1963) J. Amer. Chem. Soc. 85:2149-2156; Merrifield (1986) Science 232: 341-347; and Atherton et al. (1989) Solid Phase Peptide Synthesis: A Practical Approach, IRL Press, Oxford; each of which is incorporated herein by reference.

Fragments of an intact receptor are also embraced herein. Various fragments include the transmembrane segment,

This Sheet Has Been Replaced

which confers a cell surface attachment function. Various fragments deleting either intracellular or extracellular segments will be produced. For example, fragments comprising a transmembrane segment, either of a PDGF receptor or another protein, in combination with ligand binding determinants, will provide membrane associated ligand binding regions. Similar constructs with intracellular regions may be prepared, producing, e.g., membrane associated tyrosine kinase segments.

In particular, soluble fragments comprising ligand binding determinants will be produced which may be used for absorbing free ligand. The soluble fragments can be used for diagnostic and therapeutic purposes to block PDGF mediated responses.

In another embodiment, intracellular fragments and analogues will be prepared. The kinase insert region (KI) within the tyrosine kinase domains is a particularly useful fragment, as described below.

A peptide analogue of a phosphorylated region of a protein is a peptide with substantial primary sequence homology to a phosphorylated region of another protein, and having at least one phosphorylated or similarly derivatized residue at positions corresponding to the position of similar phosphorylated residues in the phosphorylated region of that other protein. Where such a region has two or more phosphorylated residues, peptide analogues will, in certain embodiments, have only one such phosphorylated or similarily derivatized residue. Such phosphorylated residues will generally include phosphotyrosine, phosphoserine, or phosphothreonine. The peptide analogue will sometimes have one or more unnatural amino acids, including, e.g., residues chemically modified by biotinylation, phosphorylation, sulfonation, or glycosylation. It will generally be in monomer form or a multimer of repeated peptide units, and often will be joined to heterologous proteins or peptide fragments. Such heterologous proteins or peptides will often include segments derived from various proteins, e.g., immunoglobulins, β-galactosidase, β-glucuronidase, luciferase, trpE, or Protein A. See, e.g., Godowski et al. (1988) Science 241: 812-816. Such

This Sheet Has Been Replaced



peptide analogues will generally be soluble or coupled to a solid phase support, e.g., nitrocellulose, nylon, column packing materials (e.g., Sepharose beads), magnetic beads, glass wool, cells, or other substrates.

A phosphorylated region of a protein important for normal protein function will often be identified by a number of approaches, e.g., analysis of mutated proteins that have deletions or point mutations resulting in a change of ability to bind to another protein, altered enzymatic activity, or modification of other protein characteristics. Methods well known to those in the art are available to determine whether a peptide region is phosphorylated in vivo. See, e.g., Kazlauskas and Cooper (1989) Cell 58: 1121-1133; Williams (1989) Science 243: 1564-1570; and Wahl et al. (1990) J. Biol Chem. 265: 3944. Direct structural determination, e.g., x-ray crystallographic or 2D-NMR, can be used to determine locations of interactions, which guide where modifications are likely to affect interactions, both ligand and effector binding activities.

Fragments, in various extracellular region and intracellular region embodiments, will generally be at least about 1200 daltons, often be at least about 2400 daltons, typically at least about 3000 daltons, more typically at least about 3600 daltons, and in some preferred embodiments, at least about 4200 daltons or more.

III. Nucleic Acids

The nucleic acid compositions of this invention will generally be in RNA or DNA forms, or even a mixed polymer. The described DNA embodiment is usually derived from genomic DNA or cDNA, prepared by synthesis, or derived from combinations thereof. The DNA compositions generally include the complete coding region encoding hPDGF-R or fragments thereof, e.g., comprising at least 8 codons (24 bp), usually at least 12 codons, more usually at least about 15 codons, typically at least about 20 codons, more typically at least about 30 codons and preferably even more. One or more introns may be present.

This Sheet Has Been Replaced

The term hPDGF-R, when loosely applied to a nucleic acid, refers to a nucleic acid which encodes a hPDGF-R polypeptide, fragment, or variant, including protein fusions or deletion variants. A nucleic acid encodes a polypeptide when a corresponding message of a sequence, or its complement, is translated, as provided by a universal code of nucleotide triplets into polypeptide primary structure.

An "isolated" nucleic acid is a nucleic acid, e.g., an RNA, DNA, or a mixed polymer, which is substantially separated from other DNA sequences which naturally accompany a native human sequence, e.g., ribosomes, polymerases, and many other human genome sequences. The term embraces a nucleic acid sequence which has been removed from its naturally occurring environment, and includes recombinant or cloned DNA isolates and chemically synthesized analogues or analogues biologically synthesized by heterologous systems. A substantially pure molecule includes isolated forms of the molecule.

An isolated nucleic acid will generally be a homogenous composition of molecules, but will, in some embodiments, contain minor heterogeneity. This heterogeneity is typically found at the polymer ends or portions not critical to a desired biological function or activity.

The term "encoding" refers generally to the sequence information being present in a translatable form, usually operably linked to a promoter. A sequence is operably linked to a promoter when the functional promoter enhances transcription or expression of that sequence. An anti-sense strand is considered to also encode the sequence, since the same informational content is present in a readily accessible form, especially when linked to a sequence which promotes expression of the sense strand. The information is convertible using the standard, or a modified, genetic code. See, e.g., Watson et al. (1987) The Molecular Biology of the Gene (4th ed.) vols. 1&2, Benjamin, Menlo Park, California.

The term "recombinant" refers to a nucleic acid sequence which is not naturally occurring, or is made by the artificial combination of two otherwise separated segments of sequence. This artificial combination is often accomplished by

either chemical synthesis means, or by the artificial manipulation of isolated segments of nucleic acids, e.g., by genetic engineering techniques. Such is usually done to replace a codon with a redundant codon encoding the same or a conservative amino acid, while typically introducing or removing a sequence recognition site. Alternatively, it is performed to join together nucleic acid segments of desired functions to generate a single genetic entity comprising a desired combination of functions not found in the common natural forms. Restriction enzyme recognition sites are often the target of such artificial manipulations, but other site specific targets, e.g., promoters, DNA replication sites, regulation sequences, control sequences, or other useful features may be incorporated by design. A similar concept is intended for a recombinant, e.g., fusion, polypeptide.

Homologous sequences, when compared, exhibit similarity. The standards for homology in nucleic acids are either measures for homology generally used in the art or hybridization conditions. The hybridization conditions are described below, but are further limited by the homology between the corresponding human and mouse segments. Homology will be limited, in addition to any stated parameters, by any similarity between the human and mouse sequences such that the stated homology specifically is limited by the conditions which are sufficient for the corresponding mouse segment of the segment being compared to match the stated human segment.

Substantial homology in the nucleic acid context means either that the segments, or their complementary strands, when compared, are identical when optimally aligned, with appropriate nucleotide insertions or deletions, in at least about 60% of the residues, usually at least about 70%, more usually at least about 80%, preferably at least about 90%, and more preferably at least about 95 to 98% of the nucleotides. Alternatively, substantial homology exists when the segments will hybridize under selective hybridization conditions, to a strand, or its complement, typically using a sequence derived from Table 2 or 3. Selectivity of hybridization exists when hybridization occurs which is more selective than total lack of

This Sheet Has Been Replaced

specificity. Typically, selective hybridization will occur when there is at least about 55% homology over a stretch of at least about 14 nucleotides, preferably at least about 65%, more preferably at least about 75%, and most preferably at least about 90%. See, Kanehisa (1984) Nuc. Acids Res. 12:203-213, which is incorporated herein by reference. The length of homology comparison, as described, may be over longer stretches, and in certain embodiments will often be over a stretch of at least about 17 nucleotides, usually at least about 20 nucleotides, more usually at least about 24 nucleotides, typically at least about 28 nucleotides, more typically at least about 32 nucleotides, and preferably at least about 36 or more nucleotides.

Stringent conditions, in referring to homology, will be stringent combined conditions of salt, temperature, organic solvents, and other parameters typically controlled in hybridization reactions. Stringent temperature conditions will generally include temperatures in excess of 30° C, typically in excess of 37°, and preferably in excess of 45°. Stringent salt conditions will ordinarily be less than 1000 mM, typically less than 500 mM, and preferably less than 200 mM. However, the combination of parameters is much more important than the measure of any single parameter. See, e.g., Wetmur and Davidson (1968) J. Mol. Biol. 31:349-370, which is hereby incorporated herein by reference.

While the wild-type sequences of these alleles will generally be employed, in some situations one or more mutations or minor modifications may be introduced, such as deletions, substitutions or insertions resulting in changes in the amino acid sequence, providing silent mutations or modifying amino acid residues or amino or carboxyl terminal groups. There will be circumstances where gene fusions between a hPDGF-R and another protein can be useful, or a fusion between the separate types A and B forms. The nucleic acid sequence, usually genomic, will often not exceed about 50 kb, more often will not exceed about 40 kb, typically will not exceed about 30 kb, more typically will not exceed about 20 kb, usually will not exceed about 10 kb, and in various embodiments, preferably will not

This Sheet Has Been Replaced

exceed about 6 kbp. Such sequences may have any or all introns removed.

A DNA fragment encoding hPDGF-R finds use to isolate DNA encoding PDGF receptors of other species which share substantial homologies with hPDGF-R. Fragments from the intracellular tyrosine kinase region can be used to isolate other tyrosine kinases. Portions of the DNA fragment having at least about 10 nucleotides, usually at least about 20 nucleotides, more usually at least about 30 nucleotides, and fewer than about 6 knt (kilonucleotides), usually fewer than about 0.5 knt, from a DNA sequence encoding a hPDGF-R find use as probes. The probes can be used to determine whether RNA encoding a hPDGF-R or a fragment thereof is present in a cell.

Additionally, the type B human PDGF receptor gene is located at a site on chromosome 5 where a number of growth control related genes are clustered. At least one genetic disease, 5q minus syndrome, has been shown to involve a deletion in this region. The type A receptor gene is located on chromosome 4 near the c-kit proto-oncogene. These sequences, often intact genes, will find use in diagnosing the integrity of these chromosomal regions. Fragments of hPDGF-R gene sequences will often be used as markers to probe the structure of these important regions of the genome and to diagnose genetic diseases associated with those areas of the genome. Alternatively, the sequences are useful to isolate other fragments for testing the integrity of the chromosome regions.

The recombinant nucleic acid sequences used to produce fusion proteins of the present invention will often be derived from natural or synthetic sequences. Many natural gene sequences are obtainable from various libraries, cDNA or genomic, using appropriate probes. See, GenBank™, National Institutes of Health. Typical probes for human PDGF receptors will be selected from the sequences of Table 2 or 3 in accordance with standard procedures, or from alleles of them. The phosphoramidite method described by Beaucage and Carruthers (1981) Tetra. Letts. 22:1859-1862, will produce suitable synthetic DNA fragments. A double stranded fragment will often

be obtained either by synthesizing the complementary strand and annealing the strand together under appropriate conditions or by adding the complementary strand using DNA polymerase with an appropriate primer sequence.

IV. Methods for Making PDGF Receptor and Fragments

The novel nucleic acids provided herein are useful for making PDGF receptor. The DNA fragment or portions thereof will be used to prepare an expression construct for a hPDGF-R. The expression construct normally comprises one or more DNA sequences encoding a hPDGF-R under the transcriptional control of a native or other promoter. When more than one sequence encoding hPDGF-R is present in the construct, the sequences will encode the same or different forms of the receptor, usually different. Usually the promoter will be a eukaryotic promoter for expression in a mammalian cell, where the mammalian cell may or may not lack PDGF receptors. The transcriptional regulatory sequences will typically include a heterologous enhancer or promoter which is recognized by the host. The selection of an appropriate promoter will depend upon the host, but promoters such as the trp, lac and phage promoters, tRNA promoters and glycolytic enzyme promoters are known. See, e.g., Sambrook et al. (1989). Conveniently available expression vectors which include the replication system and transcriptional and translational regulatory sequences together with the insertion site for the fibroblast growth factor receptor DNA sequence may be employed. Examples of workable combinations of cell lines and expression vectors are described in Sambrook et al. (1989); see also, Metzger et al. (1988), Nature 334:31-36, each of which is hereby incorporated herein by reference. In particular, non-fungal promoters will be preferred where expression occurs in non-fungal cells. Occasionally, it might be useful to express the sequences in other types of cells, and appropriate promoters may be selected. And in some circumstances, an inducible promoter may be preferred. In other circumstances, it will be desired to coexpress a glycosylation enzyme which will provide

This Sheet Has Been Replaced



This Sheet Has Been Replaced

a glycosylation pattern similar to that provided by a different cell type.

In cases where one wishes to expand the DNA sequence or produce the receptor protein or fragments thereof in a prokaryotic host, a preferred promoter is a prokaryotic promoter, e.g., trp, lac, and lambda, and see Sambrook et al. (1989) for other useful prokaryotic promoters. Usually a strong promoter will be employed to provide for high level transcription and expression.

The expression construct will often be contained in a vector capable of stable extrachromosomal maintenance in an appropriate cellular host or may be integrated into the host genome. The expression construct may be bordered by sequences which allow for insertion into a host, such as transposon sequences, lysogenic viral sequences, or the like. Normally, markers are provided with the expression construct which allow for selection of host cells containing the construct. The marker may be on the same or a different DNA molecule, preferably on the same DNA molecule.

In mammalian cells, the receptor gene itself will often provide a convenient marker. However, in prokaryotic cells, markers such as a resistance to a cytotoxic agent, complementation of an auxotrophic host to prototrophy, production of a detectable product, etc., will be more convenient.

The expression construct can be joined to a replication system recognized by the intended host cell. Various replication systems include viral replication systems such as retroviruses, simian virus, bovine papilloma virus, or the like. In addition, the construct may be joined to an amplifiable gene, e.g., DHFR gene, so that multiple copies of the desired hPDGF-R gene may be made. See, e.g., Schimke, R. (1984) Cell 37:705-713; and Kaufman et al. (1985) Mol. Cell Biol. 5:1750-1759; each of which is hereby incorporated herein by reference.

In particular, it will often be desired to express a receptor polypeptide in a system which provides a non-fungal glycosylation pattern. In this case, the usual pattern will be

This Sheet Has Been Replaced

that provided naturally by the expression system. However, the pattern will be modifiable by exposing the polypeptide, e.g., an unglycosylated form, to appropriate glycosylating proteins introduced into a heterologous expression system. For example, the PDGF receptor gene may be co-transformed with one or more genes encoding mammalian or other glycosylating enzymes, preferably originating from a non-fungal species, and in some embodiments, non-human species. Using this approach, certain mammalian glycosylation patterns will be achievable in prokaryote or other cells.

The means of introduction of the expression construct into a host cell will vary depending upon the particular construction and the target host. Introduction can be achieved by any convenient means, including fusion, conjugation, transfection, transduction, electroporation, injection, or the like. See, e.g., Sambrook, et al. (1989) Molecular Cloning: A Laboratory Guide, Vols 1-3, Cold Spring Harbor Press, which is hereby incorporated herein by reference. Introduction of constructs encoding different forms of the receptor into a single host cell is also contemplated. The host cells will normally be immortalized cells, i.e., cells that can be continuously passaged in culture. For the most part, these cells will be convenient mammalian cell lines which are able to express a hPDGF-R and, where desirable, process the polypeptide so as to provide an appropriate mature polypeptide. By processing is intended glycosylation, ubiquitination, disulfide bond formation, general post-translational modification, or the like. Usually the host will be able to recognize the signal sequence for inserting hPDGF-R into the membrane of the cell. If secretion is desired, the transmembrane sequence will generally be deleted or mutated to prevent membrane localization of the protein.

A wide variety of hosts will be employed for expression of the peptides, both prokaryotic and eukaryotic. Useful hosts include bacteria, such as E. coli, yeast, filamentous fungi, insect cells, mammalian cells, typically immortalized, e.g., various mouse cell lines, monkey cell lines, Chinese hamster ovary cell lines, human cell lines,

This Sheet Has Been Replaced

derivatives of them, or the like. In some cases, the cells will be derived from a neoplastic host cell or wild-type cells will be transformed with oncogenes, tumor causing viruses or the like.

Under some circumstances, it will be desirable to transfect mammalian cells which lack a PDGF receptor, e.g., with a construct where a signal sequence and transmembrane region direct the receptor peptide to the cell membrane. Progeny of transformed or transfected cells are also intended to be encompassed. Lymphocytes and cardiac myocytes are examples of primary cells which lack a receptor. Chinese hamster ovary cells (CHO), epithelial cells lines, and a number of human tumor cell lines also lack PDGF receptors.

The compositions and cells comprising hPDGF receptors and fragments can be used for diagnostic purposes and to study and treat diseases or medical conditions associated with PDGF receptors. Use of the soluble extracellular ligand binding fragments has already been described generally. In addition, various segments of the intracellular region include phosphorylated amino acid residues. The sites of phosphorylation are important in interaction of segments with other polypeptides. The nature and specificity of the interactions are highly dependent upon the presence or absence of the phosphorylation, and fragments of the intact receptor, or related sequences, will be useful in modulating the interactions. Thus, intracellular region fragments will also find important uses.

Cells expressing cloning vehicles containing defined sequences can be used to define specific sites of a PDGF receptor necessary for effecting a particular activity. Alternatively, these cells will be useful to assess the ability of a selected receptor to bind different ligands (PDGFs and analogues) thereby providing a powerful tool for evaluating the potential of drugs for promoting or inhibiting specific PDGF-induced cellular responses.

Cells transfected, injected, infected or electroporated with DNA or mRNA containing a full length natural PDGF-R sequence will often express the native or wild

This Sheet Has Been Replaced

type receptor and respond accordingly less than full length segments will often have desired equivalent functions. Specific concentrations of a purified receptor or a receptor polypeptide fragment can be used to block the binding of the ligand (PDGF) to native PDGF receptors. Alternatively, antibodies to the receptor or fragment can have the same effect. In particular, it has been demonstrated herein that antibodies against epitopes of the extracellular region can block ligand-receptor binding. Other antibodies will block dimerization of receptor polypeptides, and thus modulate receptor function.

Homogeneous and defined polypeptides and DNA sequences will find use in raising antibodies and defining specificity of their binding. In particular, antibodies against specific regions of the receptor, e.g., the ligand-binding segments, will find use in diagnostic testing or therapeutics. The reagents PDGF-R, PDGF-R polypeptides, and antibodies to specific regions of the receptor can be used to study regulation of PDGF mediated activities. Intracellular fragments will also have important uses, especially, e.g., the kinase insert segment.

PDGF has roles in tissue repair, embryogenesis, and likely is involved in atherosclerosis, myeloproliferative disease, and some carcinomas. Treatment of these conditions responds to attenuation by therapeutic administration of the soluble extracellular fragments or antibodies. For example, PDGF agonists stimulate cell proliferative development, an effect particularly beneficial in wound healing, muscle regeneration, and arterial wall proliferation. PDGF antagonists will be used, in some cases, to block excess response, or to modulate PDGF response.

Compositions containing a soluble PDGF-R polypeptide having between about five and two hundred, preferably about 10 or 15 to 50, contiguous amino acids from a human PDGF-R extracellular region are described. In one embodiment, the polypeptide contains at least about 80 amino acids from residues 1 to 499 of a type B human PDGF receptor of Table 2,

This Sheet Has Been Replaced

or from residues 1 to 501 of a type A human receptor of Table 3.

In another embodiment, fragments of the intracellular region will find various uses. In particular, the KI region, and fragments or analogues thereof, will find use in blocking or modulating interaction of a phosphorylated residue with a recognition interaction, usually binding by another protein. These interactions are often important in further signal transduction and cellular responses.

The hPDGF-R protein expressed by transfected cells also finds many uses. If the peptide is secreted, the peptide will typically be isolated from the supernatant in which the expression host is grown. If not secreted, the peptide will be typically be isolated from the expression host, e.g., from a lysate. The peptide will generally be isolated by convenient techniques employing HPLC, electrophoresis, gradient centrifugation, affinity chromatography, e.g., using PDGF, column chromatography and other methods of protein purification used in protein biochemistry to provide a substantially pure product, e.g., particularly free of cell component contaminants. See, e.g., Jacoby (1984) Methods in Enzymology, Vol. 104, Academic Press, New York; Scopes (1987) Protein Purification: Principles and Practice, (2nd Ed.) Springer-Verlag, New York; Deutscher (ed.) (1990) Guide to Protein Purification, Methods in Enzymology, Vol. 182; each of which is hereby incorporated herein by reference.

The receptor protein or amino acids beginning at about leu(1) through lys(499) of the amino terminal sequence of the type B receptor, and about gln(1) through glu(501) of the sequence of the type A receptor, which form the entire extracellular regions, and particularly the PDGF ligand binding portions of the receptor proteins, will find use to affinity purify PDGF. The extracellular region can also be used affixed to a solid substrate or free in solution to determine drugs useful as PDGF agonists and antagonists.

The intracellular region of the protein, beginning at about val(500) through the carboxyl terminal amino acid of the type B receptor, and about leu(502) through the carboxyl

This Sheet Has Been Replaced

terminus of the type A receptor, also find use as tyrosine kinases for protein phosphorylatian in accordance with well known techniques. Additionally, amino acids met(-32) through gly(-1) of the amino terminal sequence of the type B receptor, and from about met(-23) through cys(-1) of the type A receptor, comprise signal sequences which direct the structural protein through the membrane of a transfected cell. These signal sequences will be used with a hPDGF-R, but also would be expected to find use with other proteins if fused to them.

Human PDGF receptor polypeptide fragments will typically be generated either by direct expression of truncated nucleic acid sequences, or by standard protease treatment of a hPDGF receptor, preferably purified. Reagents are provided herein for either approach.

As a diagnostic use, these reagents provide a method for measuring a PDGF or a PDGF receptor in a target sample, said method comprising the steps of:

combining said target sample with a hPDGF receptor polypeptide segment; and

determining the extent of binding between said polypeptide segment and said sample.

This invention also provides a transformed cell, which is also includes progeny of the primary transformant, capable of expressing a polypeptide homologous to at least a segment of human PDGF receptor. A preferred embodiment is where the cell expresses a polypeptide homologous to substantially the entire extracellular region of a human PDGF receptor, including soluble proteins.

V. Antibodies

Polyclonal and/or monoclonal antibodies to the various PDGF receptors and peptide fragments may also be prepared. Synthetic peptide fragments may be prepared in a peptide synthesizer and coupled to a carrier molecule (e.g., keyhole limpet hemocyanin) and injected into rabbits at selected times over several months. The rabbit sera is tested for immunoreactivity to the PDGF receptor protein or fragment. Monoclonal antibodies may be made by injecting mice with hPDGF-

R protein, hPDGF-R polypeptides or mouse cells expressing high levels of the cloned PDGF receptor on its cell surface. Monoclonal antibodies will be screened by ELISA and tested for specific immunoreactivity with the PDGF receptor protein or polypeptides thereof. See, e.g., Harlow and Lane (1988) Antibodies: A Laboratory Manual, Cold Spring Harbor Press, New York, which is hereby incorporated herein by reference. These antibodies will be useful in assays, or as pharmaceuticals.

Once a sufficient quantity of the desired hPDGF receptor polypeptide has been obtained, the protein will be used for various purposes. A typical use is the production of antibodies specific for binding to these receptors. These antibodies will usually be either polyclonal or monoclonal and will usually be produced by in vitro or in vivo techniques, e.g., directed towards the goal of defining or recognizing particular antigenic determinants, or epitopes. Usually the epitopes will be provided by molecular shapes of contiguous amino acid residues, but may be sequentially non-contiguous but in close spatial proximity due to secondary or tertiary structure. In particular, a polypeptide epitope homologous to a sequence of at least six contiguous amino acids described in Table 2 or Table 3 will be a useful immunogen. The epitopes of most interest will be those from a signal segment or immunoglobulin domains found in the extracellular region, particularly those surrounding the PDGF ligand binding regions. However, other segments will also be important, including phosphorylation sites in the intracellular region, e.g., the kinase insert segment, phosphorylated or not.

For production of polyclonal antibodies, an appropriate target immune system is selected, typically a mouse or rabbit. The substantially purified antigen is presented to the immune system in a fashion determined by methods appropriate for the animal and other parameters well known to immunologists. Typical sites for injection are in the footpads, intramuscularly, intraperitoneally, or intradermally. Of course, another species will sometimes be substituted for a mouse or rabbit, including goats, sheep, cows, guinea pigs, and rats.

This Sheet Has Been Replaced



This Sheet Has Been Replaced

An immunological response is usually assayed with an immunoassay. Normally such immunoassays involve some purification of a source of antigen, for example, produced by the same cells and in the same fashion as the antigen was produced. The immunoassay will, in some instances, be a radioimmunoassay, an enzyme-linked assay (ELISA), a fluorescent assay, or any of many other choices, most of which are functionally equivalent but may exhibit advantages under specific conditions.

Monoclonal antibodies with affinities of 10^8 M^{-1} preferably 10^9 to 10^{10}, or stronger will typically be made by standard procedures as described, e.g., in Harlow and Lane, Antibodies: A Laboratory Manual, CSH Laboratory (1988); or Goding (1986) Monoclonal Antibodies: Principles and Practice (2d ed) Academic Press, New York; each of which is hereby incorporated herein by reference. Briefly, appropriate animals will be selected and the desired immunization protocol followed. After the appropriate period of time, the spleens of such animals are excised and individual spleen cells fused, typically, to immortalized myeloma cells under appropriate selection conditions. Thereafter the cells are clonally separated and the supernatants of each clone are tested for their production of an appropriate antibody specific for the desired region of the antigen.

Other suitable techniques involve in vitro exposure of lymphocytes to the antigenic polypeptides or alternatively to selection of libraries of antibodies in phage or similar vectors. See, Huse et al. (1989) "Generation of a Large Combinatorial Library of the Immunoglobulin Repertoire in Phage Lambda," Science 246:1275-1281, which is hereby incorporated herein by reference. The polypeptides and antibodies of the present invention may be used with or without modification. Frequently, the polypeptides and antibodies will be labeled by joining, either covalently or non-covalently, a substance which provides for a detectable signal. A wide variety of labels and conjugation techniques are known and are reported extensively in both the scientific and patent literature. Suitable labels include radionuclides, enzymes, substrates, cofactors,

inhibitors, fluorescent moieties, chemiluminescent moieties, magnetic particles, and the like. Patents, teaching the use of such labels include U.S. Patent Nos. 3,817,837; 3,850,752; 3,939,350; 3,996,345; 4,277,437; 4,275,149; and 4,366,241. Also, recombinant immunoglobulins may be produced, see Cabilly, U.S. Patent No. 4,816,567.

Full length peptides or portions thereof will, in particular embodiments, be used for producing antibodies, either polyclonal or monoclonal. Antibodies are produced by immunizing an appropriate vertebrate host, e.g., mouse, with the peptide or fragment itself, or in conjunction with an adjuvant. Usually two or more immunizations will be involved, and the blood or spleen will be harvested a few days after the last injection.

For polyclonal antisera, the immunoglobulins may be precipitated, isolated and purified, including affinity purification. For monoclonal antibodies, the splenocytes normally will be fused with an immortalized lymphocyte, e.g., a myeloid line, under selective conditions for hybridomas. The hybridomas will generally then be cloned under limiting dilution conditions and their supernatants screened for antibodies having the desired specificity. Techniques for producing antibodies are well known in the literature, see, e.g., Goding (1986) Monoclonal Antibodies: Principles and Practice (2d ed.) Academic Press, N.Y., and Harlow and Lane (1988) Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory, New York, and are exemplified by U.S. Patent Nos. 4,381,292, 4,451,570 and 4,618,577, which are each incorporated herein by reference. The antibodies generated therefrom may have many uses in dissecting the portions of the receptor responsible for various cellular responses and in the general processing and maturation of the receptor itself. Antibody agonists or antagonists might even be produced.

Both proteins and fragments to serve as immunogens, and as reagents to determine the specificity of binding, are provided herein.

VI. Methods for Use

This Sheet Has Been Replaced



This Sheet Has Been Replaced

The present invention provides a human platelet derived growth factor receptor (hPDGF-R) purification method as well as a method for synthesizing PDGF receptors within cells. Also provided are homogeneous receptors produced by these methods, nucleic acid sequences encoding the receptors or portions of the receptors, expression vehicles containing these sequences, cells comprising the PDGF-receptors, and antibodies to the receptors. Of particular interest are fragments of the receptors, which have functional binding sites which compete with receptors to bind particular ligands. See, e.g., Orchansky et al. (1988) "Phosphatidylinositol Linkage of a Truncated Form of the Platelet-derived Growth Factor Receptor" J. Biol. Chem. 263:15159-15165, which provides evidence that an extracellular region of the PDGF receptor can bind to the PDGF ligands. Also provided are phosphorylated fragments or sites for phosphorylation which interact with other proteins important in cellular response to ligand binding. In particular, soluble extracellular region segments are provided.

Transfected cells find use as a model for studying cellular responses to PDGF. For controlled investigation, mammalian cells which lack a PDGF receptor can be transfected with an expression construct comprising a DNA sequence encoding a hPDGF-R. Cells are produced that encode a receptor that is often functionally equivalent to the wild-type receptor and confer a PDGF-sensitive mitogenic response on the cell. In this way, the binding properties of the naturally-occurring PDGF will be analyzed, as well as fragments or synthetic compounds, both proteinaceous and non-proteinaceous. As demonstrated in the Experimental section, transfected cells were used to determine that the AA form of PDGF activates the type B receptor tyrosine kinase. The presence of the type A and type B receptor polypeptides in a single cell will facilitate the study of receptor binding properties and perhaps even receptor interactions.

In addition to studying PDGF-mediated mitogenesis, the transfected cells can be used to evaluate a drug's ability to function as a PDGF agonist or antagonist. In particular, transfected cells can be contacted with the test drug, and the

This Sheet Has Been Replacec

amount of response determined, e.g., receptor tyrosine kinase activation or the rate of DNA synthesis, as compared to control cells in the presence or absence of PDGF or analogs thereof. Alternatively, in a non-therapeutic environment, a method is provided for inhibiting binding between a PDGF analogue and a PDGF receptor present in a solution. This method will contain a step of adding a PDGF receptor peptide, e.g., a peptide homologous to a sequence described in Table 2 or Table 3 to the solution, or a mutation or modification of one. Binding affinity to variants of the PDGF will also be evaluated by such cells. The inhibition of binding will usually occur by competition or by interfering with binding, on either the receptor or the ligand. The assay will often make use of comparisons with and without test ligand, or by a displacement assay, where the displacing ligand is added after binding occurs.

However, as indicated above, the PDGF receptor likely functions in a dimer state. The soluble forms of the receptor may interfere with the dimerization and, in some embodiments, will be effective in blocking signal transduction by a different mechanism from competitive affinity for the PDGF ligands. The soluble, or intracellular or transmembrane fragments of the various receptor forms would interfere with dimer formation and thus blocks at least some types of, or some fraction of, signal transduction.

This observation provides a method for modifying <u>in vivo</u> a PDGF receptor modulated activity comprising administering to a patient an amount of a PDGF receptor blocking agent effective to inhibit PDGF binding to PDGF receptors. Sometimes, the blockage wil occur at the level of ligand binding, by blocking the functional assembly of the receptor complex, or by blocking further interaction of the receptor with other proteins important in effecting cellular response, e.g., phosphotyrosine binding interactions. As discussed above, the PDGF family of proteins have a significant role in regulating many important physiological processes. The soluble PDGF-R polypeptides will generaly be effective in modifying the extent of PDGF modulation of these processes.

This Sheet Has Been Replaced

For this reason, the soluble forms of the receptors are useful as competitive binding sites for PDGF. Likewise, truncated PDGF binding sites or binding sites which have been mutated, particularly those from the human forms described, will sometimes be equally effective as natural forms, but at a lesser cost, both in monetary terms of and in terms of medical side-effects, upon administration.

The reagents provided herein will also find use in the quantitative detection or diagnosis of PDGF analogues, or PDGF-like ligands for the receptors, or for PDGF receptor polypeptide production. Various medical conditions are indicated by an abnormal level of production of either of these proteins, including, e.g., various tumor conditions. Thus, diagnostic tests dependent upon these reagents are now available.

With the different PDGF types, combining segments into chimeric receptors will form different types of receptors having variations in affinities for the various ligands. With the genes and proteins of the present invention, distinctions between various patterns and receptor types will be found, specific for various tissue types. Thus, tissue markers based upon differences in PDGF receptor expression would become available.

Soluble fragments containing various regions of the extracellular region of the human PDGF receptor have been shown to retain high affinity specific binding as in the intact type B PDGF receptor. The extracellular region of the human receptor has been expressed at high levels in CHO cells and secreted into the medium. In the presence of ligand, the soluble extracellular fragments block the ability of BB-PDGF to initiate responses characteristic of a physiological mitogen response. The soluble fragments also retain high specificity in binding to only the BB PDGF, not altering the mitogenic effect by the AA PDGF. Additional experiments using the human extracellular region have shown that the fragments may be used as binding segments to assay, e.g., by competition, for the presence of ligands. These results suggest the possibility of treating various conditions which excessively respond to PDGF

This Sheet Has Been Replaced

such as atherosclerosis, osteosarcoma, and glioblastoma. The mitogenic response may be attenuated through use of the soluble extracellular fragments.

The blocking of physiological response to PDGF results from the inhibition of binding of the ligand to the receptor, likely through competitive inhibition. Thus, in vitro assays of the present invention will generally use soluble fragments comprising the ligand binding segments of these receptors, or fragments attached to solid phase substrates. These assays will also allow for the diagnostic determination of the effects of either binding segment mutations and modifications, or ligand mutations and modifications, e.g., ligand analogues.

The soluble fragments will also find use in testing interaction of the receptor with modified ligands, e.g., biotinylated AA isoform or other PDGF forms. Another means for blocking ligand action will be to interfere with proper multi-protein receptor association, e.g., dimerization, or intracellular region interactions with other proteins. In particular, it is demonstrated that particular phosphorylation events control interactions with other protein which mediate other cellular functions. Various assays for this are well known to those skilled in the art, and see below.

Receptor components may be substituted by equivalent or corresponding soluble fragments from the other type polypeptide, e.g., exchanging fragments from the type A and type B polypeptide forms, or from other related polypeptides, e.g., mouse receptors, or other related receptors.

Moreover, because the type A homodimer binds all three forms of PDGF, the type A binding fragments exhibit substantial generality of ligand binding. The type A binding fragments are useful for preparing general PDGF binding reagents.

The quantities of reagents necessary for effective therapy will depend upon many different factors, including means of administration, target site, physiological state of the patient, and other medicants administered. Thus, treatment dosages should be titrated to optimize safety and efficacy.

This Sheet Has Been Replaced

Typically, dosages used in vitro may provide useful guidance in the amounts useful for in situ administration of these reagents. Animal testing of effective doses for treatment of particular disorders will provide further predictive indication of human dosage. Various considerations are described, e.g., in Gilman et al. (eds) (1990) Goodman and Gilman's: The Pharmacological Bases of Therapeutics, 8th Ed., Pergamon Press; and Remington's Pharmaceutical Sciences, 17th ed. (1990), Mack Publishing Co., Easton, Penn.; each of which is hereby incorporated herein by reference. Methods for administration are discussed therein, e.g., for oral, intravenous, intraperitoneal, or intramuscular administration, transdermal diffusion, and others. Pharmaceutically acceptable carriers will include water, saline, buffers, and other compounds described, e.g., in the Merck Index, Merck & Co., Rahway, New Jersey. Because of the high affinity binding between PDGF and its receptors, low dosages of these reagents would be initially expected to be effective. Thus, dosage ranges would ordinarily be expected to be in amounts lower than 1 mM concentrations, typically less than about 10 μM concentrations, usually less than about 100 nM, preferably less than about 10 pM (picomolar), and most preferably less than about 1 fM (femtomolar), with an appropriate carrier. Slow release formulations, or slow release apparatus will often be utilized for continuous administration. The intracellular segments of the receptors, both the PDGF receptor and related receptors will find additional uses as described in detail below.

The pharmaceutical compositions will be administered by parenteral, topical, oral, or local administration, such as by aerosol or transdermally, for prophylactic and/or therapeutic treatment. The pharmaceutical compositions can be administered in a variety of unit dosage forms depending upon the method of administration. For example, unit dosage forms suitable for oral administration include powder, tablets, pills, capsules and dragees.

The pharmaceutical compositions will often be administered intravenously. Thus, this invention provides compositions for intravenous administration which comprise a

IThis Sheet Has Been Replacec

solution of the compound dissolved or suspended in an acceptable carrier, preferably an aqueous carrier. A variety of aqueous carriers may be used, e.g., water, buffered water, 0.4% saline, and the like. These compositions will sometimes be sterilized by conventional, well known sterilization techniques, or may be sterile filtered. The resulting aqueous solutions may be packaged for use as is, or lyophilized, the lyophilized preparation being combined with a sterile aqueous solution prior to administration. The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions, such as pH adjusting and buffering agents, tonicity adjusting agents, wetting agents and the like, for example, sodium acetate, sodium lactate, sodium chloride, potassium chloride, calcium chloride, sorbitan monolaurate, triethanolamine oleate, etc.

For solid compositions, conventional nontoxic solid carriers may be used which include, for example, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharin, talcum, cellulose, glucose, sucrose, magnesium carbonate, and the like. For oral administration, a pharmaceutically acceptable nontoxic composition is formed by incorporating any of the normally employed excipients, such as those carriers previously listed, and generally 10-95% of active ingredient, preferably about 20% (see, Remington's, supra).

For aerosol administration, the compounds are preferably supplied in finely divided form along with a surfactant and propellant. The surfactant should, of course, be non-toxic, and preferably soluble in the propellant. Representative of such agents are the esters or partial esters of fatty acids containing from 6 to 22 carbon atoms, such as caproic, octanoic, lauric, palmitic, stearic, linoleic, linolenic, olesteric and oleic acids with an aliphatic polyhydric alcohol or its cyclic anhydride such as, for example, ethylene glycol, glycerol, erythritol, arabitol, mannitol, sorbitol, the hexitol anhydrides derived from sorbitol, and the polyoxyethylene and polyoxypropylene derivatives of these esters. Often mixed esters, such as

This Sheet Has Been Replaced

mixed or natural glycerides will be employed. The surfactant, in some embodiments, will constitute 0.1%-20% by weight of the composition, preferably 0.25-5%. The balance of the composition is ordinarily propellant. Liquefied propellants are typically gases at ambient conditions, and are condensed under pressure. Among suitable liquefied propellants are the lower alkanes containing up to 5 carbons, such as butane and propane; and preferably fluorinated or fluorochlorinated alkanes. Mixtures of the above will sometimes also be employed. In producing the aerosol, a container equipped with a suitable valve is filled with the appropriate propellant, containing the finely divided compounds and surfactant. The ingredients are thus maintained at an elevated pressure until released by action of the valve.

The compositions containing the compounds can be administered for prophylactic and/or therapeutic treatments. In therapeutic applications, compositions are administered to a patient already suffering from a disease, as described above, in an amount sufficient to cure or at least partially arrest the symptoms of the disease and its complications. An amount adequate to accomplish this is defined as "therapeutically effective dose." Amounts effective for this use will depend on the severity of the disease and the weight and general state of the patient.

In prophylactic applications, compositions containing the compounds of the invention are administered to a patient susceptible to or otherwise at risk of a particular disease. Such an amount is defined to be a "prophylactically effective dose." In this use, the precise amounts again depend on the patient's state of health and weight.

In a particularly important aspect of the present invention, it was recognized that many growth factors mediate their pleiotropic actions by binding to and activating cell surface receptors with an intrinsic protein tyrosine kinase activity. Growth factor receptors with tyrosine kinase activity, or receptor tyrosine kinases, typically possess similarities in molecular topology. For example, they possess a large extracellular ligand binding region, a hydrophobic

This Sheet Has Been Replaced

transmembrane region, and a cytoplasmic, i.e., intracellular, region that contains a tyrosine kinase catalytic domain. Receptor tyrosine kinases typically have a topology dictating that the ligand binding segments and protein tyrosine kinase activity are separated by the plasma membrane. Therefore, receptor activation due to extracellular ligand binding is translated across the membrane barrier into activation of intracellular region functions.

The present invention takes advantage of the discovery that phosphorylation of particular amino acid residues in a polypeptide segment is important in certain protein-protein interactions. The phosphorylation state of residues within the regions of interaction, or coupling regions, modulate the interactions. In the case of the PDGF receptor and phosphatidylinositol 3' kinase (PI3 kinase) interactions, the presence of a phosphotyrosine in the defined kinase insert sequence is important in the interacting between, e.g., binding between, the receptor and the PI3 kinase, thereby activating the PI3 kinase enzyme. An unphosphorylated KI segment fails to bind a PI3 kinase activity. These observations will be generally applicable to the interaction of phosphorylated segments with other binding proteins. With the elucidation of the nature and specificity of these interactions, analogues, e.g., organic molecules, are prepared which serve as analogues to the peptides or phosphorylated peptides.

Tyrosine kinases can be broadly classified into two groups, the transmembrane receptor kinases and the cytoplasmic non-receptor kinases, with most of the members of the second group belonging to the so-called src family of tyrosine kinases. The receptor kinases genes, including the PDGF receptor, share certain structural features, e.g., an amino-terminal signal peptide which is characteristic of membrane glycoproteins and a transmembrane segment which defines and separates the extracellular and the intracellular regions of the receptor protein. The amino-terminal portion of the receptor protein makes up the extracellular region which contains the ligand binding sites. The carboxy-terminal

This Sheet Has Been Replaced

portion of the protein makes up the intracellular region of the receptor, on which the kinase activity is located. Receptor kinases are responsible for transducing a signal provided by ligand binding to the extracellular region into the intracellular compartment, and through cytoplasmic second messages, ultimately to the nucleus.

Detailed comparison of established receptor tyrosine kinase (RTK) primary sequences has led to the identification of both shared and unique structural subdomains, or segments, which permit classification of the RTK family into distinct groups, see, e.g., Yarden and Ullrich (1988) Ann. Rev. Biochem. 57:443-478; and Ullrich and Schlessinger (1990) Cell 61:203-212; each of which is incorporated herein by reference.

The PDGF receptor is a member of one such subclass. These receptors, including macrophage growth factor (CSF-1-R) and the putative receptor c-kit share at least two distinct structural features. They lack cysteine-rich repeat clusters within their extracellular regions, and they possess another conserved repeat structure that includes five immunoglobulin-like repeats, suggesting a common architecture for the ligand-binding regions of the members of this receptor subclass.

Furthermore, when compared with RTKs of other subclasses, the catalytic domains of members of this group are interrupted by long, structurally unique, hydrophilic, proline-rich insertion sequences of about 77-107 amino acid residues. This "kinase insert" (KI), also referred to as a kinase insert region, divides the catalytic domain into two segments and is defined by homology to other tyrosine kinase domains. These KI sequences are highly divergent, even within each receptor subfamily, but are strongly conserved between evolutionarily distant species, such as chickens, cats and humans. Members of a related subclass, including the FGF receptor, flg, and bek, similarly have immunoglobulin-like repeats in their extracellular region, although only three in number, and kinase insert sequences of 10-19 residues located in the corresponding position of the tyrosine kinase segments.

RTK ligands induce pleiotropic cellular responses, which in many cell types culminate in cell cycle progression,

IThis Sheet Has Been Replaced

DNA synthesis, and cellular replication. Upon ligand binding, numerous responsive membrane events are initiated, including stimulation of ion transport, glucose transport, membrane kinases, pinocytosis, membrane ruffling, and other cytoskeletal and morphological changes. These events are paralleled by activation of a number of cytoplasmic pathways, including glycolysis, polyamine synthesis, and ribosomal protein S6 phosphorylation. Alterations in the pattern of specific gene transcription (e.g., c-myc and c-fos) are detectable within minutes, and increased macromolecular synthesis of protein, RNA and DNA is observed within about 3-20 hours after ligand binding. PDGF has the unique capability of stimulating both protein kinase C, through an increased turnover of phosphatidylinositol, and protein kinase A, through production of type E prostaglandin.

The importance of the correct regulation of receptor function is emphasized by the fact that a large variety of structural alterations found in receptor-derived oncogene products lead to constitutive activation and, consequently, subversion of molecular control mechanisms and alteration of receptor signals. The most common cellular lesion found in human cancers involves autocrine activation in conjunction with receptor overexpression. Many tumors and tumor cell lines have been found to coexpress growth factors and their receptors, including PDGF-A chain, PDGF-B chain, and PDGF receptor polypeptides. Autocrine receptor activation represents one scenario of subversion of normal growth control. In principle, every receptor with tyrosine kinase activity has oncogenic potential. Many more types of activating mutations, as well as specific instances of receptor tyrosine kinase overexpression, would be anticipated to be detected in animal and human tumors. The understanding and control of such defects in normal cellular metabolism and signal transduction will play an important role in the diagnosis and therapy of oncogenesis.

When activated by ligand, the PDGF β-receptor becomes phosphorylated on tyrosine residues. See, e.g., Ek and Heldin (1982) J. Biol. Chem. 257: 10486; Frackelton et al. (1984) J. Biol. Chem. 259: 7909; Kazlauskas and Cooper (1989) Cell 58:

This Sheet Has Been Replacec

1121; and Yarden et al. (1986) Nature 323: 226-232. The mechanism of this "autophosphorylation" reaction appears to depend on ligand-induced formation of receptor dimers and a transphosphorylation reaction in which each of the two polypeptide chains of the dimer phosphorylates the other chain. The major sites of PDGF stimulated receptor phosphorylation are located in the carboxyl terminal portion of the tyrosine kinase domain, about tyr(825) in both the human and mouse type B receptor, and in the kinase insert region, about tyr(719) in both the human and mouse type B, that splits the kinase coding sequences into two parts. It is likely that additional sites of autophosphorylation have not yet been identified.

One of the consequences of ligand-induced receptor autophosphorylation appears to be a change in conformation of the intracelluar region of the receptor. In its activated state the receptor can physically interact with several cytoplasmic molecules that are likely to be important in signal transduction.

Activation is also accompanied by complex formation with, and tyrosine phosphorylation of, a variety of proteins. The first to be described was a protein of 85 kD, which is a putative PI kinase and identical to a protein referred to as p81, see Kaplan et al. (1987) Cell 50:1021. Subsequently, a number of other proteins have been shown to complex with PDGF receptors and to become phosphorylated on tyrosine following PDGF stimulation. These include the serine/threonine kinase Raf-1, which becomes activated in the complex; see, e.g., Morrison et al. (1989) Cell 58: 649-657; and phospholipase C-γ, see, e.g., Morrison et al. (1990) Mol. Cell. Biol. 10: 2359-2366; and GAP, the GTPase activating protein involved in the control of ras activity, see, e.g., Kaplan et al. (1990) Cell 58: 1121-1133. PDGF treatment also enhances the kinase activities of some of these proteins.

Recently, transient complex formation also has been shown to occur between three src family tyrosine kinases (pp60-c-src, p59-fyn, and pp62-c-yes), p81, and PDGF receptors. In addition, PDGF treatment stimulates the tyrosine kinase activates of these proteins, suggesting that src family kinases

This Sheet Has Been Replaced

may play a role in the response to PDGF. Phosphorylation on tyrosine residues likely activates the mitogenic potential of the target protein either by enhancing its enzymatic activity or by altering its interactions with other cellular proteins.

The SH2 system provides possible guidance to particular mechanisms and properties which could be applicable to the PDGF receptors. Some non-receptor tyrosine kinases share a conserved noncatalytic region of approximately 100 amino acids called the src homology region 2 (SH2) domain. The SH2 domains of the Fps and Src tyrosine kinases are thought to possibly interact with and regulate the adjacent kinase domain and may also form binding sites for proteins phosphorylated by the kinase domain.

Phospholipase C-γ (PLC-γ) and p21-ras GTPase-activating protein (GAP) each contain two adjacent SH2 domains. Recent experiments have shown that SH2 domains endow proteins such as GAP, v-Crk and p60-v-src with the potential to form complexes with specific tyrosine phosphorylated ligands. The relevance of these results to the PDGF-R was, until now, unclear. In the PDGF receptor embodiments, ligand-activated PDGF receptor induces the formation of a complex of signaling molecules including PLC-γ, Raf-1, and the 85 kDa PI-3 kinase.

One of the first of the signaling molecules to be identified as a receptor-associated protein was phosphatidyl-inositol-3' kinase (PI3 kinase). This enzyme was originally found to be a kinase that co-immunoprecipitated with the complex of polyoma middle T antigen and the c-src protein, and with transforming v-src proteins. PI3 kinase activity was also found in phosphotyrosine or receptor immunoprecipitates of lysates from PDGF-stimulated cells. The specific role of PI3 kinase in mediating cell proliferation has not been determined. However, the enzyme is regulated by a number of growth factors and growth factor-treated cells contain increased levels of phosphoinositides and inositol phosphates phosphorylated on the 3 position of the inositol ring. Mutants of middle T antigen or the PDGF receptor that do not associate with PI3 kinase are defective in their mitogenic activities. The protein responsible for the PI3 kinase activity has not been well

This Sheet Has Been Replaced

characterized. However the close correlation of the enzymatic activity with the presence of an 81-85 kD protein in co-immunoprecipitation experiments with the middle T/c-src complex and with the PDGF receptor has strongly suggested that the 85 kD protein is either the PI3 kinase or an important subunit of the kinase.

Definable regions of the PDGF receptor were capable of mediating the interaction with PI3 kinase, as evidenced by an analysis of a mutated type B PDGF receptor having a deletion of the kinase insert (KI) region. This receptor mutant (ΔKI) was defective in stimulating cell proliferation and in binding PI3 kinase, even though it had tyrosine kinase activity and stimulated several other responses to PDGF binding, including phospholipase C-mediated PI hydrolysis. Recent studies have shown that the ligand-activated ΔKI mutant receptor fails to bind PI3 kinase, but binds as much PLC-γ and the c-Raf-1 proteins as the wild type receptor. However, the mutant receptor is unable to phosphorylate c-Raf-1 on tyrosine residues and the binding of PLC-γ required tyrosine phosphorylation of the receptor. A mutant of the human type B PDGF receptor that had one of the phosphorylation sites in the kinase insert domain, tyr(719) in both the human type B receptor, converted to phenylalanine was also defective in binding PI3 kinase. These experiments indicate that either the PI3 kinase binds directly to a portion of the kinase insert region containing tyr(719) or that the binding interaction involves other portions of the receptor that are conformationally dependent on sequences in the kinase insert region.

To directly study the interaction between the type B PDGF receptor and PI3 kinase, an in vitro system was established. With this system, it was possible to test the ability of synthetic polypeptides derived from receptor sequences in the kinase insert domain to block interaction of the PI3 kinase with the PDGF receptor. The interaction could be blocked by a tyrosine-phosphorylated peptide representing a highly conserved region of the kinase insert domain that included tyr(719). However the peptide blocked PI3 kinase

This Sheet Has Been Replaced

binding to the PDGF receptor only when the peptide was phosphorylated on tyrosine. Scrambled versions of the peptide, even when phosphorylated on tyrosine, had no blocking activity. These studies show that phosphotyrosine in a specific sequence context serves as a recognition site for the binding of a cytoplasmic signaling molecule.

An in vitro system was used to study the interaction of PDGF receptors and PI3 kinase. The association of phosphoproteins with the PDGF receptor is demonstrated by stimulating 3T3 cells with PDGF, immunoprecipitating cell lysates with type B PDGF receptor antibodies and by examining the immunoprecipitates for the presence of receptor-associated proteins that can be phosphorylated in the immunoprecipitates. See Fig. 1a "+" lanes. These phosphoproteins include the 85 kDa protein and a 110 kDa protein that have recently been correlated with PI3 kinase activity. See Fig. 1c. In in vitro experiments, the type B PDGF receptor was expressed in an insect cell expression system, partially purified, and immobilized using anti-receptor antibodies and protein A Sepharose. The receptor was allowed to autophosphorylate *in vitro*, so that it would be in a conformation that mimicked the ligand-activated state of the receptor. Cytoplasmic cell lysates from density-arrested BALB/c 3T3 cells were mixed with the immobilized receptor and the complexes were washed extensively. PI3 kinase activity was found in the complex of proteins associated with the receptor (Fig. 1d). The receptor-associated phosphoproteins were detected by an *in vitro* kinase assay using ^{32}P-ATP. See Fig. 1b "-" lane. At least four receptor-associated proteins with apparent molecular weights of 140 kD, 120/110 kD (sometimes a doublet), 85 kD and 74 kD were radiolabeled (the bands at 160 kD and 150 kD are the baculovirus-expressed receptor and its precursor, respectively). The 140 kD band has been previously identified as PLC-γ and the band at 74 kD includes the *raf*-1 kinase and probably another molecule as well. The 85 kD protein co-migrated with the 85 kD band, previously considered to be PI3 kinase, in anti-phosphotyrosine immunoprecipitates of cell lysates prepared from PDGF-stimulated cells. The association

This Sheet Has Been Replaced

of 85 and 110 kD proteins with the baculovirus-expressed receptor was also observed by silver stain analysis. When cell lysate was incubated with immobilized kinase insert deletion mutant of the PDGF receptor there was no associated PI3 kinase activity, and the 110 kD and 85 kD phosphoproteins did not associate with the mutant receptor. See Fig. 2a and A2b. This in vitro result is consistent with previous indications that this receptor mutant does not associate with PI3 kinase in intact cells.

When 3T3 cells were stimulated with PDGF prior to making the lysates, there was a dramatic reduction in the amount of PI3 kinase available for association with the receptor in vitro (Fig. 1d) and a concomitant reduction in the association of the 85 kD and 110 kD phosphoproteins with the receptor (Fig. 1b "+" lane). Although the explanation of this phenomenon is not entirely clear, the observation that PDGF pretreatment of cells influences the subsequent in vitro association of cellular proteins with the receptor indicates that the associations are specific PDGF-regulated processes.

When the autophosphorylated PDGF receptor was dephosphorylated in vitro using potato acid phosphates (PAP) it lost the ability to associate with PI3 kinase activity or 84 kD protein derived from the BALB/c 3T3 cell lysates (Fig. 3b and c). This finding indicates that phosphorylation of the PDGF receptor was required for these protein interactions to occur.

To identify the site of interaction between the receptor and the PI3 kinase, phosphorylated synthetic peptides were prepared to compete for the binding of the 85 kD protein and the PI3 kinase activity to the receptor in vitro. Previous data suggested that the kinase insert region was involved in the association of the PI3 kinase activity with the PDGF receptor. In scanning sequences from the kinase insert regions of the mouse and human PDGF type B and type A receptors, a 20 amino acid region of particularly high sequence homology (8 out of 20 identities for the human and mouse PDGF type B receptor compared to 12% identity for the entire kinase insert region) was found. A peptide (Y719) containing amino acids 705 to 724

of the mouse type B PDGF receptor sequence was synthesized. This peptide sequence contained two tyrosine residues, Y708 and Y719. The tyrosine at position 719 of the mouse sequence also corresponds to tyr(719) in the human type B PDGF receptor, a known autophosphorylation site of the receptor.

To determine the ability of a series of synthetic peptides derived from this sequence to block the interaction between the receptor and the 85 kD/PI3 kinase activity, 3T3 lysates were incubated with the different peptides prior to mixing with immobilized receptor. The results of these experiments are shown in Fig. 4. The first lane of Fig. 4a shows phosphorylated proteins that associated with the wild type receptor in the absence of peptides. The arrow indicates the position of the 85 kD protein that co-purified with the PI3 kinase activity. The other lanes show the proteins that associated with the receptor in the presence of derivatives of the Y719 peptide. See Table 4. No change in the pattern of receptor-associated protein was seen when the unphosphorylated 719 peptide (Y719) was preincubated with the 3T3 lysate prior to the association with the receptor. By contrast when a derivative of this peptide that was phosphorylated at position 719 (Y719P) was added to the incubation, it blocked the binding of the 85 kD phosphoprotein (Fig. 4a) and inhibited the association of PI3 kinase activity (Fig. 4b) with the receptor. A scrambled version of this peptide that contained phosphotyrosine at a position corresponding to 719 but had a rearranged primary sequence failed to block binding of the 85 kD protein and did not prevent the association of PI3 kinase activity with the receptor.

This Sheet Has Been Replaced



TABLE 4

PDGF Receptor Synthetic Peptides

Peptides	Sequence
Y719	GGYMDMSKDESIDYVPMLDM
Y719P	GGYMDMSKDESIDYVPMLDM (* at position 15)
Y708P	GGYMDMSKDESIDYVPMLDM (* at position 9)
Y719P short	MDMSKDESIDYVPMLDM (* at position 14)
Y708P short	GGYMDMSKDESID (* at position 9)
Y708P/F719	GGYMDMSKDESIDFVPMLDM (* at position 9)
Y708/Y719P	GGFMDMSKDESIDYVPMLDM (* at position 15)
Y708P/Y719P	GGYMDMSKDESIDYVPMLDM (* at positions 9 and 15)
Y719P scrambled	MMDIKVPMDEYMSDYSDLGG (* at position 15)

The asterisks (*) indicate the position of a phosphate group

This Sheet Has Been Replaced



A shorter version of peptide Y719P that includes only 14 amino acids (Y719P short) and lacked tyrosine 708 also blocked binding of the 85 kD protein (Fig. 4a, lane 4) and inhibited the association of PI3 kinase activity with the receptor. Surprisingly, a peptide that includes tyrosine at position 719 and phosphotyrosine at position 708 blocked the interaction of PI3 kinase with the receptor (Fig. 4, lane 6). This finding suggests the possibility that tyrosine 708 is one of the autophosphorylation sites of the receptor that has not yet been mapped. A peptide that included phosphotyrosine at both positions 708 and 719 (Y708P/Y719P) also blocked binding of the 85 kD protein (Fig. 4, lane 8). Short forms of Y708P and Y719P also had blocking activity (Fig. 4 lanes 4 and 9).

To determine the amount of peptide necessary to block the association of the 85 kD and the PI3 kinase activity to the receptor, the 3T3 lysates were preincubated with a range of concentrations of phosphorylated peptide (Y719P). As shown in Fig. 5, approximately 5 μM of Y719P was sufficient to block more than 50% of the association of the 85 kD protein (Fig. 5a) and PI3 kinase activity (Fig. 6B) with the receptor. Concentrations of up to 100 μM of unphosphorylated peptide did not affect association between the receptor and the PI3 kinase. Thus the coincident blocking of 85 kD and PI3 kinase activity association with the receptor by peptide Y719P supports the hypothesis that the 85 kD protein is a subunit of the PI3 kinase enzyme.

Next, the ability of peptides to block the association of other signaling molecules with the receptor was assessed. Lysates from 3T3 cells were incubated in the absence or the presence of peptide Y719P prior to association with the receptor immunoprecipitates. The association of PLC-γ and GAP with the receptor was determined by immunoblot analysis using specific antibodies (Fig. 6), confirming previous studies that these molecules specifically associate with autophosphorylated receptors. The association of these proteins with the receptor was not affected by the addition of the phosphorylated peptide, Y719P. Thus the Y719P peptide specifically blocked association

This Sheet Has Been Replaced



This Sheet Has Been Replaced

of 85 kD/PI3 kinase activity with the receptor and did not affect the interaction of the receptor with GAP or PLC-γ.

To determine the nature of the interaction of the receptor with the 85 kD protein, a modification of Western blotting methodology was used. PDGF receptor from Sf9 cells infected with PDGF receptor baculovirus was immuno precipitated with receptor antibodies, labeled with ^{32}P in vitro, and used as a probe to bind proteins from 3T3 cell lysates that had been separated electrophoretically and transferred to nitrocellulose. The radiolabeled PDGF receptor bound directly to an 85 kDa protein in unstimulated 3T3 cell lysate (Fig. 7, lane 1). Cell lysates from PDGF-treated 3T3 cells did not show any binding when incubated with the radiolabeled PDGF receptor, (Fig. 7, lane 2) consistent with the finding that the 85 kD protein from PDGF-stimulated lysates was not available for association with the immobilized PDGF receptor (Fig. 1b). When the nitrocellulose paper that contained cell lysate proteins was pre-incubated with peptide Y719P, the radiolabeled receptor failed to associate with the 85 kD protein (Fig. 7, lane 4). The unphosphorylated peptide (Y719) or the scrambled peptide (Y719P scrambled) did not interfere with the association of the receptor with the 85 kD protein (Fig. 7, lanes 3 and 5). This experiment showed that the 85 kD protein (Fig. 7, lanes 3 and 5). This experiment showed that the 85 kD protein binds directly to the PDGF receptor and does not require the presence of other molecules. The direct binding interaction appears to involve the 20 amino acid segment in the kinase insert region containing tyrosine 719, and tyrosine phosphorylation of this segment appears to be necessary for the interaction.

These results indicate that the intracellular region of the type B PDGF receptor interacts with molecules that are likely to play a role in signal transduction. The physical association between the receptor and signaling molecules such as PI3 kinase, PLC-γ, GAP, and raf-1 only occurs when the receptor is phosphorylated on tyrosine. See Fig. 1a. A region of the receptor that is involved in the binding of the receptor to the PI3 kinase has been defined. Short peptides were used to block the in vitro association of PI3 kinase with the

This Sheet Has Been Replaced

phosphorylated receptor. The most likely explanation for the ability of the peptides to block the association is that they mimic the receptor and bind directly to the PI3 kinase. In this way they act as competitive antagonists. The observation that only tyrosine-phosphorylated peptides had this blocking activity suggests that phosphotyrosine is directly involved in the binding of the receptor to PI3 kinase. However phosphotyrosine alone is not sufficient for binding, since scrambled peptides containing phosphotyrosine did not interact with the PI3 kinase. Therefore the primary sequence of this portion of the receptor has essential structural information that is required for the binding of the receptor to PI3 kinase. It is somewhat surprising that peptides as short as 13 amino acids contained enough structural information to mimic the native receptor region that normally binds to the PI3 kinase (Table 4).

Tyrosine 719 of the type B PDGF receptor is one of the autophosphorylation sites of the receptor in intact cells as well as *in vitro*. Thus, it appears that the portion of the KI domain around tyrosine 719 is directly involved in the association of PI3 kinase with the receptor *in vivo*. The data presented herein indicate that peptide derivatives of Y719 containing one of the two tyrosine residues phosphorylated (Y708P or Y719P) efficiently prevented the association of the receptor with the PI3 kinase in *in vitro* assay. Similar sequences and interactions are subject to blockage by the appropriate fragment sequences. In particular, sequences known to be phosphorylation sites will be used to select for analogues which block interaction with other mediating proteins. The PI3 kinase is known to associate with the c-*fms* protein and with the middle T antigen, and both of these proteins are phosphorylated on tyrosine *in vivo*. A phosphorylation site of the middle T antigen (tyrosine 315) is in a sequence somewhat homologous (5 out of 10 amino acids are identical) to the peptide Y719P used in our experiments. There is no obvious homology of peptide Y719P to any of the tyrosine phosphorylation sites on the c-*fms* protein. However peptide Y719P blocked association of PI3 kinase with the c-*fms* protein

in vitro. Thus an autophosphorylation site of the c-fms protein is in a region that has a secondary structure that is similar to the corresponding domain of the PDGF receptor.

These findings indicate that signaling molecules recognize phosphotyrosine in a specific sequence context. The pattern of interactions of the kinase insert deletion mutant with GAP, PLC-γ, and c-raf-1 and the lack of ability of the peptides used in this report to block GAP and PLC-γ association with the receptor shows that different regions of the receptor, possibly containing different autophosphorylation sites, are involved in the binding of specific signaling molecules. The binding of these molecules to the receptor localize the signaling molecules and regulate their activities. Tyrosine phosphorylation of other cellular proteins by the receptor kinase targets them as proteins for binding signaling molecules and the present invention provides the methods and materials for generating reagents which will successfully mimic or interfere with natural signals. For example, fragments from the hPDGF-receptor, or related tyrosine kinase proteins, will be useful to block the natural interactions between them and function-mediating proteins. See, e.g., Williams (1989) Science 243: 1564-1570; Yarden et al. (1986) Nature 323: 226-232; and GenBank™, for other sequences and proteins which show homology, both structural and functional, with the phosphorylated inserts described herein.

This discovery leads the way to the production of analogues of the KI segments. For example, short peptides having non-hydrolyzable moieties will often be produced with, e.g., sulfonated moieties substituted for phosphorylated moieties. Alternatively, other organic molecules exhibiting sufficient structural homology will often be selected for their functional interaction with the PI3 kinase or other function mediating proteins. In addition, chimeric analogues, portions of which are peptide, or modified peptide, and other portions of which are organic molecules which have similar structural features which are important for interaction, will be constructed. Thus, molecules exhibiting particular interacting

This Sheet Has Been Replaced



features become available, including both natural or synthetically generated compounds.

The invention will better be understood by reference to the following illustrative examples. The following examples are offered by way of illustration and not by way of limitation.

This Sheet Has Been Replaced



This Sheet Has Been Replaced

EXPERIMENTAL

In general, standard techniques of recombinant DNA technology are described in various publications, e.g., Sambrook et al. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory; Ausubel, et al. (1987) Current Protocols in Molecular Biology, vols. 1 and 2 and supplements; and Wu and Grossman (eds.) (1987) Methods in Enzymology Vol. 53 (Recombinant DNA Part D); each of which is incorporated herein by reference. Variations, if any, on those methods have generally been minor.

I. Screening of Human Kidney λGT11 cDNA Library and Human Placenta λGT10 cDNA Library

A full-length DNA sequence encoding the mouse PDGF receptor (mPDGF-R) protein was used as a probe to screen 250,000 plaques of a human kidney cDNA library. Nick translation was used to prepare a probe with specific activity of 12 x 10^8 cpm per μg. The filters were incubated with the probe (10^5 cpm per ml) in hybridization buffer containing 30% formamide, 1X Denhardt's solution, 5X SSC, 0.02 M sodium phosphate pH 6.5, and 500 μg per ml of salmon sperm DNA. After 14 h of hybridization at 40°C, the filters were washed four times at 55°C with 0.2X SSC and 0.1% SDS and two additional times at 65°C with 0.2X SSC. The filters were then air dried and exposed for 16 h.

Ten positive clones were obtained which were rescreened with the full-length mPDGF-R probe. Individual clones were isolated and analyzed by restriction analysis using EcoRI endonuclease. The clone containing the largest insert (2.3 kb), designated clone HK-6, was further characterized and sequenced using dideoxy terminators. Clone HK-6 contained the receptor sequence from nucleotide 3554 to nucleotide 5691 plus nine bases from the poly-A tail.

A nick-translated probe, prepared from the 2.3 kb HK-6 DNA, was used to screen 250,000 plaques of a human placenta cDNA library. This screening was performed at high hybridization stringency (50% formamide in the hybridization buffer described above). The filters were incubated with 5 x

10^5 cpm per ml of probe for 14-16 h at 42°C. The filters were than washed at 65°C in 0.1% SSC and 0.1% SDS four times.

After secondary screening with the HK-6 probe, seven clones were selected and analyzed by restriction digestion with EcoRI endonuclease. A clone (HP-7) that contained a 4.5 kb insert was selected and characterized. The sequence of that clone is described in Table 2 and encodes the type B human PDGF receptor (B-hPDGF-R).

II. Construction of Expression Vector

The 4.5 kb DNA fragment containing the complete coding sequence for the type B human PDGF receptor was isolated from the HP-7 clone by EcoRI digestion. The gel purified fragment was cloned into the EcoRI site in the polylinker region of SV40 expression vector PSV7C. The pSV7d expression vector, obtained from P. Luciw, at the University of California, Davis, was a pML derivative containing the SV40 early promoter region (SV40 nucleotides 5190-5270), a synthetic polylinker with restriction sites for EcoRI, SmaI, XbaI, and SalI followed by three translation terminator codons (TAA) and the SV40 polyadenylation signal (SV40 nucleotides 2556-2770) (Truett et al. (1984) DNA 4:333-349). The EcoRI fragment containing the cDNA sequence obtained from the HP-7 clone was inserted at the EcoRI site of the pSV7d. In the resulting expression vector, the B-hPDGF-receptor gene was under transcriptional control of the SV40 promoter.

To ensure the proper orientation of the PDGF receptor insert (4.5 kb) with respect to the SV40 promoter, the positive clones were digested with SmaI endonuclease which cuts at position 573 of the receptor sequence and in the polylinker region of the expression vector.

Clones containing the receptor in the proper transcriptional orientation released a 4.0 kb insert in addition to the 3.2 kb fragment containing the expression vector plus 573 base pairs of the 5' end of the receptor. This plasmid, PSVRH5 was used to co-transfect cells with PSV2 neo plasmid that confers resistance to the antibiotic neomycin.

This Sheet Has Been Replaced



III. Cell Culture and Transfection of CHO Cells

CHO cell clone KI, obtained from the U.C.S.F. Tissue Culture Facility, were grown in Ham's F-12 media supplemented with 10% FCS (UCSF Tissue Culture Facility) and penicillin and streptomycin at 37°C in 5% CO_2/95% air.

pSVRH5 plasmid DNA (10 μg) and pSV2 neo (1 μg) were used to co-transfect 1 x 10^6 CHO cells by the calcium precipitation technique of Van der Eb et al. (1980), Methods in Enzymology (1980) 65:826-839, with the addition of 10 μg chloroquinone diphosphate (CDP) to prevent degradation of the transfected DNA. After 12 h of exposure to the DNA, the cells were trypsinized and replated at 1:5 dilution. Twenty-four hours later, the antibiotic G418 (GIBCO), an analog of neomycin, was added to the cultures at a concentration of 400 μg/ml.

After two weeks under selection, independent colonies were picked and transferred to 24-well plates. Confluent cultures were assayed for the presence of PDGF receptor by immunoblot using anti-receptor antibodies. Colonies that were positive by this assay were single-cell cloned by end-limiting dilution.

Stable transfected clones were tested for the expression of the type B PDGF receptor message measured by RNA protection assays and for the presence of PDGF-stimulated receptor protein detected by anti-phosphotyrosine antibodies.

IV. Expression of B-hPDGF-R cDNA in CHO Cells

CHO cells transfected with plasmid DNA containing the human receptor cDNA under the transcriptional control of the SV40 early promoter (CHO-HR5) and CHO cells transfected with a similar plasmid containing the mouse receptor cDNA (CHO-R18) were solubilized as previously described by Escobedo et al. (1988) J. Biol. Chem. 263:1482-1487. Extracts were analyzed by Western blot analysis using an antibody that specifically recognizes sequences in the receptor carboxyl-terminal region as previously described by Escobedo et al. (1988) J. Biol. Chem.; and Keating et al. (1987) J. Biol. Chem. 262:7932-7937.

This Sheet Has Been Replaced



This Sheet Has Been Replaced

The 195 kDa protein is the mature receptor and the 160 kDa protein is the receptor precursor.

The expression of the receptor protein in the transfectants was demonstrated by using antibodies that recognize an intracellular sequence in the receptor. The clone that had the highest level of human receptor expression was chosen for further study. This transfectant had receptors that were labeled with ^{125}I-PDGF as shown by the competitive binding studies described below.

V. Competitive Binding of the Different Forms of PDGF to the Type B Receptor

The ability of the human recombinant AA and BB homodimers to compete for the type B receptor sites and displace ^{125}I-labeled PDGF (prepared as described below) was studied. Each homodimer was produced selectively by a yeast expression system and was purified from yeast media that is devoid of other mesenchymal cell growth factors, thus avoiding the artifact of contamination by factors that might be present in mammalian expression systems.

BALB/c 3T3 cells and CHO transfectants (CHO-HR5) were incubated with ^{125}I-PDGF in the presence of increasing concentrations of AA or BB. Binding was carried out at 37°C for 45 min in whole cell suspension. Unbound, radiolabeled PDGF was removed by centrifugation on a Ficoll gradient Non-specific binding, determined by incubating CHO cells with ^{125}I-PDGF, accounted for 25 percent of the bound radioactivity.

The binding study demonstrated that the transfected cells can be used as a model to study the interaction of hPDGF with its receptor. In particular, this study demonstrated that the transfected type B human receptor was functionally equivalent to the native mouse receptor as indicated by the following results. Both AA and BB forms of PDGF competed for the ^{125}I-PDGF labeled sites in the human receptor transfectants. For the transfected type B human receptor as well as the native mouse receptor, the BB form was of higher affinity than the AA form. When expressed in yeast, the AA form of PDGF may be processed aberrantly, giving it a lower affinity than the BB form for both the transfected cells and mouse 3T3 cells. The

This Sheet Has Been Replaced

consistency of the pattern of competition shows that the AA form interacts with the transfected type B human receptor in the same way as it does with the native mouse receptor and demonstrates that these receptors are functionally equivalent.

VI. Activation of the PDGF Receptor Tyrosine Kinase

The ability of recombinant AA and BB homodimers and of human partially purified AB PDGF to activate the type B receptor tyrosine kinase was studied. The yeast-derived AA and BB homodimeric forms and the platelet-derived AB form stimulated autophosphorylation of the transfected human receptor.

BALB/c 3T3 cells and CHO cells transfected with the human PDGF receptor cDNA (CHO-HR5) were incubated with increasing amounts of the different forms of PDGF (AA, BB and AB). Following polyacrylamide-SDS electrophoresis, the phosphorylated receptor was identified by Western blot using an anti-phosphotyrosine antibody.

The receptor protein co-migrated with the 200 kDa molecular weight marker. The concentration of each form that was effective in stimulating autophosphorylation of the transfected human receptor was equivalent to the concentration that gave a similar autophosphorylation to the native mouse 3T3 receptor or the transfected mouse receptor.

These results showed for the first time that the AA form of PDGF activates the receptor tyrosine kinase of the type B receptor. Prior to use of the transfected cells, there was no demonstration that the AA form had hPDGF activity or that a single receptor, the type B receptor, was capable of recognizing all three forms of PDGF. Further, the results demonstrate that the human cDNA encodes a type B receptor that is functionally equivalent to the wild-type receptor that is responsible for PDGF-stimulated tyrosine kinase activity in mouse 3T3 cells.

Thus, the transfected cells are useful models for studying PDGF-induced mitogenic responses.

VII. Rate of DNA Synthesis in CHO Transfected Cells

This Sheet Has Been Replaced

BALB/c 3T3 cells and CHO cells transfected with the type B human PDGF receptor cDNA (CHO-HR5) were incubated with saturating concentrations of the three forms of PDGF. Untreated cells and cells treated with fetal calf serum (FCS) were used as negative and positive controls, respectively. The level of ^{3}H-thymidine incorporation into DNA was determined by measuring the radioactivity of the acid-precipitable material as previously described.

Transfection of CHO cells with either type B human or mouse PDGF receptor conferred a PDGF-sensitive mitogenic response. All forms of PDGF stimulated DNA synthesis in both the type B human receptor transfectant and the mouse cells bearing the native receptor.

These data showed that the A chain homodimer and the B chain homodimer, like the AB platelet-derived form, were mitogens that can act through the receptor encoded by the type B human cDNA sequence. The mitogenic action of these forms of PDGF on mouse 3T3 cells and CHO cells containing the transfected type B human receptor demonstrate that the responses were mediated by functionally equivalent receptors.

VIII. Isolation and Expression of the Type A PDGF Receptor

The type A receptor was isolated as described for the type B receptor, above, except that different probes were used and hybridization and screening were performed under low stringency conditions, as described below. In particular, a region in the type B receptor tyrosine kinase sequence having a high degree of homology to published tyrosine kinase amino acid sequences was identified and had the amino acid sequence, HRDLAARN. Oligonucleotide probes encoding the tyrosine kinase consensus sequence were prepared having the following sequences:

GTT(G/C)CGXGCXGCCAGXTC(G/C)CGXTG,

where G/C indicates either G or C was used and X indicates any of A, T, C or G was used. The human placenta λGT10 cDNA library was screened as described above but with low stringency conditions using a buffer with 6X SSC 0.1% SDS and 5X Denhardt's solution at 42°C as follows. Filters were screened

This Sheet Has Been Replaced

by washing at 52°C in 2X SSC. A clone encoding the type A receptor was isolated and sequenced by the procedure described for the type B receptor gene.

The DNA sequence of the gene encoding the type A receptor (A-hPDGF-R) together with the deduced amino acid sequence are shown in Table 3, above.

The clone encoding A-hPDGF-R was digested, gel purified and inserted into the SV40 expression vector, pSV7C, as described for the type B receptor clone.

That vector is used to transfect CHO cells as described above for the type B receptor. With expression of the vector coding sequence, transfected CHO cells produce a functional receptor that binds all three hPDGF forms, preferentially binding the AA homodimer.

IX. Extracellular Murine PDGF-R Fragments Construction of pSV-SRX1d Expression Vector and Transfection of DUKX-B11 Cells With a Murine Extracellular Region

To express the secreted extracellular region (XR) of PDGF type B receptor (type B PDGF-R), a cDNA clone of the murine PDGF-R was mutagenized to introduce a Stu I restriction site between codons 500 and 501, changing codon 500 from valine to arginine. The 1.7 kb EcoRI-Stu I fragment was inserted into pIBI-25 (IBI) which added an in-frame proline codon followed by a stop codon at the C-terminus. The 1.7 kb Eco RI-Xba I fragment was transferred to pSV-7DHFR in which the XR expression was driven by the SV-40 early promoter and the amplifiable marker, a dihydrofolate reductase (dhfr) transcriptional unit, was driven by an Adenovirus major late promoter. The complete plasmid encodes the signal sequence and the first 499 amino acids (the extracellular region) of the PDGF-R, followed by arginine, proline and stop codons.

The cDNA was expressed in dhfr-deficient CHO mutant cells, DUKX-B11. DUKX-B11 cells were transfected to 10-cm tissue culture plates with 5 μg of pSV-SRX1d plasmid and selected for expression with nucleoside-free MEM-α (Gibco) supplemented with 10% dialyzed calf serum, 200 μg/ml proline, 100 U/ml penicillin, and 100 μg/ml streptomycin. Colonies were

picked and screened by analyzing the conditioned media by SDS-PAGE and Western blotting with a polyclonal PDGF-R antibody directed against the extracellular region of the receptor (Ab77).

Amplification of murine extracellular region expression by methotrexate

Positive transfectants were treated sequentially with increasing concentrations of methotrexate starting at 1, 2, 5, and 10 nM. Colonies from the highest concentrations were then treated with 10 fold higher concentrations of methotrexate (20, 50, and 100 nM). Colonies from the highest concentrations were again picked and treated with increasing concentrations of methotrexate. The conditioned media from colonies at each stage were analyzed by SDS-PAGE and Western blotting with an anti-PDGF-R Ab (Ab77) for expression of the extracellular region protein. Methotrexate-resistant (5 μM) cells that secreted highest amount of the extracellular region protein were named DPXR (DUK-PDGF-R extracellular region) cells.

Enzymatic treatments of the extracellular region protein with O-glycanase (Genzyme Co.) and N-glycanase (Genzyme Co.) were performed. Briefly, extracellular region protein partially purified with wheat germ agglutinin (WGA) affinity chromatography (50 nM, in 20 mM sodium phosphate, pH 7.4) was incubated with N-glycanase (10 U/ml) overnight at 37°C and then with O-glycanase (2 mU/ml) for an additional 2 h. The sample was then boiled for 5 min and analyzed with SDS-PAGE and Western blotting with Ab77.

Conditioned media

DPXR cells at 95% confluence were washed twice with serum-free DME H21 medium. Four ml of DME H21 medium supplemented with 10% protein-free Serum Substitute (UCSF Cell Culture Facility) was added to each 10-cm culture plate. Conditioned media containing murine extracellular region polypeptide which were used for various assays were collected from DPXR cells over 48 h unless otherwise mentioned.

This Sheet Has Been Replaced



This Sheet Has Been Replaced



Preparation of ^{125}I-BB-PDGF

BB-PDGF (gift from Chiron Corporation) was iodinated. Briefly, 2.5 µg of BB-PDGF were rotary-evaporated in a silanized polypropylene tube. BB-PDGF was resuspended in 10 µl of 0.1 M sodium borate (pH 8.5). ^{125}I-mono-iodo Bolton Hunter reagent (500 µCi; Amersham) was dried under nitrogen. The BB-PDGF was added to the dry Bolton Hunter reagent and incubated for 15 min at 4°C. Fifty µl of quench solution (0.1 M sodium borate, 0.2 M glycine, pH 8.5) was added to the reaction mixture, which was incubated for an additional 10 min at 4°C. The entire reaction mixture was loaded on a PD-10 column (Pharmacia) equilibrated with 0.1 M acetic acid containing 1 mg/ml BSA (ICN Biochemicals), and eluted with the same buffer.

X. Binding Assays

Of relevance to the production of soluble fragments of PDGF receptor polypeptides, and assays for their use, are techniques and results reported in Kimball and Warren (1984) Biochim. Biophys. Acta 771:82-88; van der Schaal et al. (1984) Anal. Biochem. 140:48-55; van Driel et al. (1989) J. Biol. Chem. 264:9533-9538; Heldin et al. (1988) EMBO J. 7:1387-1393; Williams et al. (1982) Proc. Nat'l Acad. Sci. USA 79:5867-5870; Williams et al. (1984) J. Biol. Chem. 259:5287-5294; and particularly, Orchansky et al. (1988) "Phosphatidylinositol Linkage of a Truncated Form of the Platelet-derived Growth Factor Receptor" J. Biol. Chem. 263:15159-15165; each of which is hereby incorporated herein by reference. In particular, the Orchansky publication provides evidence that a whole extracellular region of a PDGF receptor, when separated from the TM and intracellular regions, still is capable of binding to PDGF ligands.

In whole cell binding assays, 2×10^4 R18 cells (PDGF type B receptor transfectant CHO cells) detached with PBS/EDTA (2 mM) were incubated with 10 µl of platelet-poor plasma, and PBS/Hepes (25 mM final, pH 7.4). Conditioned media, 12.5 µl to 200 µl, from DPXR cells (collected as described above), which contain the extracellular region protein, were added to the

binding mixtures in a final volume of 250 µl to form 20 to 1.25 fold dilutions. The mixtures were shaken overnight at 4°C and spun through 750 µl of Ficoll gradient (28.5% Ficoll-Paque (Pharmacia) in PBS) at 4°C. The supernatants were aspirated and the radioactivity in the cell pellets was determined with a gamma-counter.

To measure its affinity for BB-PDGF, extracellular region protein was immobilized by adsorption onto a plastic surface. 50 µl of WGA affinity chromatography purified extracellular region (approximately 80 nM, estimated by silver straining) was diluted in 10 ml of 25 mM Tris Cl, 75 mM NaCl, 20 mM NH_4HCO_3, pH 7.5, and 100 µl aliquots were plated in each well of 96-well ELISA microtiter plates (Dynatech Products Co.). After overnight incubation at 4°C, the plate was washed once and blocked with 0.5% gelatin in 100 mM NaCl, 25 mM Hepes, pH 7.35, for 3 h at 4°C. The plate was then washed twice with binding buffer (0.3% gelatin, 100 mM NaCl, 25 mM Hepes, pH 7.35). ^{125}I-BB-PDGF and/or unlabeled BB-PDGF were added to the wells in a final volume of 100 µl and the plate was incubated at 4°C for 16 h for steady state binding. The plate was washed three times again with binding buffer and stripped with 200 µl of 1% SDS, 0.5% BSA for counting in a gamma-counter.

Crosslinking experiments

Extracellular region polypeptide partially purified by WGA chromatography (25 µl, ~50 nM) was incubated with 0.2 µCi of 125I-BB-PDGF (2 nM final concentration) and various concentrations of unlabeled BB-PDGF in a final volume of 100 µl at 4°C for 3 h. The extracellular region protein was then crosslinked to the ligand by 1 mM bis(sulfosuccinimidyl) suberate (BS^3) (cat. #21579, Pierce Chemicals, Rockford, Illinois) at room temperature for 30 min. The reaction was stopped by 25 mM Tris buffer, pH 7.4, for 5 min. The extracellular region protein was then immunoprecipitated with a receptor antibody (Ab77) and analyzed by SDS-PAGE.

Autophosphorylation assay

This Sheet Has Been Replaced



This Sheet Has Been Replaced

Conditioned media containing the extracellular region protein (1 ml) were incubated with BB-PDGF for 3 h at 4°C. The mixtures were then added to the 6-well Falcon tissue culture plates (1 ml/well) which contained monolayers of quiescent human PDGF A-receptor transfectant CHO cells or BALB/c 3T3 cells. The cells were incubated with the mixtures for 10 min at 37°C and then washed with serum-free DME medium and lysed with Ripand lysis buffer. Cell lysates were analyzed by SDS-PAGE and Western blotting with an anti-phosphotyrosine mAb.

Mitogenesis assay

BALB/c 3T3 cells were plated in 96-well tissue culture plates for 3 days and made quiescent with Q-media (DMEM with 1 mg/ml insulin, 2 µg/ml transferrin and 0.5 mg/ml BSA) for 24 h. Conditioned media containing the extracellular region protein was diluted in 2-fold serial dilutions with fresh DME H21 medium, preincubated with 2 nM BB-PDGF for 3 h at 4°C and then added to BALB/c 3T3 cells (200 µl/well). The cells were incubated for 18 h at 37°C. One µCi [^{3}H]-thymidine (in 50 µl) was then added to each well for 4 h. The cells were washed twice with cold PBS and fixed with cold trichloracetic acid (TCA, 5%). Precipitated cell debris were then washed extensively with cold TCA (5%) and dissolved in 0.25 N NaOH for scintillation counting.

XI. Human Extracellular Region

Equivalent techniques for construction, expression, and determination of the physiological effect of truncation or deletion analogues of the soluble extracellular receptor fragments from the human receptor may be performed using the nucleic acid, polypeptide, and other reagents provided herein.

Human Deletion and Truncation Constructs

PDGF-R CONSTRUCTS

The 3.9 kb EcoRI-Hind III cDNA fragment of the human type B hPDGF-R was subcloned into the EcoRI-Hind III site of M13 Mp18 to produce a vector Mp18PR. For techniques, see

Maniatis et al., Molecular Cloning: A Laboratory Manual (1982), Cold Spring Harbor, N.Y., which is incorporated herein by reference. Verification of subcloning was performed by restriction enzyme digestion analysis and dideoxy chain termination sequencing, as described by Sanger et al. (1977) Proc. Nat'l Acad. Sci. USA 74:5463. Oligonucleotide directed in vitro mutagenesis was performed according to the method described by Kunkel et al. (1987) Methods in Enzymol., 154:367. The strategy for oligonucleotide directed in vitro deletion mutagenesis of Mp18PR is outlined in Fig. 8. In brief, an oligonucleotide was designed to create a soluble type B hPDGF receptor extracellular region by deletion mutagenesis. A mutagenic oligonucleotide aligned with the appropriate region of the human PDGF receptor can be used to generate deletions. The antisense strand was used for mutagenesis throughout. Mutagenesis of PΔ1 utilized Mp18PR as the template. PΔ1, a 41 bp oligomer, introduced a TAG stop codon after Lysine$_{499}$ (K_{499}) of D5 and removed the transmembrane (TM) as well as the entire intracellular kinase domain (K), producing an Mp18 PΔ1. PΔ1 codes for 530_{aa} 148_{aa} precursor proteins.

The human PDGF receptor constructs were subsequently subcloned into the EcoRI-Hind III site of pBJ1 a derivation of pCDL-SRα296, as described in Takabe et al. (1988) Molec. Cell Biol. 8:466, and co-transfected with pSV2NEO, as described by Southern and Berg (1982) J. Mol. Appl. Gen., 1: 327, into Chinese hamster ovary cells (CHO).

Function of the construct was demonstrated as follows:

A sample of 0.33 nM PDGF BB ligand is preincubated for 1 hr at 4°C under the following conditions:

1. a polyclonal antibody to human PDGF (this antibody recognizes human PDGF AA, PDGF BB and PDGF AB);
2. 18 nM (60 fold molar excess to PDGF BB) human type B PDGF receptor;
3. phosphate buffered saline solution that the receptor and antibody are in; or
4. no additions but the ligand itself.

This Sheet Has Been Replaced



This Sheet Has Been Replaced

In a duplicate set of experiments, 0.33 nM PDGF AA is incubated with three of the above preincubation conditions, e.g., 2, 3, and 4 above. The human type B PDGF receptor does not appreciably recognize PDGF AA but this ligand will still activate cell-associated human type A PDGF receptor from NIH3T3 cells and so is a control for human type B PDGF receptor specificity and PDGF BB-dependent activation versus non-specific general cellular effect, e.g., cytotoxicity.

The preincubated materials were in a final volume of 0.5 ml. They were placed in one well each of a six well tissue culture dish containing a confluent layer of serum starved (quiescent) NIH3T3 cells which were chilled to 4°C. The cells and incubation mixtures were agitated, e.g., rocked, at 4°C for 2 h. They were then washed twice with 4°C phosphate buffered saline. Forty μl of 125 mM Tris(hydroxymethyl)amino methane (Tris), pH 6.8, 20% (v/v) glycerol, 2% (w/v) sodium dodecyl sulfate (SDS), 2% (v/v) 2-mercaptoethanol, and 0.001% bromphenol blue, (known as SDS sample buffer), was added per microtiter well followed by 40 μl of 100 mM Tris, pH 8.0, 30 mM sodium pyrosphoshate, 50 mM sodium fluoride, 5 mM ethylenediaminetetraacetic acid (EDTA), 5 mM ethylenebis(oxyethylenenitrilio)tetraacetic acid, 1% (w/v) SDS, 100 mM dithiothreitol, 2 mM phenylmethylsulfonylfluoride (PMSF), and 200 μM sodium vanadate was added to the cells. The cells were solubilized and 40 μl additional SDS sample buffer was added to the solubilizate. This material was boiled 5 minutes and loaded onto a single gel sample well of a 7.5% sodium dodecyl sulfate polyacrylamide gel. Cellular proteins were separated by electrophoresis.

The separated proteins were transferred to nitrocellulose by electrotransfer and the resulting "Western blot" was incubated with 3 changes of 0.5% (w/v) sodium chloride, 5 mg/ml bovine serum albumin, 50 mM Tris, pH 7.5, (designated blocking buffer) for 20 minutes each at room temperature. A 1/1000 dilution of PY20 (a commercially available monoclonal antibody to phosphotyrosine [ICN]) in blocking buffer was incubated with the blot overnight at 4°C. The blot was washed 3 times for 20 minutes each at room

This Sheet Has Been Replaced

temperature in blocking buffer. The blot was incubated with 4 μCi/40 ml of ^{125}I-Protein A [Amersham] in blocking buffer for 1 hour at room temperature and washed 3 times for 20 minutes each at room temperature in blocking buffer. The blot was exposed to X-ray film for 48 h with one intensifying screen at -70°C and developed with standard reagents.

XII. PDGF Plate Assay

Polystyrene microtiter plates (Immulon, Dynatech Laboratories) were coated with the extracellular region fragment of the type B human PDGF receptor (described above) by incubating approximately 10-100 ng of this protein per well in 100 μl of 25 mM Tris, 75 mM NaCl, pH 7.35 for 12 to 18 h at 4°C. The protein was expressed in transfected CHO cells and collected in serum-free media (Gibco MEMα) at a concentration of 0.2 - 1 μg/ml, with a total protein concentration of 150 - 300 μg/ml.

The human type B PDGF receptor extracellular region fragment was concentrated and partially purified by passing the media over wheat germ-agglutinin-sepharose at 4°C (at 48 ml/h) in the presence of 1 mM PMSF. After extensive washing, the protein was eluted in 0.3 M N-acetyl-glucosamine, 25 mM Hepes, 100 mM NaCl, 1 mM PMSF, pH 7.4. This fraction was then applied to Sephacryl S-200 HR (Pharmacia) equilibrated in 0.15 M ammonium bicarbonate pH 7.9. The fractions containing receptor (3 - 10 ng/μl) were detected by SDS-PAGE and Western blotting with a polyclonal rabbit antibody against a peptide from the receptor external region. These fractions (3 - 10 ng/μl) were used to coat the microtiter wells as described above. The wells were then drained, rinsed once with 200 μl each of 0.5% gelatin (Bio-Rad, EIA grade), 25 mM Hepes, 100 mM NaCl, pH 7.4, and incubated for 3 h at 4°C with 150 μl of this same solution. The wells were drained and rinsed twice with 0.3% gelatin, 25 mM Hepes, 100 mM NaCl, pH 7.4 (150 μl each). The plate was put on ice and 90 μl of the 0.3% gelatin solution was put in each well (wells used to test nonspecific binding received just 80 μl and then 10 μl of 0.01 mg/ml non-labeled PDGF in the 0.3% gelatin solution). PDGF BB (Amgen) was iodinated at 4°C to

This Sheet Has Been Replaced

52,000 CPM/ng with di-iodo Bolton-Hunter reagent (Amersham) and approximately 40,000 CPM was added per well in 10 µl, containing 0.024% BSA, 0.4% gelatin, 20 mM Hepes, 80 mM NaCl, 70 mM acetic acid, pH 7.4. The plate was incubated for 4 h at 4°C, after which wells were washed three times with 150 µl each with 0.3% gelatin, 25 mM Hepes, 100 mM NaCl, pH 7.4 at 4°C. The bound radioactivity remaining was solubilized from the wells in 200 µl 1% SDS, 0.05% BSA, and counted in a gamma-counter. The nonspecific binding was determined in the presence of a 150-fold excess of unlabeled PDGF BB (Amgen) and was about 7% of the total bound ^{125}I-PDGF.

These studies were made possible by the availability of growth factor preparations devoid of contamination with other growth factors and by the use of a receptor expression system in which all of the measured PDGF responses could be attributed to this single transfected receptor cDNA.

XIII. Intracellular Region

Cell Culture and recombinant baculovirus.

BALB c/3T3 cells clone A31 from C.D. Scher, Children's Hospital of Philadelphia, PA., were cultured in Dulbecco Modified Eagle's medium (DMEM) supplemented with 10% bovine serum and penicillin and streptomycin (50 µg/ml each). Spodoptera frugiperda (Sf9) cells (from M. Summers, Texas A&M, TX) were grown in Grace's medium supplemented with 10% fetal bovine serum, 3.3 g/l yeastolate, 3.3 g/l lactalbumin hydrolysate, and penicillin and streptomycin 50 µg/ml each. Recombinant type B PDGF receptor and PDGF type B receptor ΔKI mutant baculovirus vectors were prepared by standard procedures. Recombinant baculovirus was collected from supernatant of Sf9 cells 48-60 hours after infection at a multiplicity of infection (MOI) of 1.

Antibodies, mitogens, and peptides

A type B PDGF receptor antibody (Ab77) directed against a synthetic peptide (amino acids 425-446) located at the extracellular region was used. PLC-γ monoclonal antibody was kindly provided by S.G. Rhee, NIH, Bethesda, MD, and GAP

This Sheet Has Been Replaced

antibodies were provided by F. McCormick, Cetus Corp., Emeryville, California. Recombinant BB-PDGF was provided by C.G. Nascimento from Chiron Corp. Emeryville, California. Peptides used in the association experiments were prepared by conventional peptide synthesis using phosphorylated tyrosine (N-Boc-Tyr-O-[PO_3Bzl_2], Peninsula Laboratory, Belmont, California) for the synthesis of tyrosine phosphorylated peptides.

Association Assays

In vivo associations were performed according to Morrison et al. (1989) Cell 58:649-657. Serum starved cultures of BALB c/3T3 cells (10^7 cells) were incubated in the presence or absence of 2 nM PDGF. Cell lysates were immunoprecipitated by using receptor antibody or phosphotyrosine antibodies. Immune complexes were used assayed for the presence of PI3 kinase activity and to determine the protein that associated with the receptor. In vitro association experiments were performed according to Morrison et al (1989) or Morrison et al. (1990) Mol. Cell. Biol. 10:2359-2366. Typically, baculovirus-expressed type B PDGF receptor was collected from Sf9 cells 48-60 hours after infection (MOI:10) by immunoprecipitation using receptor antibodies. Infected cells were washed twice with cold PBS and lysed in 1 ml of lysis buffer (1% NP-40, 20 mM Tris (pH 8.0), 173 mM NaCl, 10% glycerol, 2 mM EDTA, 1 mM phenylmethylsulfonylfluoride (PMSF), aprotinin (0.15 U/ml), 20 μM leupeptin, 1 mM sodium orthovanadate) at 4°C for 20 min with rocking. In most experiments (except where indicated in the legend of Fig. 3) the immunoprecipitated receptor was autophosphorylated in vitro by incubating the immune complexes in a buffer containing 20 mM Tris (pH 7.5), 20 mM MnCl, 100 μM ATP for 15 min at 25°C. Lysates were cleared of insoluble material by centrifugation at 13,000 x g for 10 min. Lysates were incubated with receptor antibodies (1:500 dilution) for 4 h at 4°C. Receptor-antibody complexes were precipitated using Protein-A sepharose (Sigma) and washed consecutively with RIPA buffer (lysis buffer with 0.1% SDS), wash buffer 1 [0.5% NP-40, 0.5 M LiCl, 50 mM Tris (pH 7.4)] and with 10 mM Tris (pH

7.4). The PI3 kinase association assay was performed by incubating the immobilized receptor with BALB c/3T3 cell lysates for 3 hours at 4°C. The immune complexes were consecutively washed with cold PBS; 0.5% NP-40, 0.5 M LiCl and 50 mM Tris (pH 7.4). In the experiment using peptides the BALB c/3T3 lysates were preincubated with the peptides (50 μM) for 30 min at 4°C prior to the incubation with the PDGF receptor protein expressed in the insect cell system.

In vitro kinase and PI3 kinase assays

In vitro protein kinase assays were performed by incubation of the immunoprecipitates in protein kinase buffer (30 mM Tris (pH 7.4), 10 mM $MnCl_2$) and 10 μCi [γ-^{32}P]-ATP 3,000 Ci/mmol, at 25°C for 15 min. The reaction was terminated by adding 4x Laemmli loading gel buffer. Samples were analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography. PI3 kinase activity was assayed as described by Kaplan et al. (1986) Proc. Nat'l Acad. Sci. USA 83:362-364. Immune complexes were incubated in PI3 kinase buffer (30 mM Hepes (pH 7.4), 30 mM $MgCl_2$, 200 μM adenosine, 40 μM ATP), 0.2 mg/ml of sonicated phosphoinositol (PI), and 10 μCi [γ-^{32}P]-ATP (3,000 Ci/mmol) at 25°C for 10 minutes. Adenosine was added in the PI3 kinase assays to inhibit any contaminating PI4 kinase activity. Reactions were terminated by the addition of 100 μl of HCl acid. The products of the reaction were extracted with chloroform and separated by thin layer chromatography. The conversion of PI into PIP was determined by exposing the TLC plate to an X-ray film for 2-3 h.

Receptor dephosphorylation

Receptor immunoprecipitates were incubated for 30 minutes at 30°C with 4 μg of potato acid phosphatase in the presence or absence of 1 mM sodium orthovanadate, see Morrison et al. (1989). After RAP treatment the immunoprecipitates were washed three times with RIPA buffer containing 1 mM sodium orthovanadate prior to the incubation with the 3T3 cell lysate.

This Sheet Has Been Replaced



"Blotting" of 85 kD protein with PDGF receptor probe

Cell lysate proteins transferred to nitrocellulose membranes were analyzed for their ability to bind directly to the PDGF receptor by probing the nitrocellulose membrane with ^{32}P-labeled PDGF receptor. To prepare the radiolabeled receptor probe, baculovirus-expressed receptor was immunoprecipitated from 10^6 Sf9 cells. The immune complexes were washed and labeled by autophosphorylation as described above. Labeled receptor was solubilized from the immunoprecipitates by repeated incubation of the immunoprecipitate in solubilization buffer (0.4% SDS, 100 mM NaCl, 2 mM EDTA, 2 mM β-mercaptoethanol, and 50 mM triethanolamine, pH 7.4) at 100°C for 2 min. The extracts were pooled, diluted in solubilization buffer without β-mercaptoethanol and brought to 10 mM iodoacetamide and 1% Triton X-100. Analysis of this material by SDS-polyacrylamide electrophoresis followed by radioautography revealed the presence of only the receptor band. BALB c/3T3 cells lysates were prepared and run on SDS-PAGE. Proteins were transferred onto nitrocellulose filter by electroblotting in the absence of SDS. Filters were incubated with radiolabeled PDGF receptor for 12 h at 4°C, and washed in the solubilization buffer containing 2% Triton X-100 without β-mercaptoethanol. For the peptide competition experiments the filters were incubated as described above with the ^{32}P-Labeled receptor probe and 2.5 μM of the indicated peptide.

All publications and patent applications herein are incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference. The invention now being fully described, it will be apparent to one of ordinary skill in the art that many changes and modifications can be made thereto without departing from the spirit or scope of the appended claims.

This Sheet Has Been Replaced



SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Williams, Lewis T.
Escobedo, Jaime A.

(ii) TITLE OF INVENTION: PLATELET-DERIVED GROWTH FACTOR RECEPTORS

(iii) NUMBER OF SEQUENCES: 5

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: TOWNSEND and TOWNSEND
(B) STREET: Steuart Street Tower, 20th Floor
(C) CITY: San Francisco
(D) STATE: California
(E) COUNTRY: US
(F) ZIP: 94105

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.24

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US 07/151,141
(B) FILING DATE: 02-FEB-1988

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US 07/309,322
(B) FILING DATE: 10-FEB-1989

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Ching, Edwin P.
(B) REGISTRATION NUMBER: 34,090
(C) REFERENCE/DOCKET NUMBER: 2307U-267-2

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: (415) 326-2400
(B) TELEFAX: (415) 326-2422

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5427 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA to mRNA

(iii) HYPOTHETICAL: N

(iv) ANTI-SENSE: N

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Homo sapiens
(B) STRAIN: lambda gt10

This Sheet Has Been Replaced



(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 187..3504
(D) OTHER INFORMATION:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

```
TGTTCTCCTG AGCCTTCAGG AGCCTGCACC AGTCCTGCCT GTCCTTCTAC TCAGCTGTTA      60

CCCACTCTGG GACCAGCAGT CTTTCTGATA ACTGGGAGAG GGCAGTAAGG AGGACTTCCT     120

GGAGGGGGTG ACTGTCCAGA GCCTGGAACT GTGCCCACAC CAGAAGCCAT CAGCAGCAAG     180

GACACC ATG CGG CTT CCG GGT GCG ATG CCA GCT CTG GCC CTC AAA GGC         228
       Met Arg Leu Pro Gly Ala Met Pro Ala Leu Ala Leu Lys Gly
         1               5                  10

GAG CTG CTG TTG CTG TCT CTC CTG TTA CTT CTG GAA CCA CAG ATC TCT       276
Glu Leu Leu Leu Leu Ser Leu Leu Leu Leu Glu Pro Gln Ile Ser
 15                  20                  25                  30

CAG GGC CTG GTC GTC ACA CCC CCG GGG CCA GAG CTT GTC CTC AAT GTC       324
Gln Gly Leu Val Val Thr Pro Pro Gly Pro Glu Leu Val Leu Asn Val
                 35                  40                  45

TCC AGC ACC TTC GTT CTG ACC TGC TCG GGT TCA GCT CCG GTG GTG TGG       372
Ser Ser Thr Phe Val Leu Thr Cys Ser Gly Ser Ala Pro Val Val Trp
             50                  55                  60

GAA CGG ATG TCC CAG GAG CCC CCA CAG GAA ATG GCC AAG GCC CAG GAT       420
Glu Arg Met Ser Gln Glu Pro Pro Gln Glu Met Ala Lys Ala Gln Asp
         65                  70                  75

GGC ACC TTC TCC AGC GTG CTC ACA CTG ACC AAC CTC ACT GGG CTA GAC       468
Gly Thr Phe Ser Ser Val Leu Thr Leu Thr Asn Leu Thr Gly Leu Asp
     80                  85                  90

ACG GGA GAA TAC TTT TGC ACC CAC AAT GAC TCC CGT GGA CTG GAG ACC       516
Thr Gly Glu Tyr Phe Cys Thr His Asn Asp Ser Arg Gly Leu Glu Thr
 95                 100                 105                 110

GAT GAG CGG AAA CGG CTC TAC ATC TTT GTG CCA GAT CCC ACC GTG GGC       564
Asp Glu Arg Lys Arg Leu Tyr Ile Phe Val Pro Asp Pro Thr Val Gly
                115                 120                 125

TTC CTC CCT AAT GAT GCC GAG GAA CTA TTC ATC TTT CTC ACG GAA ATA       612
Phe Leu Pro Asn Asp Ala Glu Glu Leu Phe Ile Phe Leu Thr Glu Ile
            130                 135                 140

ACT GAG ATC ACC ATT CCA TGC CGA GTA ACA GAC CCA CAG CTG GTG GTG       660
Thr Glu Ile Thr Ile Pro Cys Arg Val Thr Asp Pro Gln Leu Val Val
        145                 150                 155

ACA CTG CAC GAG AAG AAA GGG GAC GTT GCA CTG CCT GTC CCC TAT GAT       708
Thr Leu His Glu Lys Lys Gly Asp Val Ala Leu Pro Val Pro Tyr Asp
    160                 165                 170

CAC CAA CGT GGC TTT TCT GGT ATC TTT GAG GAC AGA AGC TAC ATC TGC       756
His Gln Arg Gly Phe Ser Gly Ile Phe Glu Asp Arg Ser Tyr Ile Cys
175                 180                 185                 190

AAA ACC ACC ATT GGG GAC AGG GAG GTG GAT TCT GAT GCC TAC TAT GTC       804
Lys Thr Thr Ile Gly Asp Arg Glu Val Asp Ser Asp Ala Tyr Tyr Val
                195                 200                 205
```

This Sheet Has Been Replaced



```
TAC AGA CTC CAG GTG TCA TCC ATC AAC GTC TCT GTG AAC GCA GTG CAG        852
Tyr Arg Leu Gln Val Ser Ser Ile Asn Val Ser Val Asn Ala Val Gln
                210                 215                 220

ACT GTG GTC CGC CAG GGT GAG AAC ATC ACC CTC ATG TGC ATT GTG ATC        900
Thr Val Val Arg Gln Gly Glu Asn Ile Thr Leu Met Cys Ile Val Ile
        225                 230                 235

GGG AAT GAT GTG GTC AAC TTC GAG TGG ACA TAC CCC CGC AAA GAA AGT        948
Gly Asn Asp Val Val Asn Phe Glu Trp Thr Tyr Pro Arg Lys Glu Ser
    240                 245                 250

GGG CGG CTG GTG GAG CCG GTG ACT GAC TTC CTC TTG GAT ATG CCT TAC        996
Gly Arg Leu Val Glu Pro Val Thr Asp Phe Leu Leu Asp Met Pro Tyr
255                 260                 265                 270

CAC ATC CGC TCC ATC CTG CAC ATC CCC AGT GCC GAG TTA GAA GAC TCG       1044
His Ile Arg Ser Ile Leu His Ile Pro Ser Ala Glu Leu Glu Asp Ser
                275                 280                 285

GGG ACC TAC ACC TGC AAT GTG ACG GAG AGT GTG AAT GAC CAT CAG GAT       1092
Gly Thr Tyr Thr Cys Asn Val Thr Glu Ser Val Asn Asp His Gln Asp
            290                 295                 300

GAA AAG GCC ATC AAC ATC ACC GTG GTT GAG AGC GGC TAC GTG CGG CTC       1140
Glu Lys Ala Ile Asn Ile Thr Val Val Glu Ser Gly Tyr Val Arg Leu
        305                 310                 315

CTG GGA GAG GTG GGC ACA CTA CAA TTT GCT GAG CTG CAT CGG AGC CGG       1188
Leu Gly Glu Val Gly Thr Leu Gln Phe Ala Glu Leu His Arg Ser Arg
    320                 325                 330

ACA CTG CAG GTA GTG TTC GAG GCC TAC CCA CCG CCC ACT GTC CTG TGG       1236
Thr Leu Gln Val Val Phe Glu Ala Tyr Pro Pro Pro Thr Val Leu Trp
335                 340                 345                 350

TTC AAA GAC AAC CGC ACC CTG GGC GAC TCC AGC GCT GGC GAA ATC GCC       1284
Phe Lys Asp Asn Arg Thr Leu Gly Asp Ser Ser Ala Gly Glu Ile Ala
                355                 360                 365

CTG TCC ACG CGC AAC GTG TCG GAG ACC CGG TAT GTG TCA GAG CTG ACA       1332
Leu Ser Thr Arg Asn Val Ser Glu Thr Arg Tyr Val Ser Glu Leu Thr
            370                 375                 380

CTG GTT CGC GTG AAG GTG GCA GAG GCT GGC CAC TAC ACC ATG CGG GCC       1380
Leu Val Arg Val Lys Val Ala Glu Ala Gly His Tyr Thr Met Arg Ala
        385                 390                 395

TTC CAT GAG GAT GCT GAG GTC CAG CTC TCC TTC CAG CTA CAG ATC AAT       1428
Phe His Glu Asp Ala Glu Val Gln Leu Ser Phe Gln Leu Gln Ile Asn
    400                 405                 410

GTC CCT GTC CGA GTG CTG GAG CTA AGT GAG AGC CAC CCT GAC AGT GGG       1476
Val Pro Val Arg Val Leu Glu Leu Ser Glu Ser His Pro Asp Ser Gly
415                 420                 425                 430

GAA CAG ACA GTC CGC TGT CGT GGC CGG GGC ATG CCG CAG CCG AAC ATC       1524
Glu Gln Thr Val Arg Cys Arg Gly Arg Gly Met Pro Gln Pro Asn Ile
                435                 440                 445

ATC TGG TCT GCC TGC AGA GAC CTC AAA AGG TGT CCA CGT GAG CTG CCG       1572
Ile Trp Ser Ala Cys Arg Asp Leu Lys Arg Cys Pro Arg Glu Leu Pro
            450                 455                 460

CCC ACG CTG CTG GGG AAC AGT TCC GAA GAG GAG AGC CAG CTG GAG ACT       1620
```

This Sheet Has Been Replaced



```
Pro Thr Leu Leu Gly Asn Ser Ser Glu Glu Glu Ser Gln Leu Glu Thr
        465                 470                 475

AAC GTG ACG TAC TGG GAG GAG GAG CAG GAG TTT GAG GTG GTG AGC ACA      1668
Asn Val Thr Tyr Trp Glu Glu Glu Gln Glu Phe Glu Val Val Ser Thr
    480                 485                 490

CTG CGT CTG CAG CAC GTG GAT CGG CCA CTG TCG GTG CGC TGC ACG CTG      1716
Leu Arg Leu Gln His Val Asp Arg Pro Leu Ser Val Arg Cys Thr Leu
495                 500                 505                 510

CGC AAC GCT GTG GGC CAG GAC ACG CAG GAG GTC ATC GTG GTG CCA CAC      1764
Arg Asn Ala Val Gly Gln Asp Thr Gln Glu Val Ile Val Val Pro His
                515                 520                 525

TCC TTG CCC TTT AAG GTG GTG GTG ATC TCA GCC ATC CTG GCC CTG GTG      1812
Ser Leu Pro Phe Lys Val Val Val Ile Ser Ala Ile Leu Ala Leu Val
            530                 535                 540

GTG CTC ACC ATC ATC TCC CTT ATC ATC CTC ATC ATG CTT TGG CAG AAG      1860
Val Leu Thr Ile Ile Ser Leu Ile Ile Leu Ile Met Leu Trp Gln Lys
        545                 550                 555

AAG CCA CGT TAC GAG ATC CGA TGG AAG GTG ATT GAG TCT GTG AGC TCT      1908
Lys Pro Arg Tyr Glu Ile Arg Trp Lys Val Ile Glu Ser Val Ser Ser
    560                 565                 570

GAC GGC CAT GAG TAC ATC TAC GTG GAC CCC ATG CAG CTG CCC TAT GAC      1956
Asp Gly His Glu Tyr Ile Tyr Val Asp Pro Met Gln Leu Pro Tyr Asp
575                 580                 585                 590

TCC ACG TGG GAG CTG CCG CGG GAC CAG CTT GTG CTG GGA CGC ACC CTC      2004
Ser Thr Trp Glu Leu Pro Arg Asp Gln Leu Val Leu Gly Arg Thr Leu
                595                 600                 605

GGC TCT GGG GCC TTT GGG CAG GTG GTG GAG GCC ACA GCT CAT GGT CTG      2052
Gly Ser Gly Ala Phe Gly Gln Val Val Glu Ala Thr Ala His Gly Leu
            610                 615                 620

AGC CAT TCT CAG GCC ACG ATG AAA GTG GCC GTC AAG ATG CTT AAA TCC      2100
Ser His Ser Gln Ala Thr Met Lys Val Ala Val Lys Met Leu Lys Ser
        625                 630                 635

ACA GCC CGC AGC AGT GAG AAG CAA GCC CTT ATG TCG GAG CTG AAG ATC      2148
Thr Ala Arg Ser Ser Glu Lys Gln Ala Leu Met Ser Glu Leu Lys Ile
    640                 645                 650

ATG AGT CAC CTT GGG CCC CAC CTG AAC GTG GTC AAC CTG TTG GGG GCC      2196
Met Ser His Leu Gly Pro His Leu Asn Val Val Asn Leu Leu Gly Ala
655                 660                 665                 670

TGC ACC AAA GGA GGA CCC ATC TAT ATC ATC ACT GAG TAC TGC CGC TAC      2244
Cys Thr Lys Gly Gly Pro Ile Tyr Ile Ile Thr Glu Tyr Cys Arg Tyr
                675                 680                 685

GGA GAC CTG GTG GAC TAC CTG CAC CGC AAC AAA CAC ACC TTC CTG CAG      2292
Gly Asp Leu Val Asp Tyr Leu His Arg Asn Lys His Thr Phe Leu Gln
            690                 695                 700

CAC CAC TCC GAC AAG CGC CGC CCG CCC AGC GCG GAG CTC TAC AGC AAT      2340
His His Ser Asp Lys Arg Arg Pro Pro Ser Ala Glu Leu Tyr Ser Asn
        705                 710                 715

GCT CTG CCC GTT GGG CTC CCC CTG CCC AGC CAT GTG TCC TTG ACC GGG      2388
Ala Leu Pro Val Gly Leu Pro Leu Pro Ser His Val Ser Leu Thr Gly
    720                 725                 730
```

This Sheet Has Been Replaced



```
GAG AGC GAC GGT GGC TAC ATG GAC ATG AGC AAG GAC GAG TCG GTG GAC      2436
Glu Ser Asp Gly Gly Tyr Met Asp Met Ser Lys Asp Glu Ser Val Asp
735                 740                 745                 750

TAT GTG CCC ATG CTG GAC ATG AAA GGA GAC GTC AAA TAT GCA GAC ATC      2484
Tyr Val Pro Met Leu Asp Met Lys Gly Asp Val Lys Tyr Ala Asp Ile
                755                 760                 765

GAG TCC TCC AAC TAC ATG GCC CCT TAC GAT AAC TAC GTT CCC TCT GCC      2532
Glu Ser Ser Asn Tyr Met Ala Pro Tyr Asp Asn Tyr Val Pro Ser Ala
            770                 775                 780

CCT GAG AGG ACC TGC CGA GCA ACT TTG ATC AAC GAG TCT CCA GTG CTA      2580
Pro Glu Arg Thr Cys Arg Ala Thr Leu Ile Asn Glu Ser Pro Val Leu
        785                 790                 795

AGC TAC ATG GAC CTC GTG GGC TTC AGC TAC CAG GTG GCC AAT GGC ATG      2628
Ser Tyr Met Asp Leu Val Gly Phe Ser Tyr Gln Val Ala Asn Gly Met
    800                 805                 810

GAG TTT CTG GCC TCC AAG AAC TGC GTC CAC AGA GAC CTG GCG GCT AGG      2676
Glu Phe Leu Ala Ser Lys Asn Cys Val His Arg Asp Leu Ala Ala Arg
815                 820                 825                 830

AAC GTG CTC ATC TGT GAA GGC AAG CTG GTC AAG ATC TGT GAC TTT GGC      2724
Asn Val Leu Ile Cys Glu Gly Lys Leu Val Lys Ile Cys Asp Phe Gly
                835                 840                 845

CTG GCT CGA GAC ATC ATG CGG GAC TCG AAT TAC ATC TCC AAA GGC AGC      2772
Leu Ala Arg Asp Ile Met Arg Asp Ser Asn Tyr Ile Ser Lys Gly Ser
            850                 855                 860

ACC TTT TTG CCT TTA AAG TGG ATG GCT CCG GAG AGC ATC TTC AAC AGC      2820
Thr Phe Leu Pro Leu Lys Trp Met Ala Pro Glu Ser Ile Phe Asn Ser
        865                 870                 875

CTC TAC ACC ACC CTG AGC GAC GTG TGG TCC TTC GGG ATC CTG CTC TGG      2868
Leu Tyr Thr Thr Leu Ser Asp Val Trp Ser Phe Gly Ile Leu Leu Trp
    880                 885                 890

GAG ATC TTC ACC TTG GGT GGC ACC CCT TAC CCA GAG CTG CCC ATG AAC      2916
Glu Ile Phe Thr Leu Gly Gly Thr Pro Tyr Pro Glu Leu Pro Met Asn
895                 900                 905                 910

GAG CAG TTC TAC AAT GCC ATC AAA CGG GGT TAC CGC ATG GCC CAG CCT      2964
Glu Gln Phe Tyr Asn Ala Ile Lys Arg Gly Tyr Arg Met Ala Gln Pro
                915                 920                 925

GCC CAT GCC TCC GAC GAG ATC TAT GAG ATC ATG CAG AAG TGC TGG GAA      3012
Ala His Ala Ser Asp Glu Ile Tyr Glu Ile Met Gln Lys Cys Trp Glu
            930                 935                 940

GAG AAG TTT GAG ATT CGG CCC CCC TTC TCC CAG CTG GTG CTG CTT CTC      3060
Glu Lys Phe Glu Ile Arg Pro Pro Phe Ser Gln Leu Val Leu Leu Leu
        945                 950                 955

GAG AGA CTG TTG GGC GAA GGT TAC AAA AAG AAG TAC CAG CAG GTG GAT      3108
Glu Arg Leu Leu Gly Glu Gly Tyr Lys Lys Lys Tyr Gln Gln Val Asp
    960                 965                 970

GAG GAG TTT CTG AGG AGT GAC CAC CCA GCC ATC CTT CGG TCC CAG GCC      3156
Glu Glu Phe Leu Arg Ser Asp His Pro Ala Ile Leu Arg Ser Gln Ala
975                 980                 985                 990

CGC TTG CCT GGG TTC CAT GGC CTC CGA TCT CCC CTG GAC ACC AGC TCC      3204
```

IThis Sheet Has Been Replaced



```
Arg Leu Pro Gly Phe His Gly Leu Arg Ser Pro Leu Asp Thr Ser Ser
                995                1000                1005

GTC CTC TAT ACT GCC GTG CAG CCC AAT GAG GGT GAC AAC GAC TAT ATC      3252
Val Leu Tyr Thr Ala Val Gln Pro Asn Glu Gly Asp Asn Asp Tyr Ile
           1010                1015                1020

ATC CCC CTG CCT GAC CCC AAA CCT GAG GTT GCT GAC GAG GGC CCA CTG      3300
Ile Pro Leu Pro Asp Pro Lys Pro Glu Val Ala Asp Glu Gly Pro Leu
      1025                1030                1035

GAG GGT TCC CCC AGC CTA GCC AGC TCC ACC CTG AAT GAA GTC AAC ACC      3348
Glu Gly Ser Pro Ser Leu Ala Ser Ser Thr Leu Asn Glu Val Asn Thr
 1040                1045                1050

TCC TCA ACC ATC TCC TGT GAC AGC CCC CTG GAG CCC CAG GAC GAA CCA      3396
Ser Ser Thr Ile Ser Cys Asp Ser Pro Leu Glu Pro Gln Asp Glu Pro
1055                1060                1065                1070

GAG CCA GAG CCC CAG CTT GAG CTC CAG GTG GAG CCG GAG CCG GAG CTG      3444
Glu Pro Glu Pro Gln Leu Glu Leu Gln Val Glu Pro Glu Pro Glu Leu
                1075                1080                1085

GAA CAG TTG CCG GAT TCG GGG TGC CCT GCG CCT CGG GCG GAA GCA GAG      3492
Glu Gln Leu Pro Asp Ser Gly Cys Pro Ala Pro Arg Ala Glu Ala Glu
           1090                1095                1100

GAT AGC TTC CTG TAGGGGGCTG GCCCCTACCC TGCCCTGCCT GAAGCTCCCC          3544
Asp Ser Phe Leu
      1105

CGCTGCCAGC ACCCAGCATC TCCTGGCCTG GCCTGGCCGG GCTTCCTGTC AGCCAGGCTG    3604

CCCTTATCAG CTGTCCCCTT CTGGAAGCTT TCTGCTCCTG ACGTGTTGTG CCCCAAACCC    3664

TGGGGCTGGC TTAGGAGGCA AGAAAACTGC AGGGGCCGTG ACCAGCCCTC TGCCTCCAGG    3724

GAGGCCAACT GACTCTGAGC CAGGGTTCCC CCAGGGAACT CAGTTTTCCC ATATGTAAGA    3784

TGGGAAAGTT AGGCTTGATG ACCCAGAATC TAGGATTCTC TCCCTGGCTG ACAGGTGGGG    3844

AGACCGAATC CCTCCCTGGG AAGATTCTTG GAGTTACTGA GGTGGTAAAT TAACTTTTTT    3904

CTGTTCAGCC AGCTACCCCT CAAGGAATCA TAGCTCTCTC CTCGCACTTT TATCCACCCA    3964

GGAGCTAGGG AAGAGACCCT AGCCTCCCTG GCTGCTGGCT GAGCTAGGGC CTAGCCTTGA    4024

GCAGTGTTGC CTCATCCAGA AGAAAGCCAG TCTCCTCCCT ATGATGCCAG TCCCTGCGTT    4084

CCCTGGCCCG AGCTGGTCTG GGGCCATTAG GCAGCCTAAT TAATGCTGGA GGCTGAGCCA    4144

AGTACAGGAC ACCCCCAGCC TGCAGCCCTT GCCCAGGGCA CTTGGAGCAC ACGCAGCCAT    4204

AGCAAGTGCC TGTGTCCCTG TCCTTCAGGC CCATCAGTCC TGGGGCTTTT TCTTTATCAC    4264

CCTCAGTCTT AATCCATCCA CCAGAGTCTA GAAGGCCAGA CGGGCCCCGC ATCTGTGATG    4324

AGAATGTAAA TGTGCCAGTG TGGAGTGGCC ACGTGTGTGT GCCAGATATG GCCCTGGCTC    4384

TGCATTGGAC CTGCTATGAG GCTTTGGAGG AATCCCTCAC CCTCTCTGGG CCTCAGTTTC    4444

CCCTTCAAAA AATGAATAAG TCGGACTTAT TAACTCTGAG TGCCTTGCCA GCACTAACAT    4504

TCTAGAGTAT CCAGGTGGTT GCACATTTGT CCAGATGAAG CAAGGCCATA TACCCTAAAC    4564

TTCCATCCTG GGGGTCAGCT GGGCTCCTGG GAGATTCCAG ATCACACATC ACACTCTGGG    4624
```

This Sheet Has Been Replaced



GACTCAGGAA CCATGCCCCT TCCCCAGGCC CCCAGCAAGT CTCAAGAACA CAGCTGCACA 4684

GGCCTTGACT TAGAGTGACA GCCGGTGTCC TGGAAAGCCC CCAGCAGCTG CCCCAGGGAC 4744

ATGGGAAGAC CACGGGACCT CTTTCACTAC CCACGATGAC CTCCGGGGGT ATCCTGGGCA 4804

AAAGGGACAA AGAGGGCAAA TGAGATCACC TCCTGCAGCC CACCACTCCA GCACCTGTGC 4864

CGAGGTCTGC GTCGAAGACA GAATGGACAG TGAGGACAGT TATGTCTTGT AAAAGACAAG 4924

AAGCTTCAGA TGGGTACCCC AAGAAGGATG TGAGAGGTGG GCGCTTTGGA GGTTTGCCCC 4984

TCACCCACCA GCTGCCCCAT CCCTGAGGCA GCGCTCCATG GGGGTATGGT TTTGTCACTG 5044

CCCAGACCTA GCAGTGACAT CTCATTGTCC CCAGCCCAGT GGGCATTGGA GGTGCCAGGG 5104

GAGTCAGGGT TGTAGCCAAG ACGCCCCCGC ACGGGGAGGG TTGGGAAGGG GGTGCAGGAA 5164

GCTCAACCCC TCTGGGCACC AACCCTGCAT TGCAGGTTGG CACCTTACTT CCCTGGGATC 5224

CCAGAGTTGG TCCAAGGAGG GAGAGTGGGT TCTCAATACG GTACCAAAGA TATAATCACC 5284

TAGGTTTACA AATATTTTTA GGACTCACGT TAACTCACAT TTATACAGCA GAAATGCTAT 5344

TTTGTATGCT GTTAAGTTTT TCTATCTGTG TACTTTTTTT TAAGGGAAAG ATTTTAATAT 5404

TAAACCTGGT GCTTCTCACT CAC 5427

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1106 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met Arg Leu Pro Gly Ala Met Pro Ala Leu Ala Leu Lys Gly Glu Leu
1 5 10 15

Leu Leu Leu Ser Leu Leu Leu Leu Leu Glu Pro Gln Ile Ser Gln Gly
20 25 30

Leu Val Val Thr Pro Pro Gly Pro Glu Leu Val Leu Asn Val Ser Ser
35 40 45

Thr Phe Val Leu Thr Cys Ser Gly Ser Ala Pro Val Val Trp Glu Arg
50 55 60

Met Ser Gln Glu Pro Pro Gln Glu Met Ala Lys Ala Gln Asp Gly Thr
65 70 75 80

Phe Ser Ser Val Leu Thr Leu Thr Asn Leu Thr Gly Leu Asp Thr Gly
85 90 95

Glu Tyr Phe Cys Thr His Asn Asp Ser Arg Gly Leu Glu Thr Asp Glu
100 105 110

Arg Lys Arg Leu Tyr Ile Phe Val Pro Asp Pro Thr Val Gly Phe Leu
115 120 125

Pro Asn Asp Ala Glu Glu Leu Phe Ile Phe Leu Thr Glu Ile Thr Glu

This Sheet Has Been Replaced

130 135 140

Ile Thr Ile Pro Cys Arg Val Thr Asp Pro Gln Leu Val Val Thr Leu
145 150 155 160

His Glu Lys Lys Gly Asp Val Ala Leu Pro Val Pro Tyr Asp His Gln
165 170 175

Arg Gly Phe Ser Gly Ile Phe Glu Asp Arg Ser Tyr Ile Cys Lys Thr
180 185 190

Thr Ile Gly Asp Arg Glu Val Asp Ser Asp Ala Tyr Tyr Val Tyr Arg
195 200 205

Leu Gln Val Ser Ser Ile Asn Val Ser Val Asn Ala Val Gln Thr Val
210 215 220

Val Arg Gln Gly Glu Asn Ile Thr Leu Met Cys Ile Val Ile Gly Asn
225 230 235 240

Asp Val Val Asn Phe Glu Trp Thr Tyr Pro Arg Lys Glu Ser Gly Arg
245 250 255

Leu Val Glu Pro Val Thr Asp Phe Leu Leu Asp Met Pro Tyr His Ile
260 265 270

Arg Ser Ile Leu His Ile Pro Ser Ala Glu Leu Glu Asp Ser Gly Thr
275 280 285

Tyr Thr Cys Asn Val Thr Glu Ser Val Asn Asp His Gln Asp Glu Lys
290 295 300

Ala Ile Asn Ile Thr Val Val Glu Ser Gly Tyr Val Arg Leu Leu Gly
305 310 315 320

Glu Val Gly Thr Leu Gln Phe Ala Glu Leu His Arg Ser Arg Thr Leu
325 330 335

Gln Val Val Phe Glu Ala Tyr Pro Pro Pro Thr Val Leu Trp Phe Lys
340 345 350

Asp Asn Arg Thr Leu Gly Asp Ser Ser Ala Gly Glu Ile Ala Leu Ser
355 360 365

Thr Arg Asn Val Ser Glu Thr Arg Tyr Val Ser Glu Leu Thr Leu Val
370 375 380

Arg Val Lys Val Ala Glu Ala Gly His Tyr Thr Met Arg Ala Phe His
385 390 395 400

Glu Asp Ala Glu Val Gln Leu Ser Phe Gln Leu Gln Ile Asn Val Pro
405 410 415

Val Arg Val Leu Glu Leu Ser Glu Ser His Pro Asp Ser Gly Glu Gln
420 425 430

Thr Val Arg Cys Arg Gly Arg Gly Met Pro Gln Pro Asn Ile Ile Trp
435 440 445

Ser Ala Cys Arg Asp Leu Lys Arg Cys Pro Arg Glu Leu Pro Pro Thr
450 455 460

Leu Leu Gly Asn Ser Ser Glu Glu Glu Ser Gln Leu Glu Thr Asn Val
465 470 475 480

Thr Tyr Trp Glu Glu Glu Gln Glu Phe Glu Val Val Ser Thr Leu Arg

This Sheet Has Been Replaced

485 490 495

Leu Gln His Val Asp Arg Pro Leu Ser Val Arg Cys Thr Leu Arg Asn
500 505 510

Ala Val Gly Gln Asp Thr Gln Glu Val Ile Val Val Pro His Ser Leu
515 520 525

Pro Phe Lys Val Val Val Ile Ser Ala Ile Leu Ala Leu Val Val Leu
530 535 540

Thr Ile Ile Ser Leu Ile Ile Leu Ile Met Leu Trp Gln Lys Lys Pro
545 550 555 560

Arg Tyr Glu Ile Arg Trp Lys Val Ile Glu Ser Val Ser Ser Asp Gly
565 570 575

His Glu Tyr Ile Tyr Val Asp Pro Met Gln Leu Pro Tyr Asp Ser Thr
580 585 590

Trp Glu Leu Pro Arg Asp Gln Leu Val Leu Gly Arg Thr Leu Gly Ser
595 600 605

Gly Ala Phe Gly Gln Val Val Glu Ala Thr Ala His Gly Leu Ser His
610 615 620

Ser Gln Ala Thr Met Lys Val Ala Val Lys Met Leu Lys Ser Thr Ala
625 630 635 640

Arg Ser Ser Glu Lys Gln Ala Leu Met Ser Glu Leu Lys Ile Met Ser
645 650 655

His Leu Gly Pro His Leu Asn Val Val Asn Leu Leu Gly Ala Cys Thr
660 665 670

Lys Gly Gly Pro Ile Tyr Ile Ile Thr Glu Tyr Cys Arg Tyr Gly Asp
675 680 685

Leu Val Asp Tyr Leu His Arg Asn Lys His Thr Phe Leu Gln His His
690 695 700

Ser Asp Lys Arg Arg Pro Pro Ser Ala Glu Leu Tyr Ser Asn Ala Leu
705 710 715 720

Pro Val Gly Leu Pro Leu Pro Ser His Val Ser Leu Thr Gly Glu Ser
725 730 735

Asp Gly Gly Tyr Met Asp Met Ser Lys Asp Glu Ser Val Asp Tyr Val
740 745 750

Pro Met Leu Asp Met Lys Gly Asp Val Lys Tyr Ala Asp Ile Glu Ser
755 760 765

Ser Asn Tyr Met Ala Pro Tyr Asp Asn Tyr Val Pro Ser Ala Pro Glu
770 775 780

Arg Thr Cys Arg Ala Thr Leu Ile Asn Glu Ser Pro Val Leu Ser Tyr
785 790 795 800

Met Asp Leu Val Gly Phe Ser Tyr Gln Val Ala Asn Gly Met Glu Phe
805 810 815

Leu Ala Ser Lys Asn Cys Val His Arg Asp Leu Ala Ala Arg Asn Val
820 825 830

Leu Ile Cys Glu Gly Lys Leu Val Lys Ile Cys Asp Phe Gly Leu Ala

IThis Sheet Has Been Replaced

```
        835                 840                 845

Arg Asp Ile Met Arg Asp Ser Asn Tyr Ile Ser Lys Gly Ser Thr Phe
    850                 855                 860

Leu Pro Leu Lys Trp Met Ala Pro Glu Ser Ile Phe Asn Ser Leu Tyr
865                 870                 875                 880

Thr Thr Leu Ser Asp Val Trp Ser Phe Gly Ile Leu Leu Trp Glu Ile
                885                 890                 895

Phe Thr Leu Gly Gly Thr Pro Tyr Pro Glu Leu Pro Met Asn Glu Gln
            900                 905                 910

Phe Tyr Asn Ala Ile Lys Arg Gly Tyr Arg Met Ala Gln Pro Ala His
        915                 920                 925

Ala Ser Asp Glu Ile Tyr Glu Ile Met Gln Lys Cys Trp Glu Glu Lys
    930                 935                 940

Phe Glu Ile Arg Pro Pro Phe Ser Gln Leu Val Leu Leu Leu Glu Arg
945                 950                 955                 960

Leu Leu Gly Glu Gly Tyr Lys Lys Lys Tyr Gln Gln Val Asp Glu Glu
                965                 970                 975

Phe Leu Arg Ser Asp His Pro Ala Ile Leu Arg Ser Gln Ala Arg Leu
            980                 985                 990

Pro Gly Phe His Gly Leu Arg Ser Pro Leu Asp Thr Ser Ser Val Leu
        995                 1000                1005

Tyr Thr Ala Val Gln Pro Asn Glu Gly Asp Asn Asp Tyr Ile Ile Pro
    1010                1015                1020

Leu Pro Asp Pro Lys Pro Glu Val Ala Asp Glu Gly Pro Leu Glu Gly
1025                1030                1035                1040

Ser Pro Ser Leu Ala Ser Ser Thr Leu Asn Glu Val Asn Thr Ser Ser
                1045                1050                1055

Thr Ile Ser Cys Asp Ser Pro Leu Glu Pro Gln Asp Glu Pro Glu Pro
            1060                1065                1070

Glu Pro Gln Leu Glu Leu Gln Val Glu Pro Glu Pro Glu Leu Glu Gln
        1075                1080                1085

Leu Pro Asp Ser Gly Cys Pro Ala Pro Arg Ala Glu Ala Glu Asp Ser
    1090                1095                1100

Phe Leu
1105
```

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 4100 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA to mRNA

(iii) HYPOTHETICAL: N

(iv) ANTI-SENSE: N

This Sheet Has Been Replaced



(vi) ORIGINAL SOURCE:
(A) ORGANISM: Homo Sapiens

(vii) IMMEDIATE SOURCE:
(A) LIBRARY: lambda gt10

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 129..3395
(D) OTHER INFORMATION:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

```
TTGGAGCTAC AGGGAGAGAA ACAGAGGAGG AGACTGCAAG AGATCATTGC AGGCCGTGGG      60

CACGCTCTTT ACTCCATGTG TGGGACATTC ATTGCGGAAT AACATCGGAG GAGAAGTTTC     120

CCAGAGCT ATG GGG ACT TCC CAT CCG GCG TTC CTG GTC TTA GGC TGT CTT       170
         Met Gly Thr Ser His Pro Ala Phe Leu Val Leu Gly Cys Leu
           1               5                  10

CTC ACA GGG CTG AGC CTA ATC CTC TGC CAG CTT TCA TTA CCC TCT ATC       218
Leu Thr Gly Leu Ser Leu Ile Leu Cys Gln Leu Ser Leu Pro Ser Ile
 15                  20                  25                  30

CTT CCA AAT GAA AAT GAA AAG GTT GTG CAG CTG AAT TCA TCC TTT TCT       266
Leu Pro Asn Glu Asn Glu Lys Val Val Gln Leu Asn Ser Ser Phe Ser
                 35                  40                  45

CTG AGA TGC TTT GGG GAG AGT GAA GTG AGC TGG CAG TAC CCC ATG TCT       314
Leu Arg Cys Phe Gly Glu Ser Glu Val Ser Trp Gln Tyr Pro Met Ser
             50                  55                  60

GAA GAA GAG AGC TCC GAT GTG GAA ATC AGA AAT GAA GAA AAC AAC AGC       362
Glu Glu Glu Ser Ser Asp Val Glu Ile Arg Asn Glu Glu Asn Asn Ser
         65                  70                  75

GGC CTT TTT GTG ACG GTC TTG GAA GTG AGC AGT GCC TCG GCG GCC CAC       410
Gly Leu Phe Val Thr Val Leu Glu Val Ser Ser Ala Ser Ala Ala His
     80                  85                  90

ACA GGG TTG TAC ACT TGC TAT TAC AAC CAC ACT CAG ACA GAA GAG AAT       458
Thr Gly Leu Tyr Thr Cys Tyr Tyr Asn His Thr Gln Thr Glu Glu Asn
 95                  100                 105                 110

GAG CTT GAA GGC AGG CAC ATT TAC ATC TAT GTG CCA GAC CCA GAT GTA       506
Glu Leu Glu Gly Arg His Ile Tyr Ile Tyr Val Pro Asp Pro Asp Val
                115                 120                 125

GCC TTT GTA CCT CTA GGA ATG ACG GAT TAT TTA GTC ATC GTG GAG GAT       554
Ala Phe Val Pro Leu Gly Met Thr Asp Tyr Leu Val Ile Val Glu Asp
            130                 135                 140

GAT GAT TCT GCC ATT ATA CCT TGT CGC ACA ACT GAT CCC GAG ACT CCT       602
Asp Asp Ser Ala Ile Ile Pro Cys Arg Thr Thr Asp Pro Glu Thr Pro
        145                 150                 155

GTA ACC TTA CAC AAC AGT GAG GGG GTG GTA CCT GCC TCC TAC GAC AGC       650
Val Thr Leu His Asn Ser Glu Gly Val Val Pro Ala Ser Tyr Asp Ser
    160                 165                 170

AGA CAG GGC TTT AAT GGG ACC TTC ACT GTA GGG CCG TAT ATC TGT GAG       698
Arg Gln Gly Phe Asn Gly Thr Phe Thr Val Gly Pro Tyr Ile Cys Glu
175                 180                 185                 190
```

This Sheet Has Been Replaced



```
GCC ACC GTC AAA GGA AAG AAG TTC CAG ACC ATC CCA TTT AAT GTT TAT          746
Ala Thr Val Lys Gly Lys Lys Phe Gln Thr Ile Pro Phe Asn Val Tyr
                195                 200                 205

GCT TTA AAA GCA ACA TCA GAG CTG GAT CTA GAA ATG GAA GCT CTT AAA          794
Ala Leu Lys Ala Thr Ser Glu Leu Asp Leu Glu Met Glu Ala Leu Lys
            210                 215                 220

ACC GTG TAT AAG TCA GGG GAA ACG ATT GTG GTC ACC TGT GCT GTT TTT          842
Thr Val Tyr Lys Ser Gly Glu Thr Ile Val Val Thr Cys Ala Val Phe
        225                 230                 235

AAC AAT GAG GTG GTT GAC CTT CAA TGG ACT TAC CCT GGA GAA GTG AAA          890
Asn Asn Glu Val Val Asp Leu Gln Trp Thr Tyr Pro Gly Glu Val Lys
    240                 245                 250

GGC AAA GGC ATC ACA ATG CTG GAA GAA ATC AAA GTC CCA TCC ATC AAA          938
Gly Lys Gly Ile Thr Met Leu Glu Glu Ile Lys Val Pro Ser Ile Lys
255                 260                 265                 270

TTG GTG TAC ACT TTG ACG GTC CCC GAG GCC ACG GTG AAA GAC AGT GGA          986
Leu Val Tyr Thr Leu Thr Val Pro Glu Ala Thr Val Lys Asp Ser Gly
                275                 280                 285

GAT TAC GAA TGT GCT GCC CGC CAG GCT ACC AGG GAG GTC AAA GAA ATG         1034
Asp Tyr Glu Cys Ala Ala Arg Gln Ala Thr Arg Glu Val Lys Glu Met
            290                 295                 300

AAG AAA GTC ACT ATT TCT GTC CAT GAG AAA GGT TTC ATT GAA ATC AAA         1082
Lys Lys Val Thr Ile Ser Val His Glu Lys Gly Phe Ile Glu Ile Lys
        305                 310                 315

CCC ACC TTC AGC CAG TTG GAA GCT GTC AAC CTG CAT GAA GTC AAA CAT         1130
Pro Thr Phe Ser Gln Leu Glu Ala Val Asn Leu His Glu Val Lys His
    320                 325                 330

TTT GTT GTA GAG GTG CGG GCC TAC CCA CCT CCC AGG ATA TCC TGG CTG         1178
Phe Val Val Glu Val Arg Ala Tyr Pro Pro Pro Arg Ile Ser Trp Leu
335                 340                 345                 350

AAA AAC AAT CTG ACT CTG ATT GAA AAT CTC ACT GAG ATC ACC ACT GAT         1226
Lys Asn Asn Leu Thr Leu Ile Glu Asn Leu Thr Glu Ile Thr Thr Asp
                355                 360                 365

GTG GAA AAG ATT CAG GAA ATA AGG TAT CGA AGC AAA TTA AAG CTG ATC         1274
Val Glu Lys Ile Gln Glu Ile Arg Tyr Arg Ser Lys Leu Lys Leu Ile
            370                 375                 380

CGT GCT AAG GAA GAA GAC AGT GGC CAT TAT ACT ATT GTA GCT CAA AAT         1322
Arg Ala Lys Glu Glu Asp Ser Gly His Tyr Thr Ile Val Ala Gln Asn
        385                 390                 395

GAA GAT GCT GTG AAG AGC TAT ACT TTT GAA CTG TTA ACT CAA GTT CCT         1370
Glu Asp Ala Val Lys Ser Tyr Thr Phe Glu Leu Leu Thr Gln Val Pro
    400                 405                 410

TCA TCC ATT CTG GAC TTG GTC GAT GAT CAC CAT GGC TCA ACT GGG GGA         1418
Ser Ser Ile Leu Asp Leu Val Asp Asp His His Gly Ser Thr Gly Gly
415                 420                 425                 430

CAG ACG GTG AGG TGC ACA GCT GAA GGC ACG CCG CTT CCT GAT ATT GAG         1466
Gln Thr Val Arg Cys Thr Ala Glu Gly Thr Pro Leu Pro Asp Ile Glu
                435                 440                 445

TGG ATG ATA TGC AAA GAT ATT AAG AAA TGT AAT AAT GAA ACT TCC TGG         1514
```

This Sheet Has Been Replaced



IThis Sheet Has Been Replacec




```
Trp Met Ile Cys Lys Asp Ile Lys Lys Cys Asn Asn Glu Thr Ser Trp
                450                 455                 460

ACT ATT TTG GCC AAC AAT GTC TCA AAC ATC ATC ACG GAG ATC CAC TCC      1562
Thr Ile Leu Ala Asn Asn Val Ser Asn Ile Ile Thr Glu Ile His Ser
        465                 470                 475

CGA GAC AGG AGT ACC GTG GAG GGC CGT GTG ACT TTC GCC AAA GTG GAG      1610
Arg Asp Arg Ser Thr Val Glu Gly Arg Val Thr Phe Ala Lys Val Glu
    480                 485                 490

GAG ACC ATC GCC GTG CGA TGC CTG GCT AAG AAT CTC CTT GGA GCT GAG      1658
Glu Thr Ile Ala Val Arg Cys Leu Ala Lys Asn Leu Leu Gly Ala Glu
495                 500                 505                 510

AAC CGA GAG CTG AAG CTG GTG GCT CCC ACC CTG CGT TCT GAA CTC ACG      1706
Asn Arg Glu Leu Lys Leu Val Ala Pro Thr Leu Arg Ser Glu Leu Thr
                515                 520                 525

GTG GCT GCT GCA GTC CTG GTG CTG TTG GTG ATT GTG ATC ATC TCA CTT      1754
Val Ala Ala Ala Val Leu Val Leu Leu Val Ile Val Ile Ile Ser Leu
            530                 535                 540

ATT GTC CTG GTT GTC ATT TGG AAA CAG AAA CCG AGG TAT GAA ATT CGC      1802
Ile Val Leu Val Val Ile Trp Lys Gln Lys Pro Arg Tyr Glu Ile Arg
        545                 550                 555

TGG AGG GTC ATT GAA TCA ATC AGC CCA GAT GGA CAT GAA TAT ATT TAT      1850
Trp Arg Val Ile Glu Ser Ile Ser Pro Asp Gly His Glu Tyr Ile Tyr
    560                 565                 570

GTG GAC CCG ATG CAG CTG CCT TAT GAC TCA AGA TGG GAG TTT CCA AGA      1898
Val Asp Pro Met Gln Leu Pro Tyr Asp Ser Arg Trp Glu Phe Pro Arg
575                 580                 585                 590

GAT GGA CTA GTG CTT GGT CGG GTC TTG GGG TCT GGA GCG TTT GGG AAG      1946
Asp Gly Leu Val Leu Gly Arg Val Leu Gly Ser Gly Ala Phe Gly Lys
                595                 600                 605

GTG GTT GAA GGA ACA GCC TAT GGA TTA AGC CGG TCC CAA CCT GTC ATG      1994
Val Val Glu Gly Thr Ala Tyr Gly Leu Ser Arg Ser Gln Pro Val Met
            610                 615                 620

AAA GTT GCA GTG AAG ATG CTA AAA CCC ACG GCC AGA TCC AGT GAA AAA      2042
Lys Val Ala Val Lys Met Leu Lys Pro Thr Ala Arg Ser Ser Glu Lys
        625                 630                 635

CAA GCT CTC ATG TCT GAA CTG AAG ATA ATG ACT CAC CTG GGG CCA CAT      2090
Gln Ala Leu Met Ser Glu Leu Lys Ile Met Thr His Leu Gly Pro His
    640                 645                 650

TTG AAC ATT GTA AAC TTG CTG GGA GCC TGC ACC AAG TCA GGC CCC ATT      2138
Leu Asn Ile Val Asn Leu Leu Gly Ala Cys Thr Lys Ser Gly Pro Ile
655                 660                 665                 670

TAC ATC ATC ACA GAG TAT TGC TTC TAT GGA GAT TTG GTC AAC TAT TTG      2186
Tyr Ile Ile Thr Glu Tyr Cys Phe Tyr Gly Asp Leu Val Asn Tyr Leu
                675                 680                 685

CAT AAG AAT AGG GAT AGC TTC CTG AGC CAC CAC CCA GAG AAG CCA AAG      2234
His Lys Asn Arg Asp Ser Phe Leu Ser His His Pro Glu Lys Pro Lys
            690                 695                 700

AAA GAG CTG GAT ATC TTT GGA TTG AAC CCT GCT GAT GAA AGC ACA CGC      2282
Lys Glu Leu Asp Ile Phe Gly Leu Asn Pro Ala Asp Glu Ser Thr Arg
        705                 710                 715
```

This Sheet Has Been Replacec



```
AGC TAT GTT ATT TTA TCT TTT GAA AAC AAT GGT GAC TAC ATG GAC ATG      2330
Ser Tyr Val Ile Leu Ser Phe Glu Asn Asn Gly Asp Tyr Met Asp Met
    720                 725                 730

AAG CAG GCT GAT ACT ACA CAG TAT GTC CCC ATG CTA GAA AGG AAA GAG      2378
Lys Gln Ala Asp Thr Thr Gln Tyr Val Pro Met Leu Glu Arg Lys Glu
735                 740                 745                 750

GTT TCT AAA TAT TCC GAC ATC CAG AGA TCA CTC TAT GAT CGT CCA GCC      2426
Val Ser Lys Tyr Ser Asp Ile Gln Arg Ser Leu Tyr Asp Arg Pro Ala
                755                 760                 765

TCA TAT AAG AAG AAA TCT ATG TTA GAC TCA GAA GTC AAA AAC CTC CTT      2474
Ser Tyr Lys Lys Lys Ser Met Leu Asp Ser Glu Val Lys Asn Leu Leu
            770                 775                 780

TCA GAT GAT AAC TCA GAA GGC CTT ACT TTA TTG GAT TTG TTG AGC TTC      2522
Ser Asp Asp Asn Ser Glu Gly Leu Thr Leu Leu Asp Leu Leu Ser Phe
        785                 790                 795

ACC TAT CAA GTT GCC CGA GGA ATG GAG TTT TTG GCT TCA AAA AAT TGT      2570
Thr Tyr Gln Val Ala Arg Gly Met Glu Phe Leu Ala Ser Lys Asn Cys
    800                 805                 810

GTC CAC CGT GAT CTG GCT GCT CGC AAC GTT CTC CTG GCA CAA GGA AAA      2618
Val His Arg Asp Leu Ala Ala Arg Asn Val Leu Leu Ala Gln Gly Lys
815                 820                 825                 830

ATT GTG AAG ATC TGT GAC TTT GGC CTG GCC AGA GAC ATC ATG CAT GAT      2666
Ile Val Lys Ile Cys Asp Phe Gly Leu Ala Arg Asp Ile Met His Asp
                835                 840                 845

TCG AAC TAT GTG TCG AAA GGC AGT ACC TTT CTG CCC GTG AAG TGG ATG      2714
Ser Asn Tyr Val Ser Lys Gly Ser Thr Phe Leu Pro Val Lys Trp Met
            850                 855                 860

GCT CCT GAG AGC ATC TTT GAC AAC CTC TAC ACC ACA CTG AGT GAT GTC      2762
Ala Pro Glu Ser Ile Phe Asp Asn Leu Tyr Thr Thr Leu Ser Asp Val
        865                 870                 875

TGG TCT TAT GGC ATT CTG CTC TGG GAG ATC TTT TCC CTT GGT GGC ACC      2810
Trp Ser Tyr Gly Ile Leu Leu Trp Glu Ile Phe Ser Leu Gly Gly Thr
    880                 885                 890

CCT TAC CCC GGC ATG ATG GTG GAT TCT ACT TTC TAC AAT AAG ATC AAG      2858
Pro Tyr Pro Gly Met Met Val Asp Ser Thr Phe Tyr Asn Lys Ile Lys
895                 900                 905                 910

AGT GGG TAC CGG ATG GCC AAG CCT GAC CAC GCT ACC AGT GAA GTC TAC      2906
Ser Gly Tyr Arg Met Ala Lys Pro Asp His Ala Thr Ser Glu Val Tyr
                915                 920                 925

GAG ATC ATG GTG AAA TGC TGG AAC AGT GAG CCG GAG AAG AGA CCC TCC      2954
Glu Ile Met Val Lys Cys Trp Asn Ser Glu Pro Glu Lys Arg Pro Ser
            930                 935                 940

TTT TAC CAC CTG AGT GAG ATT GTG GAG AAT CTG CTG CCT GGA CAA TAT      3002
Phe Tyr His Leu Ser Glu Ile Val Glu Asn Leu Leu Pro Gly Gln Tyr
        945                 950                 955

AAA AAG AGT TAT GAA AAA ATT CAC CTG GAC TTC CTG AAG AGT GAC CAT      3050
Lys Lys Ser Tyr Glu Lys Ile His Leu Asp Phe Leu Lys Ser Asp His
    960                 965                 970

CCT GCT GTG GCA CGC ATG CGT GTG GAC TCA GAC AAT GCA TAC ATT GGT      3098
```

This Sheet Has Been Replaced




```
Pro Ala Val Ala Arg Met Arg Val Asp Ser Asp Asn Ala Tyr Ile Gly
975                 980                 985                 990

GTC ACC TAC AAA AAC GAG GAA GAC AAG CTG AAG GAC TGG GAG GGT GGT      3146
Val Thr Tyr Lys Asn Glu Glu Asp Lys Leu Lys Asp Trp Glu Gly Gly
                995                1000                1005

CTG GAT GAG CAG AGA CTG AGC GCT GAC AGT GGC TAC ATC ATT CCT CTG      3194
Leu Asp Glu Gln Arg Leu Ser Ala Asp Ser Gly Tyr Ile Ile Pro Leu
           1010                1015                1020

CCT GAC ATT GAC CCT GTC CCT GAG GAG GAG GAC CTG GGC AAG AGG AAC      3242
Pro Asp Ile Asp Pro Val Pro Glu Glu Glu Asp Leu Gly Lys Arg Asn
      1025                1030                1035

AGA CAC AGC TCG CAG ACC TCT GAA GAG AGT GCC ATT GAG ACG GGT TCC      3290
Arg His Ser Ser Gln Thr Ser Glu Glu Ser Ala Ile Glu Thr Gly Ser
 1040                1045                1050

AGC AGT TCC ACC TTC ATC AAG AGA GAG GAC GAG ACC ATT GAA GAC ATC      3338
Ser Ser Ser Thr Phe Ile Lys Arg Glu Asp Glu Thr Ile Glu Asp Ile
1055                1060                1065                1070

GAC ATG ATG GAC GAC ATC GGC ATA GAC TCT TCA GAC CTG GTG GAA GAC      3386
Asp Met Met Asp Asp Ile Gly Ile Asp Ser Ser Asp Leu Val Glu Asp
                1075                1080                1085

AGC TTC CTG TAACTGGCGG ATTCGAGGGG TTCCTTCCAC TTCTGGGGCC              3435
Ser Phe Leu

ACCTCTGGAT CCCGTTCAGA AAACCACTTT ATTGCAATGC GGAGGTTGAG AGGAGGACTT    3495

GGTTGATGTT TAAAGAGAAG TTCCCAGCCA AGGGCCTCGG GGAGCCTTTC TAAATATGAA    3555

TGAATGGGAT ATTTTGAAAT GAACTTTGTC AGTGTTGCCT CTTGCAATGC CTCAGTAGCA    3615

TCTCAGTGGT GTGTGAAGTT TGGAGATAGA TGGATAAGGG AATAATAGGC CACAGAAGGT    3675

GAACTTTCTG CTTCAAGGAC ATTGGTGAGA GTCCAACAGA CACAATTTAT ACTGCGACAG    3735

AACTTCAGCA TTGTAATTAT GTAAATAACT CTAACCACGG CTGTGTTTAG ATTGTATTAA    3795

CTATCTTCTT TGGACTTCTG AAGAGACCAC TCAATCCATC CATGTACTTC CCTCTTGAAA    3855

CCTGATGTCA GCTGCTGTTG AACTTTTTAA AGAAGTGCAT GAAAAACCAT TTTTGACCTT    3915

AAAAGGTACT GGTACTATAG CATTTTGCTA TCTTTTTTAG TGTTAAAGAG ATAAAGAATA    3975

ATAATTAACC AACCTTGTTT AATAGATTTG GGTCATTTAG AAGCCTGACA ACTCATTTTC    4035

ATATTGTAAT CTATGTTTAT AATACTACTA CTGTTATCAG TAATGCTAAA TGTGTAATAA    4095

TGTAA                                                                4100
```

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1069 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

This Sheet Has Been Replaced



Met Gly Thr Ser His Pro Ala Phe Leu Val Leu Gly Cys Leu Leu Thr
1 5 10 15

Gly Leu Ser Leu Ile Leu Cys Gln Leu Ser Leu Pro Ser Ile Leu Pro
20 25 30

Asn Glu Asn Glu Lys Val Val Gln Leu Asn Ser Ser Phe Ser Leu Arg
35 40 45

Cys Phe Gly Glu Ser Glu Val Ser Trp Gln Tyr Pro Met Ser Glu Glu
50 55 60

Glu Ser Ser Asp Val Glu Ile Arg Asn Glu Glu Asn Asn Ser Gly Leu
65 70 75 80

Phe Val Thr Val Leu Glu Val Ser Ser Ala Ser Ala Ala His Thr Gly
85 90 95

Leu Tyr Thr Cys Tyr Tyr Asn His Thr Gln Thr Glu Glu Asn Glu Leu
100 105 110

Glu Gly Arg His Ile Tyr Ile Tyr Val Pro Asp Pro Asp Val Ala Phe
115 120 125

Val Pro Leu Gly Met Thr Asp Tyr Leu Val Ile Val Glu Asp Asp Asp
130 135 140

Ser Ala Ile Ile Pro Cys Arg Thr Thr Asp Pro Glu Thr Pro Val Thr
145 150 155 160

Leu His Asn Ser Glu Gly Val Val Pro Ala Ser Tyr Asp Ser Arg Gln
165 170 175

Gly Phe Asn Gly Thr Phe Thr Val Gly Pro Tyr Ile Cys Glu Ala Thr
180 185 190

Val Lys Gly Lys Lys Phe Gln Thr Ile Pro Phe Asn Val Tyr Ala Leu
195 200 205

Lys Ala Thr Ser Glu Leu Asp Leu Glu Met Glu Ala Leu Lys Thr Val
210 215 220

Tyr Lys Ser Gly Glu Thr Ile Val Val Thr Cys Ala Val Phe Asn Asn
225 230 235 240

Glu Val Val Asp Leu Gln Trp Thr Tyr Pro Gly Glu Val Lys Gly Lys
245 250 255

Gly Ile Thr Met Leu Glu Glu Ile Lys Val Pro Ser Ile Lys Leu Val
260 265 270

Tyr Thr Leu Thr Val Pro Glu Ala Thr Val Lys Asp Ser Gly Asp Tyr
275 280 285

Glu Cys Ala Ala Arg Gln Ala Thr Arg Glu Val Lys Glu Met Lys Lys
290 295 300

Val Thr Ile Ser Val His Glu Lys Gly Phe Ile Glu Ile Lys Pro Thr
305 310 315 320

Phe Ser Gln Leu Glu Ala Val Asn Leu His Glu Val Lys His Phe Val
325 330 335

Val Glu Val Arg Ala Tyr Pro Pro Pro Arg Ile Ser Trp Leu Lys Asn
340 345 350

This Sheet Has Been Replaced



Asn Leu Thr Leu Ile Glu Asn Leu Thr Glu Ile Thr Thr Asp Val Glu
355 360 365

Lys Ile Gln Glu Ile Arg Tyr Arg Ser Lys Leu Lys Leu Ile Arg Ala
370 375 380

Lys Glu Glu Asp Ser Gly His Tyr Thr Ile Val Ala Gln Asn Glu Asp
385 390 395 400

Ala Val Lys Ser Tyr Thr Phe Glu Leu Leu Thr Gln Val Pro Ser Ser
405 410 415

Ile Leu Asp Leu Val Asp Asp His His Gly Ser Thr Gly Gly Gln Thr
420 425 430

Val Arg Cys Thr Ala Glu Gly Thr Pro Leu Pro Asp Ile Glu Trp Met
435 440 445

Ile Cys Lys Asp Ile Lys Lys Cys Asn Asn Glu Thr Ser Trp Thr Ile
450 455 460

Leu Ala Asn Asn Val Ser Asn Ile Ile Thr Glu Ile His Ser Arg Asp
465 470 475 480

Arg Ser Thr Val Glu Gly Arg Val Thr Phe Ala Lys Val Glu Glu Thr
485 490 495

Ile Ala Val Arg Cys Leu Ala Lys Asn Leu Leu Gly Ala Glu Asn Arg
500 505 510

Glu Leu Lys Leu Val Ala Pro Thr Leu Arg Ser Glu Leu Thr Val Ala
515 520 525

Ala Ala Val Leu Val Leu Leu Val Ile Val Ile Ile Ser Leu Ile Val
530 535 540

Leu Val Val Ile Trp Lys Gln Lys Pro Arg Tyr Glu Ile Arg Trp Arg
545 550 555 560

Val Ile Glu Ser Ile Ser Pro Asp Gly His Glu Tyr Ile Tyr Val Asp
565 570 575

Pro Met Gln Leu Pro Tyr Asp Ser Arg Trp Glu Phe Pro Arg Asp Gly
580 585 590

Leu Val Leu Gly Arg Val Leu Gly Ser Gly Ala Phe Gly Lys Val Val
595 600 605

Glu Gly Thr Ala Tyr Gly Leu Ser Arg Ser Gln Pro Val Met Lys Val
610 615 620

Ala Val Lys Met Leu Lys Pro Thr Ala Arg Ser Ser Glu Lys Gln Ala
625 630 635 640

Leu Met Ser Glu Leu Lys Ile Met Thr His Leu Gly Pro His Leu Asn
645 650 655

Ile Val Asn Leu Leu Gly Ala Cys Thr Lys Ser Gly Pro Ile Tyr Ile
660 665 670

Ile Thr Glu Tyr Cys Phe Tyr Gly Asp Leu Val Asn Tyr Leu His Lys
675 680 685

Asn Arg Asp Ser Phe Leu Ser His His Pro Glu Lys Pro Lys Lys Glu
690 695 700

This Sheet Has Been Replaced

Leu Asp Ile Phe Gly Leu Asn Pro Ala Asp Glu Ser Thr Arg Ser Tyr
705 710 715 720

Val Ile Leu Ser Phe Glu Asn Asn Gly Asp Tyr Met Asp Met Lys Gln
725 730 735

Ala Asp Thr Thr Gln Tyr Val Pro Met Leu Glu Arg Lys Glu Val Ser
740 745 750

Lys Tyr Ser Asp Ile Gln Arg Ser Leu Tyr Asp Arg Pro Ala Ser Tyr
755 760 765

Lys Lys Lys Ser Met Leu Asp Ser Glu Val Lys Asn Leu Leu Ser Asp
770 775 780

Asp Asn Ser Glu Gly Leu Thr Leu Leu Asp Leu Leu Ser Phe Thr Tyr
785 790 795 800

Gln Val Ala Arg Gly Met Glu Phe Leu Ala Ser Lys Asn Cys Val His
805 810 815

Arg Asp Leu Ala Ala Arg Asn Val Leu Leu Ala Gln Gly Lys Ile Val
820 825 830

Lys Ile Cys Asp Phe Gly Leu Ala Arg Asp Ile Met His Asp Ser Asn
835 840 845

Tyr Val Ser Lys Gly Ser Thr Phe Leu Pro Val Lys Trp Met Ala Pro
850 855 860

Glu Ser Ile Phe Asp Asn Leu Tyr Thr Thr Leu Ser Asp Val Trp Ser
865 870 875 880

Tyr Gly Ile Leu Leu Trp Glu Ile Phe Ser Leu Gly Gly Thr Pro Tyr
885 890 895

Pro Gly Met Met Val Asp Ser Thr Phe Tyr Asn Lys Ile Lys Ser Gly
900 905 910

Tyr Arg Met Ala Lys Pro Asp His Ala Thr Ser Glu Val Tyr Glu Ile
915 920 925

Met Val Lys Cys Trp Asn Ser Glu Pro Glu Lys Arg Pro Ser Phe Tyr
930 935 940

His Leu Ser Glu Ile Val Glu Asn Leu Leu Pro Gly Gln Tyr Lys Lys
945 950 955 960

Ser Tyr Glu Lys Ile His Leu Asp Phe Leu Lys Ser Asp His Pro Ala
965 970 975

Val Ala Arg Met Arg Val Asp Ser Asp Asn Ala Tyr Ile Gly Val Thr
980 985 990

Tyr Lys Asn Glu Glu Asp Lys Leu Lys Asp Trp Glu Gly Gly Leu Asp
995 1000 1005

Glu Gln Arg Leu Ser Ala Asp Ser Gly Tyr Ile Ile Pro Leu Pro Asp
1010 1015 1020

Ile Asp Pro Val Pro Glu Glu Glu Asp Leu Gly Lys Arg Asn Arg His
1025 1030 1035 1040

Ser Ser Gln Thr Ser Glu Glu Ser Ala Ile Glu Thr Gly Ser Ser Ser
1045 1050 1055

This Sheet Has Been Replaced

Ser Thr Phe Ile Lys Arg Glu Asp Glu Thr Ile Glu Asp Ile Asp Met
1060 1065 1070

Met Asp Asp Ile Gly Ile Asp Ser Ser Asp Leu Val Glu Asp Ser Phe
1075 1080 1085

Leu

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6375 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA to mRNA

(iii) HYPOTHETICAL: N

(iv) ANTI-SENSE: N

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Homo Sapiens

(vii) IMMEDIATE SOURCE:
(A) LIBRARY: lambda gt10

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 129..3395
(D) OTHER INFORMATION: /note= "nucleotide number 1 of this sequence is identical to the nucleotide number 1 of the previous 4100 long sequence."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

TTGGAGCTAC AGGGAGAGAA ACAGAGGAGG AGACTGCAAG AGATCATTGG AGGCCGTGGG 60

CACGCTCTTT ACTCCATGTG TGGGACATTC ATTGCGGAAT AACATCGGAG GAGAAGTTTC 120

CCAGAGCTAT GGGGACTTCC CATCCGGCGT TCCTGGTCTT AGGCTGTCTT CTCACAGGGC 180

TGAGCCTAAT CCTCTGCCAG CTTTCATTAC CCTCTATCCT TCCAAATGAA AATGAAAAGG 240

TTGTGCAGCT GAATTCATCC TTTTCTCTGA GATGCTTTGG GGAGAGTGAA GTGAGCTGGC 300

AGTACCCCAT GTCTGAAGAA GAGAGCTCCG ATGTGGAAAT CAGAAATGAA GAAAACAACA 360

GCGGCCTTTT TGTGACGGTC TTGGAAGTGA GCAGTGCCTC GGCGGCCCAC ACAGGGTTGT 420

ACACTTGCTA TTACAACCAC ACTCAGACAG AAGAGAATGA GCTTGAAGGC AGGCACATTT 480

ACATCTATGT GCCAGACCCA GATGTAGCCT TTGTACCTCT AGGAATGACG GATTATTTAG 540

TCATCGTGGA GGATGATGAT TCTGCCATTA TACCTTGTCG CACAACTGAT CCCGAGACTC 600

CTGTAACCTT ACACAACAGT GAGGGGGTGG TACCTGCCTC CTACGACAGC AGACAGGGCT 660

TTAATGGGAC CTTCACTGTA GGGCCCTATA TCTGTGAGGC CACCGTCAAA GGAAAGAAGT 720

TCCAGACCAT CCCATTTAAT GTTTATGCTT TAAAAGCAAC ATCAGAGCTG GATCTAGAAA 780

This Sheet Has Been Replaced



TGGAAGCTCT TAAAACCGTG TATAAGTCAG GGGAAACGAT TGTGGTCACC TGTGCTGTTT 840
TTAACAATGA GGTGGTTGAC CTTCAATGGA CTTACCCTGG AGAAGTGAAA GGCAAAGGCA 900
TCACAATGCT GGAAGAAATC AAAGTCCCAT CCATCAAATT GGTGTACACT TTGACGGTCC 960
CCGAGGCCAC GGTGAAAGAC AGTGGAGATT ACGAATGTGC TGCCCGCCAG GCTACCAGGG 1020
AGGTCAAAGA AATGAAGAAA GTCACTATTT CTGTCCATGA GAAAGGTTTC ATTGAAATCA 1080
AACCCACCTT CAGCCAGTTG GAAGCTGTCA ACCTGCATGA AGTCAAACAT TTTGTTGTAG 1140
AGGTGCGGGC CTACCCACCT CCCAGGATAT CCTGGCTGAA AAACAATCTG ACTCTGATTG 1200
AAAATCTCAC TGAGATCACC ACTGATGTGG AAAAGATTCA GGAAATAAGG TATCGAAGCA 1260
AATTAAAGCT GATCCGTGCT AAGGAAGAAG ACAGTGGCCA TTATACTATT GTAGCTCAAA 1320
ATGAAGATGC TGTGAAGAGC TATACTTTTG AACTGTTAAC TCAAGTTCCT TCATCCATTC 1380
TGGACTTGGT CGATGATCAC CATGGCTCAA CTGGGGGACA GACGGTGAGG TGCACAGCTG 1440
AAGGCACGCC GCTTCCTGAT ATTGAGTGGA TGATATGCAA AGATATTAAG AAATGTAATA 1500
ATGAAACTTC CTGGACTATT TTGGCCAACA ATGTCTCAAA CATCATCACG GAGATCCACT 1560
CCCGAGACAG GAGTACCGTG GAGGGCCGTG TGACTTTCGC CAAAGTGGAG GAGACCATCG 1620
CCGTGCGATG CCTGGCTAAG AATCTCCTTG GAGCTGAGAA CCGAGAGCTG AAGCTGGTGG 1680
CTCCCACCCT GCGTTCTGAA CTCACGGTGC CTGCTGCAGT CCTGGTGCTG TTGGTGATTG 1740
TGATCATCTC ACTTATTGTC CTGGTTGTCA TTTGGAAACA GAAACCGAGG TATGAAATTC 1800
GCTGGAGGGT CATTGAATCA ATCAGCCCAG ATGGACATGA ATATATTTAT GTGGACCCGA 1860
TGCAGCTGCC TTATGACTCA AGATGGGAGT TTCCAAGAGA TGGACTAGTG CTTGGTCGGG 1920
TCTTGGGGTC TGGAGCGTTT GGGAAGGTGG TTGAAGGAAC AGCCTATGGA TTAAGCCGGT 1980
CCCAACCTGT CATGAAAGTT GCAGTGAAGA TGCTAAAACC CACGGCCAGA TCCAGTGAAA 2040
AACAAGCTCT CATGTCTGAA CTGAAGATAA TGACTCACCT GGGGCCACAT TTGAACATTG 2100
TAAACTTGCT GGGAGCCTGC ACCAAGTCAG GCCCCATTTA CATCATCACA GAGTATTGCT 2160
TCTATGGAGA TTTGGTCAAC TATTTGCATA AGAATAGGGA TAGCTTCCTG AGCCACCACC 2220
CAGAGAAGCC AAAGAAAGAG CTGGATATCT TTGGATTGAA CCCTGCTGAT GAAAGCACAC 2280
GGAGCTATGT TATTTTATCT TTTGAAAACA ATGGTGACTA CATGGACATG AAGCAGGCTG 2340
ATACTACACA GTATGTCCCC ATGCTAGAAA GGAAAGAGGT TTCTAAATAT TCCGACATCC 2400
AGAGATCACT CTATGATCGT CCAGCCTCAT ATAAGAAGAA ATCTATGTTA GACTCAGAAG 2460
TCAAAAACCT CCTTTCAGAT GATAACTCAG AAGGCCTTAC TTTATTGGAT TTGTTGAGCT 2520
TCACCTATCA AGTTGCCCGA GGAATGGAGT TTTTGGCTTC AAAAAATTGT GTCCACCGTG 2580
ATCTGGCTGC TCGCAACGTT CTCCTGGCAC AAGGAAAAAT TGTGAAGATC TGTGACTTTG 2640
GCCTGGCCAG AGACATCATG CATGATTCGA ACTATGTGTC GAAAGGCAGT ACCTTTCTGC 2700
CCGTGAAGTG GATGGCTCCT GAGAGCATCT TTGACAACCT CTACACCACA CTGAGTGATG 2760

This Sheet Has Been Replaced


```
TCTGGTCTTA TGGCATTCTG CTCTGGGAGA TCTTTTCCCT TGGTGGCACC CCTTACCCCG   2820
GCATGATGGT GGATTCTACT TTCTACAATA AGATCAAGAG TGGGTACCGG ATGGCCAAGC   2880
CTGACCACGC TACCAGTGAA GTCTACGAGA TCATGGTCAA ATGCTGGAAC AGTGAGCCGG   2940
AGAAGAGACC CTCCTTTTAC CACCTGAGTG AGATTGTGGA GAATCTGCTG CCTGGACAAT   3000
ATAAAAAGAG TTATGAAAAA ATTCACCTGG ACTTCCTGAA GAGTGACCAT CCTGCTGTGG   3060
CACGCATGCG TGTGGACTCA GACAATGCAT ACATTGGTGT CACCTACAAA AACGAGGAAG   3120
ACAAGCTGAA GGACTGGGAG GGTGGTCTGG ATGAGCAGAG ACTGAGCGCT GACAGTGGCT   3180
ACATCATTCC TCTGCCTGAC ATTGACCCTG TCCCTGAGGA GGAGGACCTG GGCAAGAGGA   3240
ACAGACACAG CTCGCAGACC TCTGAAGAGA GTGCCATTGA GACGGGTTCC AGCAGTTCCA   3300
CCTTCATCAA GAGAGAGGAC GAGACCATTG AAGACATCGA CATGATGGAC GACATCGGCA   3360
TAGACTCTTC AGACCTGGTG GAAGACAGCT TCCTGTAACT GGCGGATTCG AGGGGTTCCT   3420
TCCACTTCTG GGGCCACCTC TGGATCCCGT TCAGAAAACC ACTTTATTGC AATGCGGAGG   3480
TTGAGAGGAG GACTTGGTTG ATGTTTAAAG AGAAGTTCCC AGCCAAGGGC CTCGGGGAGC   3540
CTTTCTAAAT ATGAATGAAT GGGATATTTT GAAATGAACT TTGTCAGTGT TGCCTCTTGC   3600
AATGCCTCAG TAGCATCTCA GTGGTGTGTG AAGTTTGGAG ATAGATGGAT AAGGGAATAA   3660
TAGGCCACAG AAGGTGAACT TTCTGCTTCA AGGACATTGG TGAGAGTCCA ACAGACACAA   3720
TTTATACTGC GACAGAACTT CAGCATTGTA ATTATGTAAA TAACTCTAAC CACGGCTGTG   3780
TTTAGATTGT ATTAACTATC TTCTTTGGAC TTCTGAAGAG ACCACTCAAT CCATCCATGT   3840
ACTTCCCTCT TGAAACCTGA TGTCAGCTGC TGTTGAACTT TTTAAAGAAG TGCATGAAAA   3900
ACCATTTTTG ACCTTAAAAG GTACTGGTAC TATAGCATTT TGCTATCTTT TTTAGTGTTA   3960
AAGAGATAAA GAATAATAAT TAACCAACCT TGTTTAATAG ATTTGGGTCA TTTAGAAGCC   4020
TGACAACTCA TTTTCATATT GTAATCTATG TTTATAATAC TACTACTGTT ATCAGTAATG   4080
CTAAATGTGT AATAATGTAA CATGATTTCC CTCCACACAA AGCACAATTT AAAAACAATC   4140
CTTACTAAGT AGGTGATGAG TTTGACAGTT TTTGACATTT ATATTAAATA ACATGTTTCT   4200
CTATAAAGTA TGGTAATAGC TTTAGTGAAT TAAATTTAGT TGAGCATAGA GAACAAAGTA   4260
AAAGTAGTGT TGTCCAGGAA GTCAGAATTT TTAACTGTAC TGAATAGGTT CCCCAATCCA   4320
TCGTATTAAA AAACAATTAA CTGCCCTCTG AAATAATGGG ATTAGAAACA AACAAAACTC   4380
TTAAGTCCTA AAAGTTCTCA ATGTAGAGGC ATAAACCTGT GCTGAACATA ACTTCTCATG   4440
TATATTACCC AATGGAAAAT ATAATGATCA GCGCANAAAG ACTGGATTTG CAGAAGTTNT   4500
TTTTTTTTTT TCTTCTTGCC TGATGAAAGC TTTGGCGACC CCAATATATG TATTTTTTGA   4560
ATCTATGAAC CTGAAAAGGG TCACAAAGGA TGCCCAGACA TCAGCCTCCT TCTTTCACCC   4620
CTTACCCCAA AGAGAAAGAG TTTGAAACTC GAGACCATAA AGATATTCTT TAGTGGAGGC   4680
TGGAAGTGCA TTAGCCTGAT CCTCAGTTCT CAAATGTGTG TGGCAGCCAG GTAGACTAGT   4740
```

```
ACCTGGGTTT CCATCCTTGA GATTCTGAAG TATGAAGTCT GAGGGAAACC AGAGTCTGTA   4800
TTTTTCTAAA CTCCCTGGCT GTTCTGATCG GCCAGGTTTC GGAAACACTG ACTTAGGTTT   4860
CAGGAAGTTG CCATGGGAAA CAAATAATTT GAACTTTGGA ACAGGGTTCT TAAGTTGGTG   4920
CGTCCTTCGG ATGATAAATT TAGGAACCGA AGTCCAATCA CTGTAAATTA CGGTAGATCG   4980
ATCGTTAACG CTGGAATTAA ATTGAAAGGT CAGAATCGAC TCCGACTCTT TCGATTTCAA   5040
ACCAAAACTG TCCAAAAGGT TTTCATTTCT ACGATGAAGG GTGACATACC CCCTCTAACT   5100
TGAAAGGGGC AGAGGGCAGA AGAGCGGAGG GTGAGGTATG GGGCGGTTCC TTTCCGTACA   5160
TGTTTTTAAT ACGTTAAGTC ACAAGGTTCA GAGACACATT GGTCGAGTCA CAAAACCACC   5220
TTTTTTGTAA AATTCAAAAT GACTATTAAA CTCCAATCTA CCCTCCTACT TAACAGTGTA   5280
GATAGGTGTG ACAGTTTGTC CAACCACACC CAAGTAACCG TAAGAAACGT TATGACGAAT   5340
TAACGACTAT GGTATACTTA CTTTGTACCC GACACTAATG ACGTTAGTGA CACGATAGCC   5400
GTCTACTACG AAACCTTCTA CGTCTTCGTT ATTATTTCAT GAACTGATGG ATGACCACAT   5460
TAGAGTTACG TTCGGGGTTC AAAGAATAGG TTGAAAAAGT ATCATTCACG CTTCTGACTC   5520
GGTCTAACCG GTTAATTTTT CTTTTGGACT GATCCAAGAC ATCTCGGTTA ATCTGAACTT   5580
TATGCAAACA CAAAGATCTT AGTGTCGAGT TCGTAAGACA AATAGCGAGT GAGAGGGAAC   5640
ATGTCGGAAT AAAACAACCA CGAAACGTAA AACTATAACG ACACTCGGAA CGTACTGTAG   5700
TACTCCGGCC TACTTTGAAG AGTCAGGTCG TCAAAGGTCA GGATTGTTTA CGAGGGTGGA   5760
CTTAAACATA TACTGACGTA AACACCCACA CACACACAAA AGTCGTTTAA GGTCTAAACA   5820
AAGGAAAACC GGAGGACGTT TCAGAGGTCT TCTTTTAAAC GGTTAGAAAG GATGAAAGAT   5880
AAAAATACTA CTGTTAGTTT CGGCCGGACT CTTTGTGATA AACACTGAAA AATTTGCTAA   5940
TCACTACAGG AATTTTACAC CAGACGGTTA GACATGTTTT ACCAGGATAA AAACACTTCT   6000
CCCTGTATTC TATTTTACTA CAATATGTAG TTATACATAT ATACATAAAG ATATATCTGA   6060
ACCTCTTATG ACGGTTTTGT AAATACTGTT CGACATAGTG ACGGAAGCAA ATATAAAAAA   6120
ATTGACACTA TTAGGGGTGT CCGTGTAATT GACAACGTGA AAACTTACAG GTTTTAAATA   6180
TAAAATCTTT ATTATTTTTC TTTCTATGAA TGTACAAGGG TTTTGTTACC ACACCACTTA   6240
CACACTCTTT TTGATTGAAC TATCCCAGAT GGTTATGTTT TACATAATGC TTACGGGGAC   6300
AAGTACAAAA ACAAAATTTT GCACATTTAC TTCTAGAAAT ATAAAGTTAT TTACTATATA   6360
TTAAATTTCC TTAAG                                                    6375
```

This Sheet Has Been Replaced



WHAT IS CLAIMED IS:

1. A purified and isolated recombinant nucleic acid of less than about 50 Kbp comprising at least about 24 contiguous nucleotides which encode a human platelet-derived growth factor receptor (hPDGF-R) polypeptide segment.

2. A nucleic acid of Claim 1, wherein said segment is a soluble polypeptide.

3. A nucleic acid of Claim 1, wherein said segment consists essentially of a full length extracellular region of a B type or an A type hPDGF receptor, and further has a sequence of a polypeptide in Table 2 or Table 3.

4. A nucleic acid of Claim 1, wherein said segment comprises a phosphorylation site.

5. A nucleic acid of Claim 1, wherein the segment is less than about 300 amino acids.

6. A nucleic acid of Claim 1, wherein said segment is capable of binding to PDGF.

7. A nucleic acid of Claim 1, wherein said segment is a substrate for phosphorylation.

8. A nucleic acid of Claim 1, wherein said segment is capable of binding to a PI3 kinase.

9. A cell transformed with a nucleic acid of Claim X1, and wherein said cell is a mammalian cell.

10. A cell of Claim 9, further comprising a glycosylation enzyme originating from a non-fungal species.

This Sheet Has Been Replaced

11. A nucleic acid of Claim 1, wherein said nucleotides encoding said segment are operably linked to a promoter.

12. A nucleic acid of Claim 1, further encoding a heterologous polypeptide which is fused to said hPDGF-R segment.

13. A method for evaluating the ability of a compound to function as a hPDGF-R agonist or antagonist comprising the step of comparing the amount of a PDGF-induced response in a cell of Claim 9 with the response from a control cell, and wherein said PDGF-induced response is compared by measuring synthesis of DNA in a cell after contacting said cell with PDGF.

14. A substantially pure hPDGF-R polypeptide fragment of at least about twenty amino acids having platelet-derived growth factor (PDGF) binding activity or tyrosine kinase activity.

15. A substantially pure polypeptide fragment of Claim 14, wherein said polypeptide fragment is soluble.

16. A hPDGF-R fragment having hPDGF-R binding activity consisting essentially of amino acids beginning at about 1 and ending at about 499 of a type B hPDGF-R, and is further derived from Table 2.

17. A hPDGF-R fragment having hPDGF-R binding activity consisting essentially of amino acids beginning about 1 and ending at about 501 of a type A hPDGF-R, and is further derived from Table 3.

18. A composition comprising an unglycosylated hPDGF-R fragment.

This Sheet Has Been Replaced

19. A composition of Claim 18, wherein said fragment is substantially pure.

20. A composition comprising a hPDGF-R fragment, which exhibits a glycosylation pattern which is non-fungal and non-human.

21. A composition of claim 20, wherein said fragment is essentially the extracellular region of a type B or a type A hPDGF-R.

22. A composition of Claim 20 having a sequence from Table 2, or from Table 3.

23. A composition comprising a combination of:

a) a recombinant nucleic acid encoding a human platelet-derived growth factor receptor polypeptide (hPDGF-R) fragment; and

b) a non-fungal glycosylation enzyme capable of glycosylating said fragment when expressed.

24. A method for introducing a hPDGF-R activity to a cell, said method comprising the step of introducing a hPDGF-R protein fragment of at least about five hundred daltons to said cell.

25. A method for assaying the presence of a ligand for a PDGF receptor in a sample, comprising the steps of:

combining said sample with a hPDGF receptor ligand binding site; and

detecting binding between said ligand and said hPDGF receptor ligand binding site.

26. An isolated polypeptide of less than about 200 amino acids comprising a receptor kinase insert region.

This Sheet Has Been Replaced

27. An isolated polypeptide of claim 26, wherein said polypeptide has a phosphorylated amino acid residue.

28. An isolated polypeptide of claim 26, wherein said polypeptide comprises a sequence substantially homologous to a kinase insert segment of a PDGF receptor, and further has a sequence from Table 2 or Table 3.

29. An isolated polypeptide of Claim 26, with a pharmaceutically acceptable carrier.

30. A method for modulating the biological activity of a first protein which binds to a phosphorylated region of a second protein, said method comprising a step of:

adding to said first protein a peptide analogue of said phosphorylated region, wherein said analogue is capable of inhibiting the binding of said first protein to said second protein.

31. A method of selecting a molecule capable of inhibiting binding of a protein which binds to a target phosphorylated polypeptide, comprising the steps of:

contacting said protein with said target phosphorylated polypeptide in the presence of said molecule in a first analysis;

contacting said protein with said target phosphorylated polypeptide in the absence of said molecule in a second analysis; and

comparing said analyses to determine the effect of said molecule on said binding.

32. A method of Claim 31, wherein said contacting steps are performed in succession.

33. A method for modulating a PI3 kinase activity comprising the step of:

This Sheet Has Been Replaced

adding a phosphorylated PDGF receptor kinase insert region polypeptide to said PI3 kinase, thereby allowing binding between said polypeptide and said PI3 kinase.

34. A method of purifying, from a sample, a protein capable of binding to a PDGF receptor kinase insert segment, comprising the step of:

contacting said sample with an analogue of a phosphorylated polypeptide substantially homologous to a PDGF receptor kinase insert region polypeptide, thereby allowing said protein to bind specifically to said phosphorylated polypeptide.

35. A method of isolating a nucleic acid encoding a protein capable of binding to a PDGF receptor, comprising the steps of:

combining a labeled and phosphorylated PDGF receptor kinase insert region polypeptide with cells expressing various proteins, thereby labeling those cells which express said nucleic acid to produce a protein which binds said phosphorylated polypeptide, and

isolating those cells which have been labeled.

36. A method of Claim 35, wherein said protein capable of binding a PDGF receptor is PI3 kinase or c-fms.

This Sheet Has Been Replaced

ABSTRACT

HUMAN PLATELET-DERIVED GROWTH FACTOR RECEPTORS

DNA sequences encoding human platelet-derived growth factor receptors (hPDGF-R), and expression constructs comprising sequences which encode a receptor that can be secreted or incorporated into the membrane of a mammalian cell, Peptide fragments with functions equivalent to the wild-type receptor, conferring a PDGF-sensitive mitogenic response on cells lacking that receptor are provided. The constructs can be used for enhancing PDGF response of cells, determining the regions involved transducing the signal in response to PDGF binding, providing mutated analogs and evaluating drugs for their physiologic activity. Soluble fragments comprising PDGF receptor sequences are also provided, including important intracellular kinase insert sequences which interact with intracellular proteins.

This Sheet Has Been Replaced



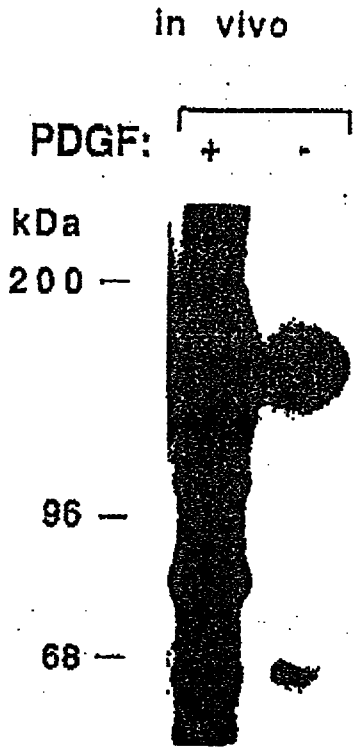


FIG. IA.

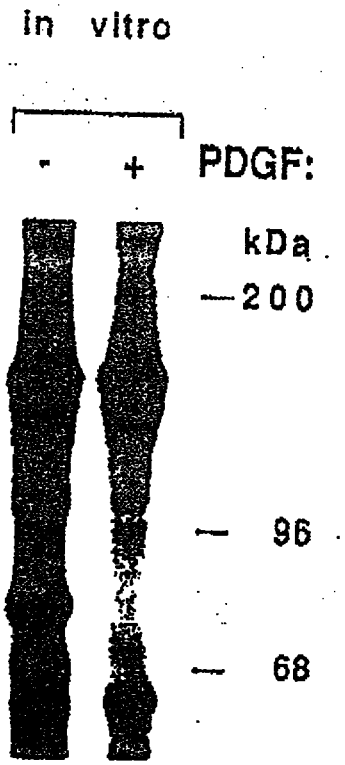


FIG. IB.

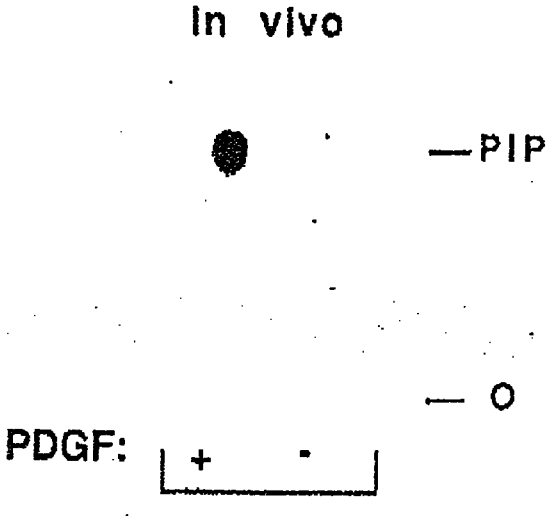


FIG. IC.

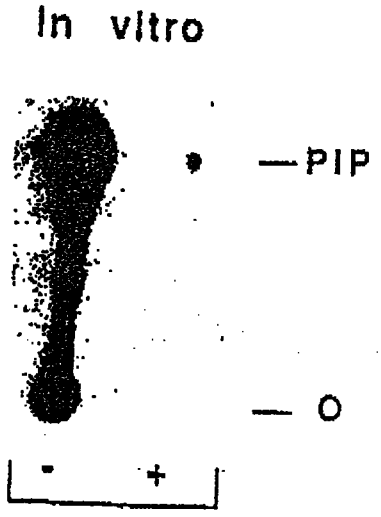


FIG. ID.

This Sheet Has Been Replaced

PCT/US92/00862

FIG. 2A.

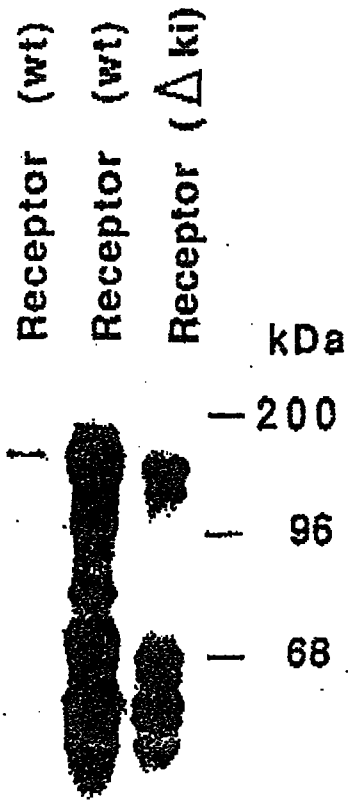
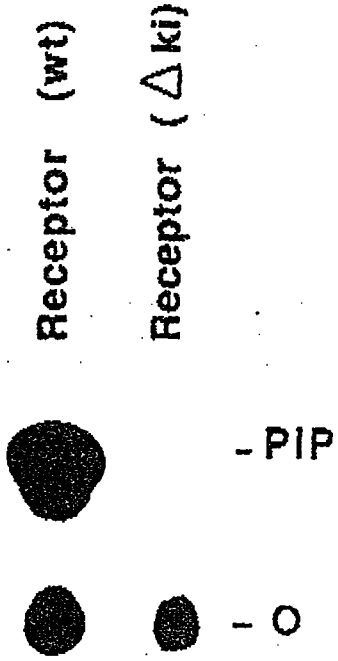


FIG. 2B.



This Sheet Has Been Replaced

PCT/US92/00862

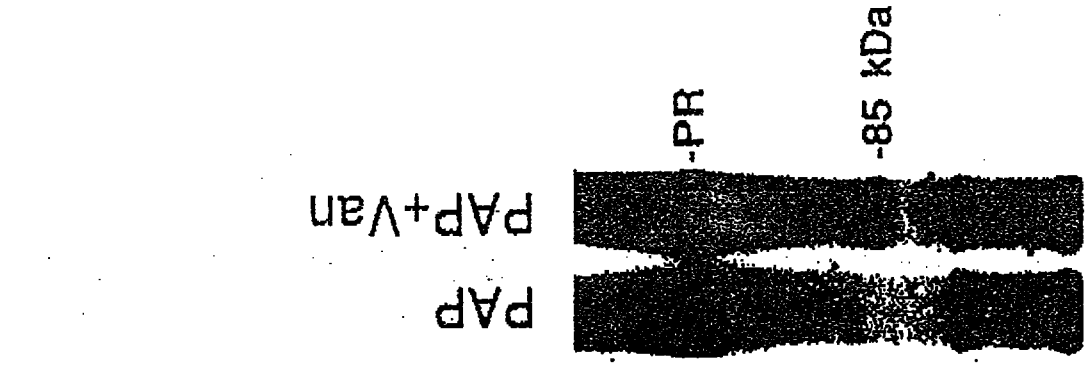


FIG. 3C.

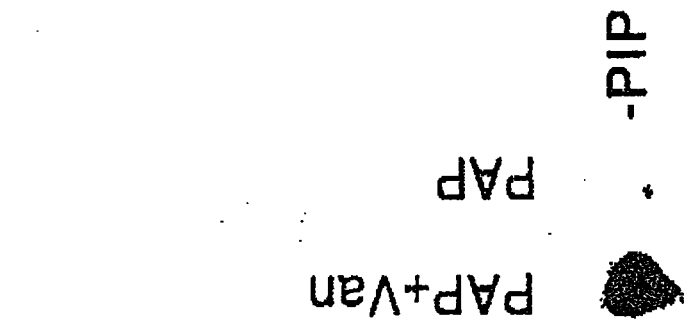


FIG. 3B.

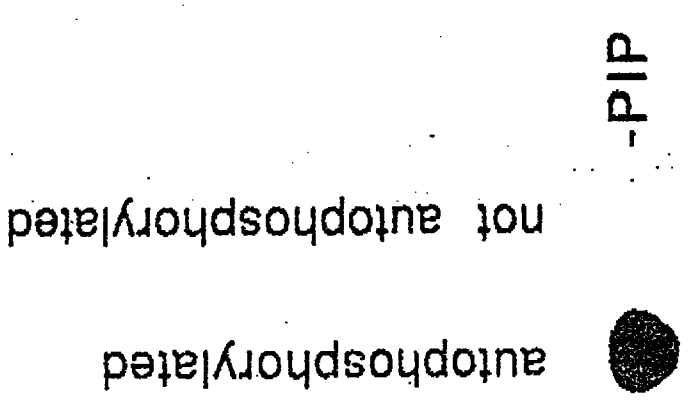


FIG. 3A.

This Sheet Has Been Replaced

SUBSTITUTE SHEET

PCT/US92/00862

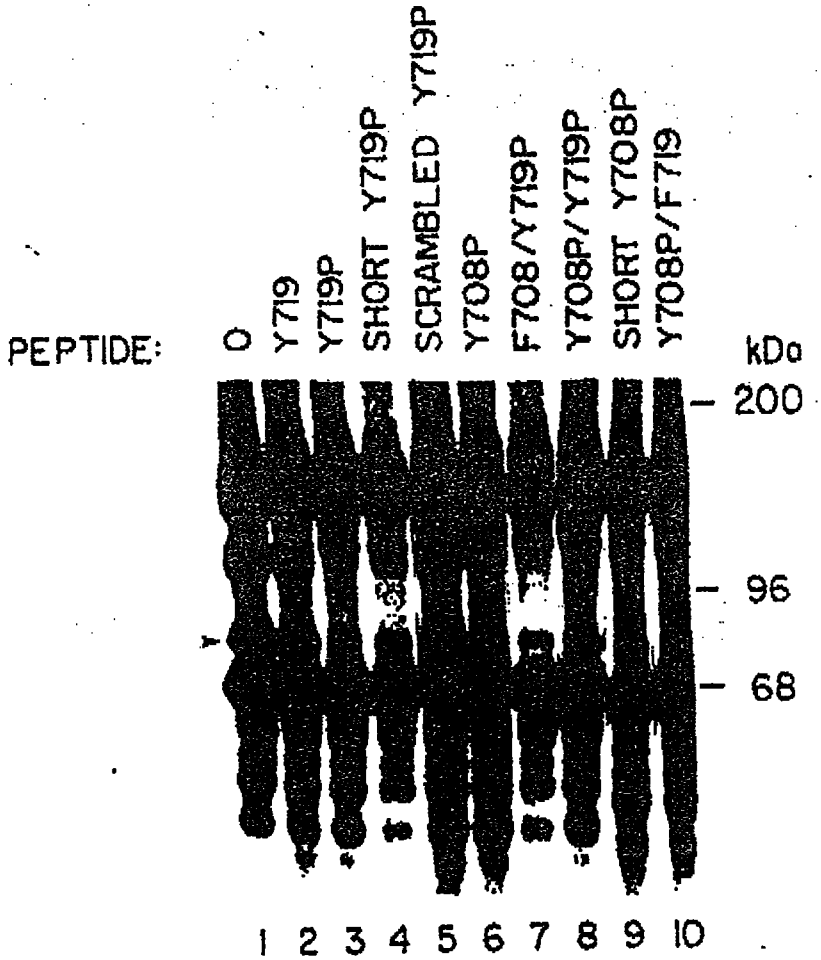
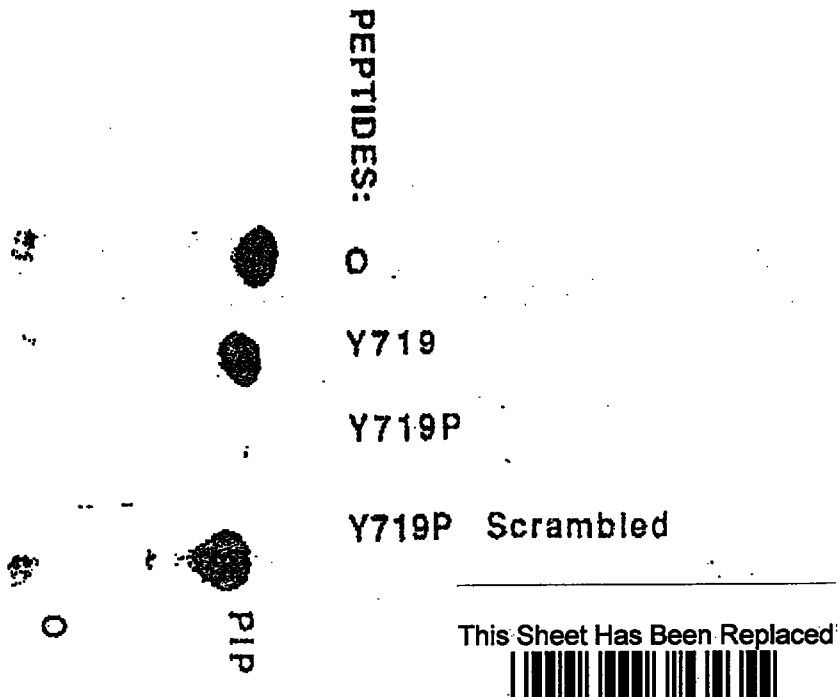


FIG. 4A.



This Sheet Has Been Replaced

PCT/US92/00862

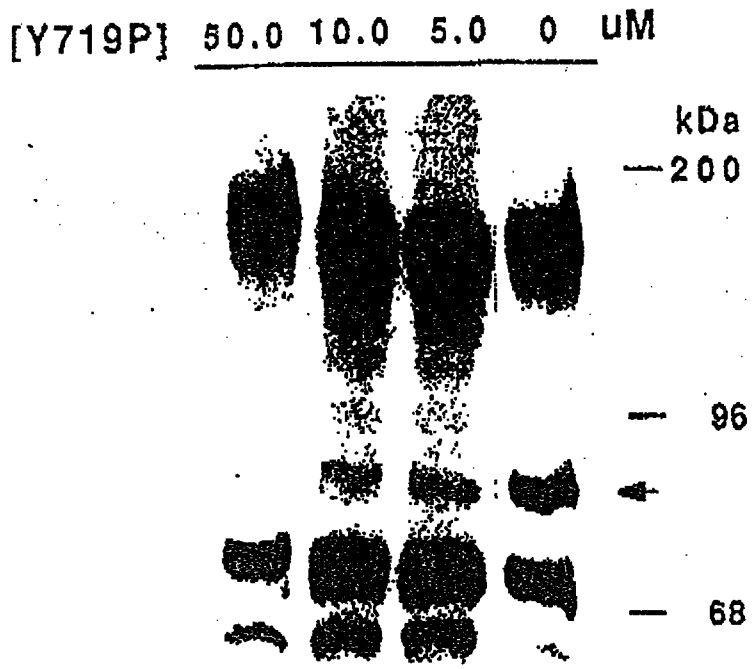


FIG. 5A.

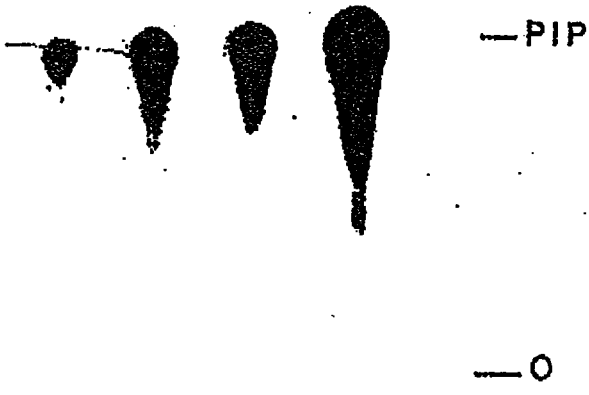


FIG. 5B.

This Sheet Has Been Replaced



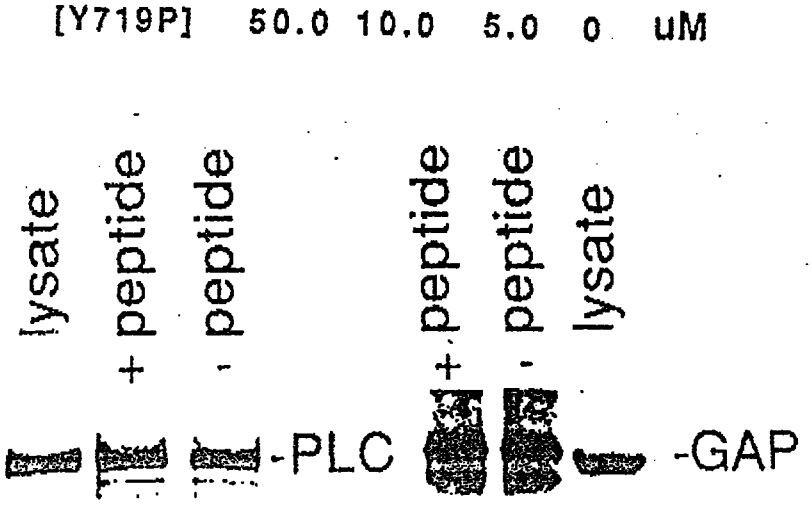


FIG. 6.

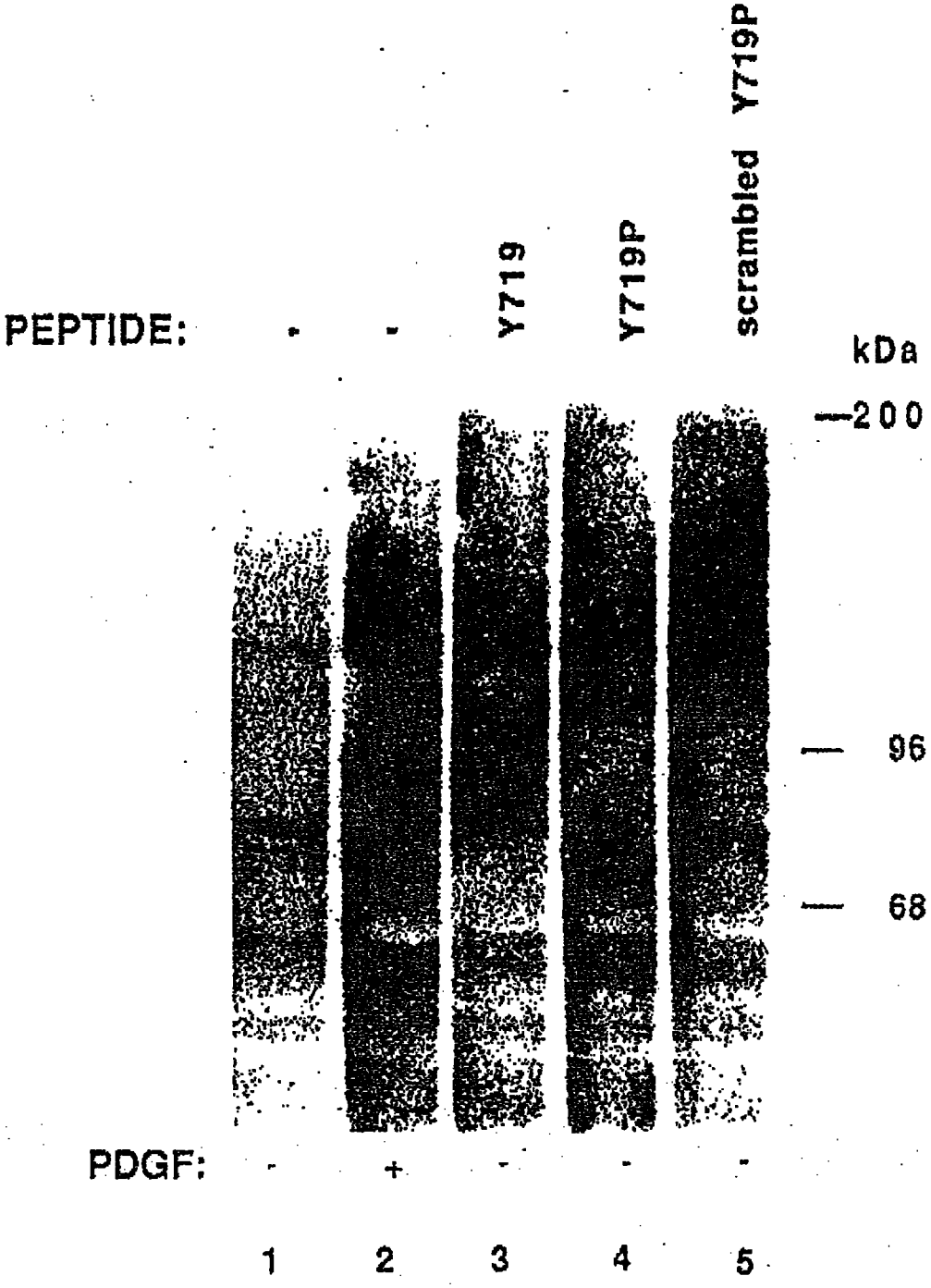


FIG. 7.

This Sheet Has Been Replaced

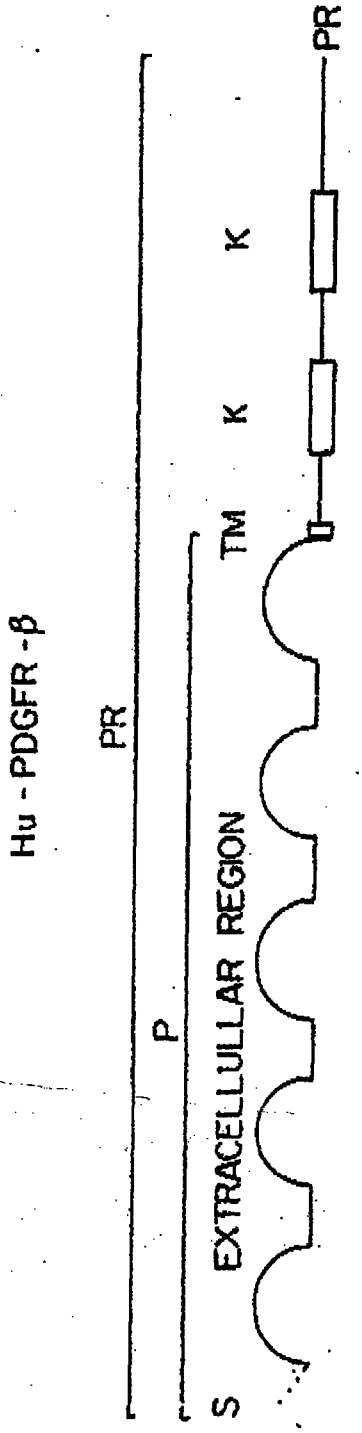


FIG. 8.

This Sheet Has Been Replaced

REPLACED SHEETS SG
☐ Description : paged ..... to paged .....
☑ Claim(s) : paged 105 to paged 114
☐ Drawing(s) : paged ..... to paged .....
☐ Abstract : paged ..... to paged .....
DATE OF RECEIPT .....

REPLACEMENT SHEETS SG
✓ Description : paged 1 to paged 104
✓ Claim(s) : paged 105 to paged 112
✓ Drawing(s) : paged 47 to paged 47
✓ Abstract : paged 113 to paged —
DATE OF RECEIPT 7/10/96

HUMAN PLATELET-DERIVED GROWTH FACTOR RECEPTORS

The present application is a continuation-in-part application of commonly assigned patent application U.S.S.N. 07/309,322, filed February 10, 1989, which is a C-I-P of U.S.S.N. 07/151,414, filed February 2, 1988, both of which are incorporated herein by reference.

The Government has rights in this invention pursuant to Grant No. R01 HL-32898 awarded by the National Institutes of Health.

Field of the Invention

The present invention relates to the development of diagnostic and therapeutic agents and, in particular, to compositions based on platelet-derived growth factor receptors.

BACKGROUND OF THE INVENTION

Platelet-derived growth factor (PDGF) is a major mitogen for cells of mesenchymal origin. The protein mitogen is usually a 32 kDa protein heterodimer usually composed of two polypeptide chains, A and B, linked by disulfide bonds. In addition to the PDGF AB heterodimer, two homodimeric forms of PDGF, denoted AA and BB, have been identified.

The first event in PDGF-mediated mitogenesis is the binding of PDGF to its receptor at the cell membrane. This interaction triggers a diversity of early cellular responses including activation of receptor tyrosine kinase, increased phosphatidylinositol turnover, enhanced expression of a group of genes, activation of phospholipase A2, changes in cell shape, an increase in cellular calcium concentration, changes in intracellular pH, and internalization and degradation of bound PDGF. These changes are followed by an increase in the rate of proliferation of the target receptor containing cells.

The ability of a polypeptide to stimulate growth of a particular cell type *in vitro* does not prove that it serves the same function *in vivo*, but the roles of many growth factors on cells are being studied to determine the roles that the factors play in the whole organism. *In vitro*, platelet-derived growth factor is a major polypeptide mitogen in serum for cells of

This is the replacement sheet
Date: - 7 OCT 1996

mesenchymal origin such as fibroblasts, smooth muscle cells, and glial cells. In vivo, PDGF does not circulate freely in blood, but is stored in the α granules of circulating blood platelets. During blood clotting and platelet adhesion, the granules are released, often at sites of injured blood vessels, thus implicating PDGF in the repair of blood vessels. PDGF may stimulate migration of arterial smooth muscle cells from the medial to the intimal layer of the artery where the muscle cells may proliferate. This is likely to be an early response to injury.

PDGF is being studied to determine how cell proliferation is controlled in the body. The growth factor has been implicated in wound healing, in atherosclerosis, in myeloproliferative disease, and in stimulating genes associated with cancerous transformation of cells, particularly c-myc and c-fos. Therefore, PDGF agonists may be useful in promoting wound healing. PDGF antagonists may also be useful in preventing atherosclerosis, in retarding blood vessel narrowing that occurs after cardiovascular intervention and in controlling cancerous proliferation.

The interaction of PDGF with cells is mediated, in part, by a receptor for the mitogen. The PDGF receptor is therefore a very important component in mitogenic stimulation by the growth factor. However, the inability to characterize the direct interaction between the PDGF and its receptor and between the PDGF receptor and intracellular components has hampered the development of reagents needed in the diagnosis or treatment of physiological conditions or disorders characterized by abnormal or undesired PDGF responses. For these reasons, a dramatic need exists to characterize the structural and physiological properties of PDGF receptors.

SUMMARY OF THE INVENTION

In accordance with the present invention, DNA sequences encoding human platelet-derived growth factor receptor (hPDGF-R) polypeptides have been isolated and sequenced. In one embodiment, expression constructs are provided comprising one or more sequences that encode

This is the replacement sheet

Date: - 7 OCT 1996

PDGF-receptor proteins that can be secreted or associated with the membrane of a mammalian cell. The membrane associated receptor should be functionally similar to or equivalent to a wild-type receptor thereby conferring a PDGF sensitive mitogenic response on cells lacking the receptor. The construct can be used, inter alia, for producing large amounts of the PDGF-receptor or fragments, for enhancing PDGF response of cells, for determining the regions of the receptor polypeptides involved in transducing the mitogenic signal in response to PDGF binding, for providing mutated analogs of the receptor, for evaluating drugs for their physiologic activity, and for probing the integrity of sequences nearby the chromosomal loci of the receptor genes. In particular, various soluble fragments of the receptor are provided, many possessing various properties of cell associated hPDGF-R proteins. Novel methods utilizing these constructs are also provided.

The present invention provides a purified and isolated recombinant nucleic acid of less than about 50 Kbp comprising at least about 24 contiguous nucleotides which encode a human platelet-derived growth factor receptor (hPDGF-R) polypeptide segment. Preferably the segment is a soluble polypeptide. In particular embodiments, the segment consists essentially of a full length extracellular region of a B type or an A type hPDGF receptor, e.g., a sequence of a polypeptide in Table 2 or Table 3. In other embodiments, the nucleic acid encodes a segment with a phosphorylation site.

Usually, the encoded segment is less than about 300 amino acids, and will preferably be capable of binding to PDGF, be a substrate for phosphorylation, or be capable of binding to a PI3 kinase. In other embodiments, the encoded segment lacks a substantially complete intracellular region.

The invention also embraces a cell transformed with the described nucleic acids, typically where the cell is a mammalian cell. In particular embodiments, the cell further contains a glycosylation enzyme originating from a non-fungal species.

Expression vectors are also provided, and in certain embodiments, the nucleic acid nucleotides encoding the segment

This is the replacement sheet

Date: - 7 OCT 1996

are operably linked to a promoter. Recombinant nucleic acids are provided which further encode a heterologous polypeptide which is fused to the hPDGF-R segment.

As another aspect of the invention, methods are provided for evaluating the ability of a compound to function as a hPDGF-R agonist or antagonist, utilizing the step of comparing the amount of a PDGF-induced response from a control cell with that in a cell transformed with a hPDGF-R peptide fragment encoding nucleic acid. In various embodiments, the PDGF-induced response is compared by measuring synthesis of DNA in the cells. After contacting the cells with cell with PDGF.

Polypeptide embodiments include a substantially pure hPDGF-R polypeptide fragment of at least about twenty amino acids having platelet-derived growth factor (PDGF) binding activity or tyrosine kinase activity. Typically, the polypeptide fragment will be soluble.

In other embodiments, hPDGF-R fragments are provided having hPDGF-R binding activity consisting essentially of amino acids beginning at about 1 and ending at about 499 of a type B hPDGF-R, e.g., derived from Table 2, or consisting essentially of amino acids beginning about 1 and ending at about 501 of a type A hPDGF-R, e.g., derived from Table 3. The invention embraces compositions having an unglycosylated hPDGF-R fragment, preferably where the fragment is substantially pure. In other embodiments, the hPDGF-R fragment exhibits a glycosylation pattern which is non-fungal and non-human. Particularly useful compositions have a hPDGF-R polypeptide fragment which is essentially the extracellular region of a type B or a type A hPDGF-R, e.g., derived from sequences of Table 2 or Table 3. An additional embodiment is a composition comprising a combination of: (a) a recombinant nucleic acid encoding a human platelet-derived growth factor receptor polypeptide (hPDGF-R) fragment; and (b) a non-fungal glycosylation enzyme capable of glycosylating said fragment when expressed.

The present invention provides various methods for introducing a hPDGF-R activity to a cell, comprising the step of introducing a hPDGF-R protein fragment of at least about

This is the replacement sheet

Date: - 7 OCT 1996

five hundred daltons to a cell. A method for assaying the presence of a ligand for a PDGF receptor in a sample is also provided, comprising the steps of: (a) combining the sample with a hPDGF receptor ligand binding site; and (b) detecting binding between the ligand and the hPDGF receptor ligand binding site.

With respect to the intracellular region of the PDGF receptors, the present invention provides an isolated polypeptide of less than about 200 amino acids comprising a receptor kinase insert region. In various embodiments, the polypeptide has a phosphorylated amino acid residue, e.g., phosphotyrosine. Usually, the polypeptide has a sequence substantially homologous to a kinase insert segment of a PDGF receptor, e.g., a sequence from Table 2 or Table 3. The invention also provides a composition with the polypeptide and a pharmaceutically acceptable carrier.

Various methods are provided by the invention, including methods for modulating the biological activity of a first protein which binds to a phosphorylated region of a second protein, the method including a step of adding to the first protein a peptide analogue of the phosphorylated region, where the analogue is capable of inhibiting the binding of the first protein to the second protein. Other methods are provided for selecting a molecule capable of inhibiting binding of a protein which binds to a target phosphorylated polypeptide. This method has steps of contacting the protein with the target phosphorylated polypeptide in the presence of the molecule in a first analysis; contacting the protein with the target phosphorylated polypeptide in the absence of the molecule in a second analysis; and comparing these analyses to determine the effect of the molecule on the binding. In particular embodiments, these contacting steps are performed in succession.

Other methods for modulating a PI3 kinase activity as provided, comprising the step of adding a phosphorylated PDGF receptor kinase insert region polypeptide to the PI3 kinase, thereby allowing binding between the polypeptide and the PI3 kinase.

This is the replacement sheet

Date: - 7 OCT 1996

The present invention provides methods for purifying, from a sample, a protein capable of binding to a PDGF receptor kinase insert segment, comprising the step of contacting the sample with an analogue of a phosphorylated polypeptide substantially homologous to a PDGF receptor kinase insert region polypeptide, thereby allowing the protein to bind specifically to the phosphorylated polypeptide.

Methods of isolating a nucleic acid encoding a protein capable of binding to a PDGF receptor are provided, comprising the steps of combining a labeled and phosphorylated PDGF receptor kinase insert region polypeptide with cells expressing various proteins, thereby labeling those cells which express the nucleic acid to produce a protein which binds the phosphorylated polypeptide; and isolating those cells which have been labeled. This method is particularly useful to isolate nucleic acids encoding PI3 kinase or c-fms.

This is the replacement sheet

Date: - 7 OCT 1996

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 illustrates the association of 85 kD/PI3 kinase with the type B PDGF receptor. The association of phosphoprotein with the PDGF receptor (a and b) and phosphatidylinositol 3' kinase (PI3 kinase) activity (c and d) were studied in intact cells (a and c) and *in vitro* (b and d). In the intact cell experiments PDGF-stimulated (+) or control cells (-) were solubilized and the receptor was immunoprecipitated using anti-receptor antibodies. The receptor-associated phosphoproteins were detected by incubating the receptor-associated protein complex with $MnCl_2$ and γ^{32}P-ATP followed by autoradiography of an SDS polyacrylamide gel. The PDGF-stimulated receptor associated with 85 kD and 110 kD phosphoproteins and PI3 kinase activity, but the unstimulated receptor was not. Equivalent amounts of receptor were immunoprecipitated from the lysates from stimulated and unstimulated cells. *In vitro* associations were performed by incubating the baculovirus-expressed receptor with lysates from PDGF-stimulated (+) or unstimulated (-) cells. Receptor-associated phosphoproteins (b) and PI3 kinase activity (d) were detected. The position of the 85 kD protein is indicated by the arrows.

Figure 2 illustrates the association of 85 kD protein and PI3 kinase activity with the wild type and kinase insert deletion mutant receptor *in vitro*. Lysates from unstimulated 3T3 cells were mixed with immunoprecipitated wild type PDGF type B receptor or kinase insert deletion mutant receptor that were prepared using a baculovirus expression system. The immunoprecipitates containing receptor-associated proteins were washed extensively. *In vitro* protein kinase assays (panel a) or PI3 kinase activity assays (panel b) were performed on the proteins. The first lane of panel (a) shows the *in vitro* protein kinase assay of the baculovirous-expressed receptor that was not mixed with cell lysate. The top two bands of the second and third lanes of panel (a) are from the baculovirus-expressed receptor (second lane) or the mutated receptor (third lane) and their respective precursors. The arrow indicates the 85 kD protein.

This is the replacement sheet

Date: - 7 OCT 1996

Figure 3 illustrates the need for requirement of receptor autophosphorylation for association with PI3 kinase activity and 85 kD protein. PDGF receptors were immunoprecipitated from Sf9 cells infected with the wild type PDGF receptor recombinant baculovirus. Immunoprecipitates containing phosphorylated receptor were incubated with solubilized 3T3 cell lysates. PI3 kinase assays (a and b) and in vitro kinase assays (c) were performed. (a) Association of PI3 kinase activity with phosphorylated PDGF receptors were incubated with potato acid phosphatase (PAP) or PAP plus orthovanadate.

Figure 4 illustrates the use of peptides to block the association of the 85 kD phosphoprotein and PI3 kinase activity with the receptor. The peptides listed in Table AJ were preincubated with unstimulated 3T3 cell lysates prior to incubation with immunoprecipitated receptor. The detection of the receptor-associated phosphoproteins (panel a) and the receptor-associated PI3 kinase activity (panel b) were performed as in Figs. 1 and 2. The 85 kD protein is indicated by the arrow.

Figure 5 illustrates the concentration-dependance of the blocking activity of peptide Y719P.. A series of concentrations of peptide Y719P were tested for the ability to block the binding of proteins (panel a) or PI3 kinase activity (panel b) to baculovirus-expressed immunoprecipitated receptor. The arrow points to the 85 kD phosphoprotein. The assays were performed as described in Figs. 1, 2 and 3.

Figure 6 illustrates that the association of PDGF receptor with PLC-γ and GAP is not affected by preincubation of 3T3 unstimulated lysates with receptor phosphopeptide, Y719P. Unstimulated 3T3 lysates were preincubated in the presence and absence of a receptor phosphopeptide (Y719P) prior to the association with the baculovirus-expressed receptor. The peptide/lysate mixture was incubated with the immunoprecipitated baculovirus-expressed receptor as described in Figs. 1, 2 and 3. The presence of PLC-γ and GAP in the receptor complex was determined by immunoblot analysis using PLC-γ and GAP antibodies as probes. In the lane marked

This is the replacement sheet

Date: - 7 OCT 1996

"lysates", crude lysates of 3T3 cells were immunoblotted as controls to show the electrophoretic position of the PLC-γ and GAP.

Figure 7 illustrates the association of soluble radiolabeled receptor with immobilized 85 kD protein ("receptor blot"). Baculovirus-expressed PDGF receptors were immunoprecipitated using receptor antibodies and the receptor was ^{32}P-labeled <u>in vitro</u> by autophosphorylation. The solubilized radiolabeled receptor was used to probe nitrocellulose filters containing SDS-polyacrylamide gel-fractionated lysates from control (lanes 1, 3, 4 and 5) or PDGF stimulated (lane 2) cells. In the peptide competition experiments (lane 3, 4, and 5) the peptides were added to the nitrocellulose paper along with the radiolabeled receptor probe. The arrow indicates the position of the 85 kD protein.

Figure 8 illustrates a strategy for oligonucleotide directed <u>in vitro</u> production of a soluble hPDGF-R extracellular region. The abbreviations used are:

PR = PDGFR; intact
P = PDGFR; extracellular region
TM = transmembrane region
K = kinase
S = signal sequence

This is the replacement sheet

Date: - 7 OCT 1996

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

Human platelet derived growth factor receptor (hPDGF-R) compositions are provided by the present invention. These compositions will be full length natural forms, fragments of the natural forms, fusion proteins with those fragments, modified forms of each, and multi-protein complexes comprising them. In particular, soluble polypeptides exhibiting hPDGF-R functions are made available, both extracellular and intracellular region fragments.

Methods for producing protein compositions based upon hPDGF receptors are provided. Nucleic acid constructs which encode the various hPDGF-R protein compositions are also disclosed. The nucleic acid constructs will be useful to transfect cells, providing an efficient and economical means to produce commercially useful quantities of the protein compositions. These cells, and others containing hPDGF receptors, will also be useful in diagnosis or for study of mechanisms of PDGF mitogenic action. They will be used to evaluate new drugs which affect signal transduction of PDGF ligand binding, and in treatment of diseases associated with hPDGF receptors. The constructs can be used to transfect cells, providing membrane-bound receptors that are functionally equivalent to wild-type receptors, and conferring a PDGF-sensitive mitogenic response on cells lacking receptors. The transfected cells can be used as a model for studying the PDGF-induced response of cells, determining the regions involved in transducing the signal in response to PDGF ligand binding, and evaluating drugs for their physiologic activities. Encoded receptors or their binding regions also find use in evaluating PDGF analogues.

The receptor fragments and their analogues will find use in determining regions of the receptor polypeptides which are involved in signal transduction in response to PDGF binding. In particular, the ability to test various combinations of structural features, e.g., ligand binding determinants, will allow dissection of receptor features to determine the importance to ligand binding affinity, ligand specificity, and physiological responses.

This is the replacement sheet

Date: - 7 OCT 1996

The hPDGF-R proteins will also find use in producing antibody reagents. These reagents should be capable of interfering with, or simulating, particular molecular interactions of the receptor polypeptides.

Soluble proteins possessing PDGF binding capacity will be useful in blocking PDGF action, as reagents for the quantitation of PDGF in diagnostic samples, for soluble testing of binding interactions between modified determinants for ligand binding and natural, modified, or derivatized ligands, and for screening for monoclonal antibodies against hPDGF-R polypeptides. They will also find therapeutic uses.

The DNA sequences will also find use as probes to detect genetic abnormalities, e.g., deletions or rearrangements in the region of chromosome 5 where a number of growth-control related genes are clustered, in the region of chromosome 4 near the c-kit oncogenes, or to detect other genes encoding tyrosine kinases or homologous genes.

Certain regions of the cytoplasmic region of the PDGF receptor, among them the kinase insert (KI) region, allow the PDGF receptor to interact with, e.g., bind with, other cellular proteins. Peptides substantially homologous to these regions, as well as organic analogue molecules, will find use in inhibiting the interaction between the PDGF receptor and other proteins, in identifying, screening for, and purifying proteins with which the PDGF receptor interacts, and in cloning genes encoding these proteins. These homologous peptides will also find use in medical diagnosis and in drug therapies, e.g., affecting PDGF receptor activities and receptor interactions with other proteins.

Moreover, the understanding of the role of phosphorylated residues to protein-protein interactions is applicable to other receptors and phosphorylated proteins. The phosphorylation of specific amino acids in particular polypeptide segments or substantially homologous segments, e.g., phosphorylation sites, has special biological significance. Identification of the phosphorylation sites and regions of protein interactions are important in preparing reagents useful in inhibiting such interactions, leading to a

This is the replacement sheet

Date: - 7 OCT 1998

modulation of the function of such proteins. The peptide analogues will also be useful in identifying and purifying proteins which interact with phosphorylated residues and in isolating and cloning genes encoding these proteins.

OUTLINE

I. General Description
  A. PDGF-R
    1. structural features
      a. extracellular region
        i. signal sequence
        ii. Ig domains
      b. transmembrane segment
      c. intracellular region
        i. tyrosine kinase
        ii. insert
    2. function
      a. bind PDGF
      b. bind to PDGF-R peptide
      c. tyrosine kinase activity
  B. Physiological Functions
    1. cellular
    2. tissue differentiation
    3. organismal
II. Polypeptides
  A. Soluble Forms
  B. Truncated Forms
  C. Fusion Proteins
  D. Genetic Variants (site-directed mutagenized)
  E. Compositions Comprising Proteins
III. Nucleic Acids
  A. Isolated Nucleic Acids
  B. Recombinant Nucleic Acids
  C. Compositions Comprising Nucleic Acids
IV. Methods for Making PDGF-R
  A. Protein Purification
    1. affinity with derivatized PDGF
    2. various ligands, same receptor
  B. Expression of Nucleic Acids
V. Antibodies
VI. Methods for Use
  A. Diagnostic
  B. Therapeutic

* * * * *

I. General Description

The isolated full-length human platelet-derived growth factor (hPDGF) receptor mRNA encodes a single hydrophobic polypeptide segment similar to a membrane-spanning segment (designated the transmembrane segment). The segments

This is the replacement sheet

Date: - 7 OCT 1996

of a PDGF-R amino proximal to the transmembrane segment make up the extracellular region, while the segments carboxy proximal to the transmembrane segment are designated the intracellular region. From the amino terminus, the extracellular region has an NH_2-terminal hydrophobic putative signal sequence, an immunoglobulin-like domain (designated IgI), and second, third, fourth, and fifth immunoglobulin-like domains (designated IgII, IgIII, IgIV, and IgV, respectively). Although various structural features are identified in the external region of the hPDGF-R, the most important functional property which defines the region is the binding to the receptor ligands, e.g., members of the PDGF family and analogues thereof.

The intracellular region is characterized, in part, by the presence of a split tyrosine kinase structural domain. In the human type B receptor polypeptide, this domain is about 244 residues long and has an insert of about 104 amino acids. See Table 2. In the human type A receptor polypeptide, the tyrosine kinase domain is also about 244 residues long with a kinase insert of about 103 amino acids. See Table 3. Functionally, this domain is defined, in part, by its tyrosine kinase activity, typically modulated by ligand binding to the extracellular region, and appears to function in a dimer state. The substrate for phosphorylation includes various tyrosine residues on the accompanying receptor polypeptide chain, and other proteins which associate with the receptor. The tyrosine kinase domain is also defined, in part, by its homology to similar domains in other tyrosine kinase activity containing proteins. See, e.g., Yarden et al. (1986) Nature 323: 226-232. As such, the actual boundaries, determined by homology to other similar domains, may vary by a few amino acid residues, perhaps as many as about 7 residues, but probably within about 4 residues, and more probably within about one or two residues. A protein substantially lacks a complete intracellular region when it lacks a prototypical intracellular region, particularly lacking a tyrosine kinase domain; a similar concept is provided with respect to lack of a complete transmembrane region.

A typical PDGF-R nucleic acid sequence encodes a transitory NH_2-terminal hydrophobic sequence, which is usually

This is the replacement sheet

Date: - 7 OCT 1996

cleaved during the membrane translocation process. The classical function of a signal sequence is to direct the nascent polypeptide chain to membrane bound ribosomes, thereby leading to membrane translocation and subsequent processing and targeting steps. Since the signal sequence is typically removed in the translocation process, the signal sequence is absent in a mature polypeptide. However, other features of the amino proximal sequence of the mature protein may also be important for expression or correct localization or targeting of receptor fragments.

The putative boundaries of the different regions of the receptors are indicated in Table 1, derived, in part, by homology to the mouse PDGF receptor. In particular, a hydrophobic segment has been identified and designated a transmembrane segment, due to its structural homology and physical properties characteristic of a membrane spanning segment. The segment divides the protein into an extracellular region and an intracellular region. The intracellular region has homology to various other receptors believed to exhibit tyrosine kinase activity, though this receptor has an insert region, as indicated, which is embedded within the region of high homology to other tyrosine kinase segments.

This is the replacement sheet

Date: - 7 OCT 1996

TABLE 1

approximate segment boundaries

	Type B	Type A
XR	leu (1) - lys (499)	gln (1) - glu (501)
TM	val (500) - trp (524)	leu (502) - trp (526)
TK1	arg (572) - his (662)	leu (572) - his (664)
KI	arg (663) - leu (766)	lys (665) - leu (767)
TK2	ser (767) - phe (919)	thr (768) - phe (920)

This is the replacement sheet

Date: - 7 OCT 1996

The intracellular region of the receptor contains a tyrosine kinase activity. PDGF receptors have a characteristic split tyrosine kinase domain, with an insert segment of about 103 or 104 amino acids. This insert segment has regulatory and other properties which are described in greater detail below.

The present invention provides isolated nucleic acids and polypeptides relating to PDGF receptors. An isolated molecule is one which is substantially separated from substances which naturally accompany the molecule when found in its natural form. For example, an isolated polypeptide is one which is substantially purified away from naturally accompanying human cell components, e.g., human nucleic acids, lipids, and other proteins. Of particular interest are contiguous segments of polypeptide which might be separated by genetic recombination or deletion techniques. Thus, an expression product of an isolated and manipulated genetic sequence is an isolated polypeptide, as used herein, even if expressed in a homologous cell type. Synthetically made forms or molecules expressed by heterologous cells are inherently isolated molecules.

Physiologically, the receptor is responsible for initiating a number of metabolic changes in, e.g., phosphatidylinositol levels, pH, calcium ion levels, cytoskeletal structure, gene expression, cAMP levels, and ligand receptor levels. Many of these metabolic changes are characteristic of a mitogenic, e.g., ligand induced, response.

The type A and type B PDGF receptor forms, when combined in the various dimer combinations into functional receptor complexes, also have characteristic binding affinities for the various forms of PDGF's, e.g., for the AA, AB and BB forms. For example, a type B isoform receptor binds the BB isoform PDGF at high affinity, the AB heteroform PDGF with lower affinity, and the AA isoform PDGF at low or no affinity. The type A isoform receptor binds to the AA isoform PDGF with high affinity, and with the AB heterodimer and BB isoform PDGF's with lower affinity.

The term "ligand" refers to molecules, usually members of the platelet-derived growth factor family, that bind

This is the replacement sheet

Date: - 7 OCT 1996

the segments involved in the growth factor binding. Also, a ligand is a molecule which serves either as a natural ligand to which the receptor, or an analogue of the receptor, binds, or a functional analogue of a natural ligand. The functional analogue may be a ligand with structural modifications, or may be a wholly unrelated molecule which has a molecular shape which interacts with the appropriate ligand binding determinants. The ligands may serve as agonists or antagonists, see, e.g., Goodman et al. (eds) (1990) Goodman & Gilman's: The Pharmacological Bases of Therapeutics (8th ed), Pergamon Press.

II. Polypeptides

The PDGF receptor is believed to be a dimer of similar polypeptides and the component polypeptides determine the dimerized receptor binding affinity for ligand.

The AA homodimer PDGF ligand is preferentially bound with high affinity by one form of the hPDGF receptor, and this form is correlated with a polypeptide chain referred to as the A receptor, the α receptor, and, as used herein, the type A receptor polypeptide (A-hPDGF-R). The receptor dimer is apparently a homodimer of the type A receptor polypeptides.

The BB homodimer PDGF ligand is preferentially bound with high affinity by two forms of the hPDGF-receptor. These forms are correlated with a second polypeptide chain referred to as the B receptor, the β receptor, and, as used herein, the type B receptor polypeptide (B-hPDGF-R). These two receptor dimer forms are apparently the homodimer of the B receptor polypeptides and the heterodimer type B/type A form.

The designations type A and type B polypeptides are each intended to also cover all alleles of each respective isoform. Thus, naturally occurring variants of each are embraced by the invention, as are other variants, analogues, and modified sequences.

The nucleotide sequence of a cDNA sequence encoding one B-hPDGF-R allele is set forth in Table 2 (See SEQ ID No.: 1) together with the deduced amino acid sequence of the receptor precursor. The following descriptions indicate

This is the replacement sheet

Date: - 7 OCT 1996

presumed gross structural and functional characterizations based upon analogy to the mouse and other growth factor receptors and proteins.

The sequence beginning at the amino acid numbered 1 corresponds to the putative amino terminus of a mature form of a human PDGF-R. The first 32 amino acids (designated -32 to -1) encode the putative signal peptide sequence. The putative transmembrane sequence corresponds to amino acid residues val(500) to trp(524). Potential N-glycosylation sites are at positions corresponding to amino acids asn(13) - ser(15); asn(57) - thr(50); asn(71) - ser(73); asn(183) - ser(185); asn(198) - thr(200); asn(260) - thr(262); asn(275) - thr(277); asn(322) - thr(324); asn(339) - ser(341); asn(436) - ser(438); and asn(447) - thr(449). The putative tyrosine kinase domain is interrupted by an amino acid insertion between about arg(663) and leu(766), see Table 1. The putative polyadenylation site is at the 3' end of the given cDNA sequence.

This is the replacement sheet

Date: - 7 OCT 1996

TABLE 2, panels A--?
sequence of the type B hPDGF-R from 12418-14 disk, file 01table
both nucleic acid and polypeptide sequence

This is the replacement sheet

Date: - 7 OCT 1996

The nucleotide sequence of a cDNA sequence encoding one allele of a type A hPDGF-R is set forth in Table 3 (See SEQ ID No. 5), together with the deduced amino acid sequence of the receptor (See SEQ ID No. 3). The structural features, as described, are again based upon analogy to the mouse PDGF receptors and other growth factor receptors and proteins.

The sequence beginning at the amino acid numbered 1 corresponds to the putative amino terminus of a mature form of a human PDGF-R. The first 23 amino acids (designated -23 to -1) encode the putative signal peptide sequence. The putative transmembrane sequence corresponds to amino acid residues leu(502) to trp(526). Potential N-glycosylation sites are at positions corresponding to amino acids asn(19) - ser(21); asn(53) - ser(55); asn(80) - thr(82); asn(156) - thr(158); asn(330) - thr(332); asn(336) - thr(338); asn(435) - thr(437); and asn(445) - ser(447). The putative tyrosine kinase domain is interrupted by an amino acid insertion between about lys(665) and leu(767), see Table 1.

This is the replacement sheet

Date: - 7 OCT 1996

TABLE 3, panel A

sequence of the type A hPDGF-R from 12418-14 disk; file 02table

This is the replacement sheet

Date: - 7 OCT 1996

As seen in Tables 2 and 3, the presumed intracellular tyrosine kinase domain of the type A and type B receptors have about 80% identical residues. The putative extracellular regions of the type A and B receptors have about 34-35% identical residues, an additional 14% of the remaining residues being conservative substitutions, i.e., substitutions with amino acids having similar chemical properties. The designated transmembrane regions of the hPDGF receptors have about 48% identical residues. Of the 52% of residues that differ, 70% are conservative substitutions. As seen in the tables, both receptor sequences have an amino acid insertion of about 103 or 104 amino acids interrupting the putative tyrosine kinase region. The overall homology between the two polypeptide sequences is about 44%.

The term platelet-derived growth factor receptor (PDGF-R) refers to a composition having properties characteristic of a PDGF-R. The native human PDGF receptors are examples of the class of PDGF-Rs, as are each of the polypeptides whose sequences are disclosed in Tables 2 and 3, and allelic variants. The natural receptor is typically a membrane glycoprotein having a molecular weight of about 180 kd. The receptor is normally found in vascular smooth muscle cell, fibroblasts, and glial cells, but not commonly observed in endothelial cells or on most hematopoietic cells.

As indicated above, the PDGF receptors have three major identifiable regions. The first is an extracellular region which contains the ligand binding determinants for the PDGF, i.e., the ligand binding segments. The extracellular region is also divided into Ig-like domains with the characteristics described above. The second major identifiable region is a transmembrane region and the third is an intracellular region. The intracellular region contains a characteristic type of tyrosine kinase domain, interrupted by a kinase insert (KI) segment.

The description "human" refers to the origin of the composition. In one use, it implies that the composition was found, at one point, within a human or human-derived cell or a minor variant from a naturally occurring human cell. With

This is the replacement sheet

Date: - 7 OCT 1996

respect to a protein or nucleic acid or sequence, it refers to a composition encoded by a natural human gene, or a closely related gene. Thus, the present invention includes proteins and nucleic acids encoding such proteins. The nucleic acids include RNA, cDNA, genomic DNA, synthetic forms, and mixed polymers, both sense and antisense strands. Thus, a protein derived from the sequence information disclosed, even if made synthetically, would be considered a human sequence. Furthermore, different alleles of each isoform are each also considered a human PDGF-R.

Thus, for the purposes used herein, the term human PDGF-R, when applied to a polypeptide, means a protein or polypeptide, which is substantially homologous to the amino acid sequences depicted in Table 2 or 3 and any alleles of them, or a fragment thereof. Ordinarily, hPDGF-R's will be at least about 80 percent homologous to the described PDGF-R sequences, preferably in excess of about 90 percent homology, and, more preferably, at least about 95 percent homology. The receptor will ordinarily also exhibit at least some biological activity in common with a hPDGF-R provided in the relevant sequences, e.g., PDGF binding, tyrosine kinase activity, phosphatidylinositol 3' kinase (PI3 kinase) binding, or immunologic properties. The hPDGF receptor embraces forms of the molecule which share the primary structural sequence, and is intended to encompass chemical and biochemical modifications, e.g., glycosylation, phosphorylation, ubiquinization, disulfide bonds, and other minor alterations in the basic primary sequence.

In particular, glycosylation alterations are included, made by modifying the glycosylation patterns of a polypeptide during its synthesis and processing, or in further processing steps. Particularly preferred means for accomplishing this are by exposing the polypeptide to glycosylating enzymes derived from cells which normally provide such processing, e.g., mammalian glycosylation enzymes. Also embraced are versions of the same primary amino acid sequence which have phosphorylated amino acid residues, e.g., phosphotyrosine, phosphoserine, or phosphothreonine.

This is the replacement sheet

Date: - 7 OCT 1996

The type B hPDGF-R is an isotype of a hPDGF-R polypeptide whose properties have been described above, and includes all alleles of that sequence and minor modified sequences derived therefrom. Generally, the term also is intended to include fragments of the protein, particularly those which are characteristic of the human type B isoform. Corresponding meaning is intended in reference to the type A hPDGF-R.

In certain uses, the term applies to the functional receptor. As described, the functional form is believed to be a dimer complex of the type A receptor polypeptide, or of the type B receptor polypeptide, or the heterodimer form.

A heterologous fusion protein is a fusion of proteins or segments which are naturally not normally fused in the same manner. Thus, the fusion product of an immunoglobulin with a hPDGF-R polypeptide is a continuous protein molecule having sequences fused in a typical peptide linkage, typically made as a single translation product and exhibiting both immunoglobulin and hPDGF-R polypeptide dependent propertis. A similar concept applies to heterologous nucleic acid sequences.

A polypeptide fragment, or segment, is a stretch of amino acid residues of at least about 5 amino acids, often at least about 7 amino acids, usually at least about 9 amino acids, more usually at least about 11 amino acids, typically at least about 13 amino acids, more typically at least about 15 amino acids, and, in various embodiments, at least about 17 or more amino acids. In a nucleic acid, a fragment or segment is a stretch of at least about 6 nucleotides, often at least about 9 nucleotides, usually at least about 12 nucleotides, more usually at least about 15 nucleotides, typically at least about 18 nucleotides, more typitally at least about 21 nucleotides, and in various preferred embodiments, at least about 24 or more nucleotides.

A polypeptide fragment is "physiologically active" when it substantially contributes to an activity characteristic of a natural polypeptide or fragment, e.g., a PDGF-R polypeptide. Typically, a hPDGF-R polypeptide, when associated into a functional receptor, has the set of activities performed

This is the replacement sheet

Date: - 7 OCT 1996

by the receptor in a biological context (e.g., in an organism or an in vitro biological context). In the case of the hPDGF-R, a functional fragment has these physiological activities, or functional activities characteristic of a hPDGF receptor, which include PDGF binding, tyrosine kinase activity and any of the mitogenic or other cellular responses mediated by the receptor. However, the protein fragment may exhibit other specific or inherent functions, such as vital conformational constraints, serving as a substrate site for glycosylation, contributing the signal sequence for cellular targeting, presenting characteristic epitopes absent in a mouse PDGF-R, or presently unrecognized functions possessed inherently.

Solubility of a polypeptide depends upon the environment and the polypeptide. Many parameters affect polypeptide solubility, including the temperature, the electrolyte environment, the size and molecular characteristics of the polypeptide, and the nature of the solvent. Typically, the temperature at which the polypeptide is used ranges from about 4°C to about 65°C. Usually the temperature at use is greater than about 18°C and more usually greater than about 22°C. For diagnostic purposes, the temperature will usually be about room temperature or warmer, but less than the denaturation temperature of components in the assay. For therapeutic purposes, the temperature will usually be body temperature, typically about 37°C for humans, though under certain situations the temperature may be raised or lowered in situ or in vitro.

The electrolytes will usually approximate in situ physiological conditions, but may be modified to higher or lower ionic strength where advantageous. The actual ions may be modified to conform to standard buffers used in physiological or analytical contexts.

The size and structure of the polypeptide should generally be in a substantially stable and globular state, and usually not in a denatured state. The polypeptide may be associated with other polypeptides in a quaternary structure, e.g., to confer solubility.

This is the replacement sheet

Date: - 7 OCT 1996

The solvent will usually be a biologically compatible buffer, of a type used for preservation of biological activities, and will usually approximate a physiological solvent. On some occasions, a detergent will be added, typically a mild non-denaturing one.

Solubility is usually measured in Svedberg units, which are a measure of the sedimentation velocity of a molecule under particular conditions. The determination of the sedimentation velocity was classically performed in an analytical ultracentrifuge, but is typically now performed in a standard ultracentrifuge. See, Freifelder (1982) Physical Biochemistry (2d ed.), W.H. Freeman, and Cantor and Schimmel (1980) Biophysical Chemistry, parts 1-3, W.H. Freeman & Co., San Francisco, each of which is hereby incorporated herein by reference. As a crude determination, a sample containing a putatively soluble polypeptide is spun in a standard full sized ultracentrifuge at about 50K rpm for about 10 minutes, and soluble molecules will remain in the supernatant. A soluble particle or polypeptide will typically be less than about 30S, more typically less than about 15S, usually less than about 10S, more usually less than about 6S, and, in particular embodiments, preferably less than about 4S, and more preferably less than about 3S.

The PDGF receptors or the specific extracellular regions of the receptors as provided herein may be used to affinity purify respective PDGFs. The full length, or entire, extracellular region, comprises the ligand-binding determinants of the type B receptor in Table 2, and extends from about leu(1) to lys(499). The full length, or entire, extracellular region comprising the ligand-binding determinants of the type A hPDGF-R shown in Table 3 extends from about gln(1) to glu(501). The ligand-binding determinants vary with different PDGF receptor alleles and may be anywhere from 5% to all of the extracellular region. The minimal amount of protein sequence essential for ligand binding may be determined by excising various segments of the extracellular region and measuring ligand binding. Studies of ligand-receptor interaction indicate that the ligand-binding region is located in the

This is the replacement sheet

Date: - 7 OCT 1996

extracellular region of the receptor. More sophisticated studies on the effects of various residues on the specificity of ligand binding are also made possible by the reagents of this invention. As used in this application, PDGF receptor or PDGF-R ligand-binding activity means having the ability to bind a PDGF, a PDGF agonist or antagonist, or other specific ligand for a hPDGF receptor. Usually these ligands will be members of the PDGF family, typically a human form. Therefore the external region has utility in establishing whether an analogue is a PDGF agonist or antagonist.

It is also likely that the PDGF-R, like many other growth factor receptors, is found naturally in a multimeric protein complex, most likely in dimer form. Thus, other important regions of a receptor will be those segments, either extracellular or otherwise, which are involved in dimerization or protein-protein interaction.

Besides substantially full-length polypeptides, the present invention provides for biologically active fragments of the polypeptides, or analogues thereof, including organic molecules which simulate the interactions of the peptides. Significant biological activities include ligand-binding, immunological properties, dimer association, serving as a kinase substrate, interacting with other mediators specific for binding to a PDGF-R protein, and other biological activities characteristic of human PDGF receptor polypeptides. Immunological activities include both immunogenic function in a target immune system, sharing of immunological epitopes for binding, and serving as either a competitor or substitute antigen for a human PDGF receptor epitope. Assays for these immunological activities are known in the art, see, e.g., below in the Experimental section; Harlow and Lane (1989) Antibodies: A Laboratory Manual, Cold Spring Harbor Press, New York; and Escobedo et al. (1988) J. Biol. Chem. 263:1482-1487, which are each hereby incorporated herein by reference. For example, ligand-binding or other segments may be "swapped" between different new fusion polypeptides or fragments. Thus, new chimeric polypeptides exhibiting new combinations of specificities will result from the functional linkage of

This is the replacement sheet

Date: - 7 OCT 1996

ligand-binding specificities and intracellular regions. For example, the Ig domains from other related polypeptides may be added or substituted for other Ig-like domains of these receptors. The resulting protein will often have hybrid function and properties.

For immunological purposes, immunogens may be produced which tandemly repeat polypeptide segments, thereby producing highly antigenic proteins. Alternatively, such polypeptides will serve as highly efficient competitors for specific binding. Production of antibodies to hPDGF receptor polypeptides is described below.

The present invention also provides for other polypeptides comprising fragments of PDGF receptors and polypeptides substantially homologous thereto. The receptor peptides of the present invention will generally exhibit at least about 80% homology with naturally occurring sequences of hPDGF receptor, typically at least about 85% homology with a natural receptor sequence, more typically at least about 90% homology, usually at least about 95% homology, and more usually at least about 97% homology. The length of comparison sequences will generally be at least about 16 amino acids, usually at least about 20 residues, more usually at least about 24 residues, typically at least about 28 residues, and preferably more than about 35 residues.

Homology, for polypeptides, is typically measured using sequence analysis software, see, e.g., Sequence Analysis Software Package of the Genetics Computer Group, University of Wisconsin Biotechnology Center, 1710 University Avenue, Madison, Wisconsin 53705. Protein analysis software matches similar sequences using measure of homology assigned to various substitutions, deletions, substitutions, and other modifications. Conservative substitutions typically include substitutions within the following groups: glycine, alanine; valine, isoleucine, leucine; aspartic acid, glutamic acid; asparagine, glutamine; serine, threonine; lysine, arginine; and phenylalanine, tyrosine.

Fusion polypeptides between the receptors and other homologous or heterologous proteins are also provided.

This is the replacement sheet

Date: - 7 OCT 1996

Homologous polypeptides may be fusions between different growth factor receptors, resulting in, for instance, a hybrid protein exhibiting ligand specificity of one receptor and the intracellular region of another, or a receptor which may have broadened or weakened specificity of binding. Likewise, heterologous fusions may be constructed which would exhibit a combination of properties or activities of the derivative proteins. Typical examples are fusions of a reporter polypeptide, e.g., luciferase, with a segment or domain of a receptor, e.g., a ligand-binding segment, so that the presence or location of a desired ligand may be easily determined. See, e.g., Dull et al., U.S. No. 4,859,609, which is hereby incorporated herein by reference. Other gene fusion partners include bacterial β-galactosidase, trpE, Protein A, β-lactamase, alpha amylase, alcohol dehydrogenase, and yeast alpha mating factor. See, e.g,., Godowski et al. (1988) Science 241:812-816.

Such polypeptides may also have amino acid residues which have been chemically modified by phosphorylation, sulfonation, biotinylation, or the addition of other moieties, particularly those which have molecular shapes similar to phosphate groups. In some embodiments, the modifications will be useful labeling reagents, or serve as purification targets, e.g., affinity ligands.

Fusion proteins will typically be made either recombinant nucleic acid methods or by synthetic polypeptide methods. Techniques for nucleic acid manipulation are described generally, for example, in Sambrook et al. (1989), Molecular Cloning: A Laboratory Manual (2d ed.), Vols. 1-3, Cold Spring Harbor Laboratory, which are incorporated hereby by reference. Techniques for synthesis of polypeptides are described, for example, in Merrifield (1963) J. Amer. Chem. Soc. 85:2149-2156; Merrifield (1986) Science 232: 341-347; and Atherton et al. (1989) Solid Phase Peptide Synthesis: A Practical Approach, IRL Press, Oxford; each of which is incorporated herein by reference.

Fragments of an intact receptor are also embraced herein. Various fragments include the transmembrane segment,

This is the replacement sheet

Date: - 7 OCT 1996

which confers a cell surface attachment function. Various fragments deleting either intracellular or extracellular segments will be produced. For example, fragments comprising a transmembrane segment, either of a PDGF receptor or another protein, in combination with ligand binding determinants, will provide membrane associated ligand binding regions. Similar constructs with intracellular regions may be prepared, producing, e.g., membrane associated tyrosine kinase segments.

In particular, soluble fragments comprising ligand binding determinants will be produced which may be used for absorbing free ligand. The soluble fragments can be used for diagnostic and therapeutic purposes to block PDGF mediated responses.

In another embodiment, intracellular fragments and analogues will be prepared. The kinase insert region (KI) within the tyrosine kinase domains is a particularly useful fragment, as described below.

A peptide analogue of a phosphorylated region of a protein is a peptide with substantial primary sequence homology to a phosphorylated region of another protein, and having at least one phosphorylated or similarly derivatized residue at positions corresponding to the position of similar phosphorylated residues in the phosphorylated region of that other protein. Where such a region has two or more phosphorylated residues, peptide analogues will, in certain embodiments, have only one such phosphorylated or similarily derivatized residue. Such phosphorylated residues will generally include phosphotyrosine, phosphoserine, or phosphothreonine. The peptide analogue will sometimes have one or more unnatural amino acids, including, e.g., residues chemically modified by biotinylation, phosphorylation, sulfonation, or glycosylation. It will generally be in monomer form or a multimer of repeated peptide units, and often will be joined to heterologous proteins or peptide fragments. Such heterologous proteins or peptides will often include segments derived from various proteins, e.g., immunoglobulins, β-galactosidase, β-glucuronidase, luciferase, trpE, or Protein A. See, e.g., Godowski et al. (1988) Science 241: 812-816. Such

This is the replacement sheet

Date: - 7 OCT 1996

peptide analogues will generally be soluble or coupled to a solid phase support, e.g., nitrocellulose, nylon, column packing materials (e.g., Sepharose beads), magnetic beads, glass wool, cells, or other substrates.

A phosphorylated region of a protein important for normal protein function will often be identified by a number of approaches, e.g., analysis of mutated proteins that have deletions or point mutations resulting in a change of ability to bind to another protein, altered enzymatic activity, or modification of other protein characteristics. Methods well known to those in the art are available to determine whether a peptide region is phosphorylated in vivo. See, e.g., Kazlauskas and Cooper (1989) Cell 58: 1121-1133; Williams (1989) Science 243: 1564-1570; and Wahl et al. (1990) J. Biol Chem. 265: 3944. Direct structural determination, e.g., x-ray crystallographic or 2D-NMR, can be used to determine locations of interactions, which guide where modifications are likely to affect interactions, both ligand and effector binding activities.

Fragments, in various extracellular region and intracellular region embodiments, will generally be at least about 1200 daltons, often be at least about 2400 daltons, typically at least about 3000 daltons, more typically at least about 3600 daltons, and in some preferred embodiments, at least about 4200 daltons or more.

III. Nucleic Acids

The nucleic acid compositions of this invention will generally be in RNA or DNA forms, or even a mixed polymer. The described DNA embodiment is usually derived from genomic DNA or cDNA, prepared by synthesis, or derived from combinations thereof. The DNA compositions generally include the complete coding region encoding hPDGF-R or fragments thereof, e.g., comprising at least 8 codons (24 bp), usually at least 12 codons, more usually at least about 15 codons, typically at least about 20 codons, more typically at least about 30 codons and preferably even more. One or more introns may be present.

This is the replacement sheet

Date: - 7 OCT 1996

The term hPDGF-R, when loosely applied to a nucleic acid, refers to a nucleic acid which encodes a hPDGF-R polypeptide, fragment, or variant, including protein fusions or deletion variants. A nucleic acid encodes a polypeptide when a corresponding message of a sequence, or its complement, is translated, as provided by a universal code of nucleotide triplets into polypeptide primary structure.

An "isolated" nucleic acid is a nucleic acid, e.g., an RNA, DNA, or a mixed polymer, which is substantially separated from other DNA sequences which naturally accompany a native human sequence, e.g., ribosomes, polymerases, and many other human genome sequences. The term embraces a nucleic acid sequence which has been removed from its naturally occurring environment, and includes recombinant or cloned DNA isolates and chemically synthesized analogues or analogues biologically synthesized by heterologous systems. A substantially pure molecule includes isolated forms of the molecule.

An isolated nucleic acid will generally be a homogenous composition of molecules, but will, in some embodiments, contain minor heterogeneity. This heterogeneity is typically found at the polymer ends or portions not critical to a desired biological function or activity.

The term "encoding" refers generally to the sequence information being present in a translatable form, usually operably linked to a promoter. A sequence is operably linked to a promoter when the functional promoter enhances transcription or expression of that sequence. An anti-sense strand is considered to also encode the sequence, since the same informational content is present in a readily accessible form, especially when linked to a sequence which promotes expression of the sense strand. The information is convertible using the standard, or a modified, genetic code. See, e.g., Watson et al. (1987) The Molecular Biology of the Gene (4th ed.) vols. 1&2, Benjamin, Menlo Park, California.

The term "recombinant" refers to a nucleic acid sequence which is not naturally occurring, or is made by the artificial combination of two otherwise separated segments of sequence. This artificial combination is often accomplished by

This is the replacement sheet

Date: 7 OCT 1996

either chemical synthesis means, or by the artificial manipulation of isolated segments of nucleic acids, e.g., by genetic engineering techniques. Such is usually done to replace a codon with a redundant codon encoding the same or a conservative amino acid, while typically introducing or removing a sequence recognition site. Alternatively, it is performed to join together nucleic acid segments of desired functions to generate a single genetic entity comprising a desired combination of functions not found in the common natural forms. Restriction enzyme recognition sites are often the target of such artificial manipulations, but other site specific targets, e.g., promoters, DNA replication sites, regulation sequences, control sequences, or other useful features may be incorporated by design. A similar concept is intended for a recombinant, e.g., fusion, polypeptide.

Homologous sequences, when compared, exhibit similarity. The standards for homology in nucleic acids are either measures for homology generally used in the art or hybridization conditions. The hybridization conditions are described below, but are further limited by the homology between the corresponding human and mouse segments. Homology will be limited, in addition to any stated parameters, by any similarity between the human and mouse sequences such that the stated homology specifically is limited by the conditions which are sufficient for the corresponding mouse segment of the segment being compared to match the stated human segment.

Substantial homology in the nucleic acid context means either that the segments, or their complementary strands, when compared, are identical when optimally aligned, with appropriate nucleotide insertions or deletions, in at least about 60% of the residues, usually at least about 70%, more usually at least about 80%, preferably at least about 90%, and more preferably at least about 95 to 98% of the nucleotides. Alternatively, substantial homology exists when the segments will hybridize under selective hybridization conditions, to a strand, or its complement, typically using a sequence derived from Table 2 or 3. Selectivity of hybridization exists when hybridization occurs which is more selective than total lack of

This is the replacement sheet

Date: - 7 OCT 1996

specificity. Typically, selective hybridization will occur when there is at least about 55% homology over a stretch of at least about 14 nucleotides, preferably at least about 65%, more preferably at least about 75%, and most preferably at least about 90%. See, Kanehisa (1984) Nuc. Acids Res. 12:203-213, which is incorporated herein by reference. The length of homology comparison, as described, may be over longer stretches, and in certain embodiments will often be over a stretch of at least about 17 nucleotides, usually at least about 20 nucleotides, more usually at least about 24 nucleotides, typically at least about 28 nucleotides, more typically at least about 32 nucleotides, and preferably at least about 36 or more nucleotides.

Stringent conditions, in referring to homology, will be stringent combined conditions of salt, temperature, organic solvents, and other parameters typically controlled in hybridization reactions. Stringent temperature conditions will generally include temperatures in excess of 30° C, typically in excess of 37°, and preferably in excess of 45°. Stringent salt conditions will ordinarily be less than 1000 mM, typically less than 500 mM, and preferably less than 200 mM. However, the combination of parameters is much more important than the measure of any single parameter. See, e.g., Wetmur and Davidson (1968) J. Mol. Biol. 31:349-370, which is hereby incorporated herein by reference.

While the wild-type sequences of these alleles will generally be employed, in some situations one or more mutations or minor modifications may be introduced, such as deletions, substitutions or insertions resulting in changes in the amino acid sequence, providing silent mutations or modifying amino acid residues or amino or carboxyl terminal groups. There will be circumstances where gene fusions between a hPDGF-R and another protein can be useful, or a fusion between the separate types A and B forms. The nucleic acid sequence, usually genomic, will often not exceed about 50 kb, more often will not exceed about 40 kb, typically will not exceed about 30 kb, more typically will not exceed about 20 kb, usually will not exceed about 10 kb, and in various embodiments, preferably will not

This is the replacement sheet

Date: - 7 OCT 1996

exceed about 6 kbp. Such sequences may have any or all introns removed.

A DNA fragment encoding hPDGF-R finds use to isolate DNA encoding PDGF receptors of other species which share substantial homologies with hPDGF-R. Fragments from the intracellular tyrosine kinase region can be used to isolate other tyrosine kinases. Portions of the DNA fragment having at least about 10 nucleotides, usually at least about 20 nucleotides, more usually at least about 30 nucleotides, and fewer than about 6 knt (kilonucleotides), usually fewer than about 0.5 knt, from a DNA sequence encoding a hPDGF-R find use as probes. The probes can be used to determine whether RNA encoding a hPDGF-R or a fragment thereof is present in a cell.

Additionally, the type B human PDGF receptor gene is located at a site on chromosome 5 where a number of growth control related genes are clustered. At least one genetic disease, 5q minus syndrome, has been shown to involve a deletion in this region. The type A receptor gene is located on chromosome 4 near the c-kit proto-oncogene. These sequences, often intact genes, will find use in diagnosing the integrity of these chromosomal regions. Fragments of hPDGF-R gene sequences will often be used as markers to probe the structure of these important regions of the genome and to diagnose genetic diseases associated with those areas of the genome. Alternatively, the sequences are useful to isolate other fragments for testing the integrity of the chromosome regions.

The recombinant nucleic acid sequences used to produce fusion proteins of the present invention will often be derived from natural or synthetic sequences. Many natural gene sequences are obtainable from various libraries, cDNA or genomic, using appropriate probes. See, GenBank™, National Institutes of Health. Typical probes for human PDGF receptors will be selected from the sequences of Table 2 or 3 in accordance with standard procedures, or from alleles of them. The phosphoramidite method described by Beaucage and Carruthers (1981) Tetra. Letts. 22:1859-1862, will produce suitable synthetic DNA fragments. A double stranded fragment will often

This is the replacement sheet

Date: - 7 OCT 1996

be obtained either by synthesizing the complementary strand and annealing the strand together under appropriate conditions or by adding the complementary strand using DNA polymerase with an appropriate primer sequence.

IV. Methods for Making PDGF Receptor and Fragments

The novel nucleic acids provided herein are useful for making PDGF receptor. The DNA fragment or portions thereof will be used to prepare an expression construct for a hPDGF-R. The expression construct normally comprises one or more DNA sequences encoding a hPDGF-R under the transcriptional control of a native or other promoter. When more than one sequence encoding hPDGF-R is present in the construct, the sequences will encode the same or different forms of the receptor, usually different. Usually the promoter will be a eukaryotic promoter for expression in a mammalian cell, where the mammalian cell may or may not lack PDGF receptors. The transcriptional regulatory sequences will typically include a heterologous enhancer or promoter which is recognized by the host. The selection of an appropriate promoter will depend upon the host, but promoters such as the trp, lac and phage promoters, tRNA promoters and glycolytic enzyme promoters are known. See, e.g., Sambrook et al. (1989). Conveniently available expression vectors which include the replication system and transcriptional and translational regulatory sequences together with the insertion site for the fibroblast growth factor receptor DNA sequence may be employed. Examples of workable combinations of cell lines and expression vectors are described in Sambrook et al. (1989); see also, Metzger et al. (1988), Nature 334:31-36, each of which is hereby incorporated herein by reference. In particular, non-fungal promoters will be preferred where expression occurs in non-fungal cells. Occasionally, it might be useful to express the sequences in other types of cells, and appropriate promoters may be selected. And in some circumstances, an inducible promoter may be preferred. In other circumstances, it will be desired to coexpress a glycosylation enzyme which will provide

This is the replacement sheet

Date: - 7 OCT 1996

a glycosylation pattern similar to that provided by a different cell type.

In cases where one wishes to expand the DNA sequence or produce the receptor protein or fragments thereof in a prokaryotic host, a preferred promoter is a prokaryotic promoter, e.g., trp, lac, and lambda, and see Sambrook et al. (1989) for other useful prokaryotic promoters. Usually a strong promoter will be employed to provide for high level transcription and expression.

The expression construct will often be contained in a vector capable of stable extrachromosomal maintenance in an appropriate cellular host or may be integrated into the host genome. The expression construct may be bordered by sequences which allow for insertion into a host, such as transposon sequences, lysogenic viral sequences, or the like. Normally, markers are provided with the expression construct which allow for selection of host cells containing the construct. The marker may be on the same or a different DNA molecule, preferably on the same DNA molecule.

In mammalian cells, the receptor gene itself will often provide a convenient marker. However, in prokaryotic cells, markers such as a resistance to a cytotoxic agent, complementation of an auxotrophic host to prototrophy, production of a detectable product, etc., will be more convenient.

The expression construct can be joined to a replication system recognized by the intended host cell. Various replication systems include viral replication systems such as retroviruses, simian virus, bovine papilloma virus, or the like. In addition, the construct may be joined to an amplifiable gene, e.g., DHFR gene, so that multiple copies of the desired hPDGF-R gene may be made. See, e.g., Schimke, R. (1984) Cell 37:705-713; and Kaufman et al. (1985) Mol. Cell Biol. 5:1750-1759; each of which is hereby incorporated herein by reference.

In particular, it will often be desired to express a receptor polypeptide in a system which provides a non-fungal glycosylation pattern. In this case, the usual pattern will be

This is the replacement sheet

Date: - 7 OCT 1996

that provided naturally by the expression system. However, the pattern will be modifiable by exposing the polypeptide, e.g., an unglycosylated form, to appropriate glycosylating proteins introduced into a heterologous expression system. For example, the PDGF receptor gene may be co-transformed with one or more genes encoding mammalian or other glycosylating enzymes, preferably originating from a non-fungal species, and in some embodiments, non-human species. Using this approach, certain mammalian glycosylation patterns will be achievable in prokaryote or other cells.

The means of introduction of the expression construct into a host cell will vary depending upon the particular construction and the target host. Introduction can be achieved by any convenient means, including fusion, conjugation, transfection, transduction, electroporation, injection, or the like. See, e.g., Sambrook, et al. (1989) Molecular Cloning: A Laboratory Guide, Vols 1-3, Cold Spring Harbor Press, which is hereby incorporated herein by reference. Introduction of constructs encoding different forms of the receptor into a single host cell is also contemplated. The host cells will normally be immortalized cells, i.e., cells that can be continuously passaged in culture. For the most part, these cells will be convenient mammalian cell lines which are able to express a hPDGF-R and, where desirable, process the polypeptide so as to provide an appropriate mature polypeptide. By processing is intended glycosylation, ubiquitination, disulfide bond formation, general post-translational modification, or the like. Usually the host will be able to recognize the signal sequence for inserting hPDGF-R into the membrane of the cell. If secretion is desired, the transmembrane sequence will generally be deleted or mutated to prevent membrane localization of the protein.

A wide variety of hosts will be employed for expression of the peptides, both prokaryotic and eukaryotic. Useful hosts include bacteria, such as E. coli, yeast, filamentous fungi, insect cells, mammalian cells, typically immortalized, e.g., various mouse cell lines, monkey cell lines, Chinese hamster ovary cell lines, human cell lines,

This is the replacement sheet

Date: - 7 OCT 1996

derivatives of them, or the like. In some cases, the cells will be derived from a neoplastic host cell or wild-type cells will be transformed with oncogenes, tumor causing viruses or the like.

Under some circumstances, it will be desirable to transfect mammalian cells which lack a PDGF receptor, e.g., with a construct where a signal sequence and transmembrane region direct the receptor peptide to the cell membrane. Progeny of transformed or transfected cells are also intended to be encompassed. Lymphocytes and cardiac myocytes are examples of primary cells which lack a receptor. Chinese hamster ovary cells (CHC), epithelial cells lines, and a number of human tumor cell lines also lack PDGF receptors.

The compositions and cells comprising hPDGF receptors and fragments can be used for diagnostic purposes and to study and treat diseases or medical conditions associated with PDGF receptors. Use of the soluble extracellular ligand binding fragments has already been described generally. In addition, various segments of the intracellular region include phosphorylated amino acid residues. The sites of phosphorylation are important in interaction of segments with other polypeptides. The nature and specificity of the interactions are highly dependent upon the presence or absence of the phosphorylation, and fragments of the intact receptor, or related sequences, will be useful in modulating the interactions. Thus, intracellular region fragments will also find important uses.

Cells expressing cloning vehicles containing defined sequences can be used to define specific sites of a PDGF receptor necessary for effecting a particular activity. Alternatively, these cells will be useful to assess the ability of a selected receptor to bind different ligands (PDGFs and analogues) thereby providing a powerful tool for evaluating the potential of drugs for promoting or inhibiting specific PDGF-induced cellular responses.

Cells transfected, injected, infected or electroporated with DNA or mRNA containing a full length natural PDGF-R sequence will often express the native or wild

This is the replacement sheet

Date: - 7 OCT 1996

type receptor and respond accordingly less than full length segments will often have desired equivalent functions. Specific concentrations of a purified receptor or a receptor polypeptide fragment can be used to block the binding of the ligand (PDGF) to native PDGF receptors. Alternatively, antibodies to the receptor or fragment can have the same effect. In particular, it has been demonstrated herein that antibodies against epitopes of the extracellular region can block ligand-receptor binding. Other antibodies will block dimerization of receptor polypeptides, and thus modulate receptor function.

Homogeneous and defined polypeptides and DNA sequences will find use in raising antibodies and defining specificity of their binding. In particular, antibodies against specific regions of the receptor, e.g., the ligand-binding segments, will find use in diagnostic testing or therapeutics. The reagents PDGF-R, PDGF-R polypeptides, and antibodies to specific regions of the receptor can be used to study regulation of PDGF mediated activities. Intracellular fragments will also have important uses, especially, e.g., the kinase insert segment.

PDGF has roles in tissue repair, embryogenesis, and likely is involved in atherosclerosis, myeloproliferative disease, and some carcinomas. Treatment of these conditions responds to attenuation by therapeutic administration of the soluble extracellular fragments or antibodies. For example, PDGF agonists stimulate cell proliferative development, an effect particularly beneficial in wound healing, muscle regeneration, and arterial wall proliferation. PDGF antagonists will be used, in some cases, to block excess response, or to modulate PDGF response.

Compositions containing a soluble PDGF-R polypeptide having between about five and two hundred, preferably about 10 or 15 to 50, contiguous amino acids from a human PDGF-R extracellular region are described. In one embodiment, the polypeptide contains at least about 80 amino acids from residues 1 to 499 of a type B human PDGF receptor of Table 2,

This is the replacement sheet

Date: - 7 OCT 1996

or from residues 1 to 501 of a type A human receptor of Table 3.

In another embodiment, fragments of the intracellular region will find various uses. In particular, the KI region, and fragments or analogues thereof, will find use in blocking or modulating interaction of a phosphorylated residue with a recognition interaction, usually binding by another protein. These interactions are often important in further signal transduction and cellular responses.

The hPDGF-R protein expressed by transfected cells also finds many uses. If the peptide is secreted, the peptide will typically be isolated from the supernatant in which the expression host is grown. If not secreted, the peptide will be typically be isolated from the expression host, e.g., from a lysate. The peptide will generally be isolated by convenient techniques employing HPLC, electrophoresis, gradient centrifugation, affinity chromatography, e.g., using PDGF, column chromatography and other methods of protein purification used in protein biochemistry to provide a substantially pure product, e.g., particularly free of cell component contaminants. See, e.g., Jacoby (1984) Methods in Enzymology, Vol. 104, Academic Press, New York; Scopes (1987) Protein Purification: Principles and Practice, (2nd Ed.) Springer-Verlag, New York; Deutscher (ed.) (1990) Guide to Protein Purification, Methods in Enzymology, Vol. 182; each of which is hereby incorporated herein by reference.

The receptor protein or amino acids beginning at about leu(1) through lys(499) of the amino terminal sequence of the type B receptor, and about gln(1) through glu(501) of the sequence of the type A receptor, which form the entire extracellular regions, and particularly the PDGF ligand binding portions of the receptor proteins, will find use to affinity purify PDGF. The extracellular region can also be used affixed to a solid substrate or free in solution to determine drugs useful as PDGF agonists and antagonists.

The intracellular region of the protein, beginning at about val(500) through the carboxyl terminal amino acid of the type B receptor, and about leu(502) through the carboxyl

This is the replacement sheet

Date: - 7 OCT 1996

terminus of the type A receptor, also find use as tyrosine kinases for protein phosphorylatian in accordance with well known techniques. Additionally, amino acids met(-32) through gly(-1) of the amino terminal sequence of the type B receptor, and from about met(-23) through cys(-1) of the type A receptor, comprise signal sequences which direct the structural protein through the membrane of a transfected cell. These signal sequences will be used with a hPDGF-R, but also would be expected to find use with other proteins if fused to them.

Human PDGF receptor polypeptide fragments will typically be generated either by direct expression of truncated nucleic acid sequences, or by standard protease treatment of a hPDGF receptor, preferably purified. Reagents are provided herein for either approach.

As a diagnostic use, these reagents provide a method for measuring a PDGF or a PDGF receptor in a target sample, said method comprising the steps of:

combining said target sample with a hPDGF receptor polypeptide segment; and

determining the extent of binding between said polypeptide segment and said sample.

This invention also provides a transformed cell, which is also includes progeny of the primary transformant, capable of expressing a polypeptide homologous to at least a segment of human PDGF receptor. A preferred embodiment is where the cell expresses a polypeptide homologous to substantially the entire extracellular region of a human PDGF receptor, including soluble proteins.

V. Antibodies

Polyclonal and/or monoclonal antibodies to the various PDGF receptors and peptide fragments may also be prepared. Synthetic peptide fragments may be prepared in a peptide synthesizer and coupled to a carrier molecule (e.g., keyhole limpet hemocyanin) and injected into rabbits at selected times over several months. The rabbit sera is tested for immunoreactivity to the PDGF receptor protein or fragment. Monoclonal antibodies may be made by injecting mice with hPDGF-

This is the replacement sheet

Date: - 7 OCT 1996

R protein, hPDGF-R polypeptides or mouse cells expressing high levels of the cloned PDGF receptor on its cell surface. Monoclonal antibodies will be screened by ELISA and tested for specific immunoreactivity with the PDGF receptor protein or polypeptides thereof. See, e.g., Harlow and Lane (1988) Antibodies: A Laboratory Manual, Cold Spring Harbor Press, New York, which is hereby incorporated herein by reference. These antibodies will be useful in assays, or as pharmaceuticals.

Once a sufficient quantity of the desired hPDGF receptor polypeptide has been obtained, the protein will be used for various purposes. A typical use is the production of antibodies specific for binding to these receptors. These antibodies will usually be either polyclonal or monoclonal and will usually be produced by in vitro or in vivo techniques, e.g., directed towards the goal of defining or recognizing particular antigenic determinants, or epitopes. Usually the epitopes will be provided by molecular shapes of contiguous amino acid residues, but may be sequentially non-contiguous but in close spatial proximity due to secondary or tertiary structure. In particular, a polypeptide epitope homologous to a sequence of at least six contiguous amino acids described in Table 2 or Table 3 will be a useful immunogen. The epitopes of most interest will be those from a signal segment or immunoglobulin domains found in the extracellular region, particularly those surrounding the PDGF ligand binding regions. However, other segments will also be important, including phosphorylation sites in the intracellular region, e.g., the kinase insert segment, phosphorylated or not.

For production of polyclonal antibodies, an appropriate target immune system is selected, typically a mouse or rabbit. The substantially purified antigen is presented to the immune system in a fashion determined by methods appropriate for the animal and other parameters well known to immunologists. Typical sites for injection are in the footpads, intramuscularly, intraperitoneally, or intradermally. Of course, another species will sometimes be substituted for a mouse or rabbit, including goats, sheep, cows, guinea pigs, and rats.

This is the replacement shee

Date: - 7 OCT 1996

An immunological response is usually assayed with an immunoassay. Normally such immunoassays involve some purification of a source of antigen, for example, produced by the same cells and in the same fashion as the antigen was produced. The immunoassay will, in some instances, be a radioimmunoassay, an enzyme-linked assay (ELISA), a fluorescent assay, or any of many other choices, most of which are functionally equivalent but may exhibit advantages under specific conditions.

Monoclonal antibodies with affinities of 10^6 M^{-1} preferably 10^9 to 10^{10}, or stronger will typically be made by standard procedures as described, e.g., in Harlow and Lane, Antibodies: A Laboratory Manual, CSH Laboratory (1988); or Goding (1986) Monoclonal Antibodies: Principles and Practice (2d ed) Academic Press, New York; each of which is hereby incorporated herein by reference. Briefly, appropriate animals will be selected and the desired immunization protocol followed. After the appropriate period of time, the spleens of such animals are excised and individual spleen cells fused, typically, to immortalized myeloma cells under appropriate selection conditions. Thereafter the cells are clonally separated and the supernatants of each clone are tested for their production of an appropriate antibody specific for the desired region of the antigen.

Other suitable techniques involve in vitro exposure of lymphocytes to the antigenic polypeptides or alternatively to selection of libraries of antibodies in phage or similar vectors. See, Huse et al. (1989) "Generation of a Large Combinatorial Library of the Immunoglobulin Repertoire in Phase Lambda," Science 246:1275-1281, which is hereby incorporated herein by reference. The polypeptides and antibodies of the present invention may be used with or without modification. Frequently, the polypeptides and antibodies will be labeled by joining, either covalently or non-covalently, a substance which provides for a detectable signal. A wide variety of labels and conjugation techniques are known and are reported extensively in both the scientific and patent literature. Suitable labels include radionuclides, enzymes, substrates, cofactors,

This is the replacement shee

Date: - 7 OCT 1996

inhibitors, fluorescent moieties, chemiluminescent moieties, magnetic particles, and the like. Patents, teaching the use of such labels include U.S. Patent Nos. 3,817,837; 3,850,752; 3,939,350; 3,996,345; 4,277,437; 4,275,149; and 4,366,241. Also, recombinant immunoglobulins may be produced, see Cabilly, U.S. Patent No. 4,816,567.

Full length peptides or portions thereof will, in particular embodiments, be used for producing antibodies, either polyclonal or monoclonal. Antibodies are produced by immunizing an appropriate vertebrate host, e.g., mouse, with the peptide or fragment itself, or in conjunction with an adjuvant. Usually two or more immunizations will be involved, and the blood or spleen will be harvested a few days after the last injection.

For polyclonal antisera, the immunoglobulins may be precipitated, isolated and purified, including affinity purification. For monoclonal antibodies, the splenocytes normally will be fused with an immortalized lymphocyte, e.g., a myeloid line, under selective conditions for hybridomas. The hybridomas will generally then be cloned under limiting dilution conditions and their supernatants screened for antibodies having the desired specificity. Techniques for producing antibodies are well known in the literature, see, e.g., Goding (1986) Monoclonal Antibodies: Principles and Practice (2d ed.) Academic Press, N.Y., and Harlow and Lane (1988) Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory, New York, and are exemplified by U.S. Patent Nos. 4,381,292, 4,451,570 and 4,618,577, which are each incorporated herein by reference. The antibodies generated therefrom may have many uses in dissecting the portions of the receptor responsible for various cellular responses and in the general processing and maturation of the receptor itself. Antibody agonists or antagonists might even be produced.

Both proteins and fragments to serve as immunogens, and as reagents to determine the specificity of binding, are provided herein.

VI. Methods for Use

This is the replacement sheet

Date: - 7 OCT 1996

The present invention provides a human platelet derived growth factor receptor (hPDGF-R) purification method as well as a method for synthesizing PDGF receptors within cells. Also provided are homogeneous receptors produced by these methods, nucleic acid sequences encoding the receptors or portions of the receptors, expression vehicles containing these sequences, cells comprising the PDGF-receptors, and antibodies to the receptors. Of particular interest are fragments of the receptors, which have functional binding sites which compete with receptors to bind particular ligands. See, e.g., Orchansky et al. (1988) "Phosphatidylinositol Linkage of a Truncated Form of the Platelet-derived Growth Factor Receptor" J. Biol. Chem. 263:15159-15165, which provides evidence that an extracellular region of the PDGF receptor can bind to the PDGF ligands. Also provided are phosphorylated fragments or sites for phosphorylation which interact with other proteins important in cellular response to ligand binding. In particular, soluble extracellular region segments are provided.

Transfected cells find use as a model for studying cellular responses to PDGF. For controlled investigation, mammalian cells which lack a PDGF receptor can be transfected with an expression construct comprising a DNA sequence encoding a hPDGF-R. Cells are produced that encode a receptor that is often functionally equivalent to the wild-type receptor and confer a PDGF-sensitive mitogenic response on the cell. In this way, the binding properties of the naturally-occurring PDGF will be analyzed, as well as fragments or synthetic compounds, both proteinaceous and non-proteinaceous. As demonstrated in the Experimental section, transfected cells were used to determine that the AA form of PDGF activates the type B receptor tyrosine kinase. The presence of the type A and type B receptor polypeptides in a single cell will facilitate the study of receptor binding properties and perhaps even receptor interactions.

In addition to studying PDGF-mediated mitogenesis, the transfected cells can be used to evaluate a drug's ability to function as a PDGF agonist or antagonist. In particular, transfected cells can be contacted with the test drug, and the

This is the replacement sheet

Date: - 7 OCT 1996

amount of response determined, e.g., receptor tyrosine kinase activation or the rate of DNA synthesis, as compared to control cells in the presence or absence of PDGF or analogs thereof. Alternatively, in a non-therapeutic environment, a method is provided for inhibiting binding between a PDGF analogue and a PDGF receptor present in a solution. This method will contain a step of adding a PDGF receptor peptide, e.g., a peptide homologous to a sequence described in Table 2 or Table 3 to the solution, or a mutation or modification of one. Binding affinity to variants of the PDGF will also be evaluated by such cells. The inhibition of binding will usually occur by competition or by interfering with binding, on either the receptor or the ligand. The assay will often make use of comparisons with and without test ligand, or by a displacement assay, where the displacing ligand is added after binding occurs.

However, as indicated above, the PDGF receptor likely functions in a dimer state. The soluble forms of the receptor may interfere with the dimerization and, in some embodiments, will be effective in blocking signal transduction by a different mechanism from competitive affinity for the PDGF ligands. The soluble, or intracellular or transmembrane fragments of the various receptor forms would interfere with dimer formation and thus blocks at least some types of, or some fraction of, signal transduction.

This observation provides a method for modifying <u>in vivo</u> a PDGF receptor modulated activity comprising administering to a patient an amount of a PDGF receptor blocking agent effective to inhibit PDGF binding to PDGF receptors. Sometimes, the blockage wil occur at the level of ligand binding, by blocking the functional assembly of the receptor complex, or by blocking further interaction of the receptor with other proteins important in effecting cellular response, e.g., phosphotyrosine binding interactions. As discussed above, the PDGF family of proteins have a significant role in regulating many important physiological processes. The soluble PDGF-R polypeptides will generaly be effective in modifying the extent of PDGF modulation of these processes.

This is the replacement sheet

Date: - 7 OCT 1996

For this reason, the soluble forms of the receptors are useful as competitive binding sites for PDGF. Likewise, truncated PDGF binding sites or binding sites which have been mutated, particularly those from the human forms described, will sometimes be equally effective as natural forms, but at a lesser cost, both in monetary terms of and in terms of medical side-effects, upon administration.

The reagents provided herein will also find use in the quantitative detection or diagnosis of PDGF analogues, or PDGF-like ligands for the receptors, or for PDGF receptor polypeptide production. Various medical conditions are indicated by an abnormal level of production of either of these proteins, including, e.g., various tumor conditions. Thus, diagnostic tests dependent upon these reagents are now available.

With the different PDGF types, combining segments into chimeric receptors will form different types of receptors having variations in affinities for the various ligands. With the genes and proteins of the present invention, distinctions between various patterns and receptor types will be found, specific for various tissue types. Thus, tissue markers based upon differences in PDGF receptor expression would become available.

Soluble fragments containing various regions of the extracellular region of the human PDGF receptor have been shown to retain high affinity specific binding as in the intact type B PDGF receptor. The extracellular region of the human receptor has been expressed at high levels in CHO cells and secreted into the medium. In the presence of ligand, the soluble extracellular fragments block the ability of BB-PDGF to initiate responses characteristic of a physiological mitogen response. The soluble fragments also retain high specificity in binding to only the BB PDGF, not altering the mitogenic effect by the AA PDGF. Additional experiments using the human extracellular region have shown that the fragments may be used as binding segments to assay, e.g., by competition, for the presence of ligands. These results suggest the possibility of treating various conditions which excessively respond to PDGF

This is the replacement sheet

Date: 7 OCT 1996

such as atherosclerosis, osteosarcoma, and glioblastoma. The mitogenic response may be attenuated through use of the soluble extracellular fragments.

The blocking of physiological response to PDGF results from the inhibition of binding of the ligand to the receptor, likely through competitive inhibition. Thus, in vitro assays of the present invention will generally use soluble fragments comprising the ligand binding segments of these receptors, or fragments attached to solid phase substrates. These assays will also allow for the diagnostic determination of the effects of either binding segment mutations and modifications, or ligand mutations and modifications, e.g., ligand analogues.

The soluble fragments will also find use in testing interaction of the receptor with modified ligands, e.g., biotinylated AA isoform or other PDGF forms. Another means for blocking ligand action will be to interfere with proper multi-protein receptor association, e.g., dimerization, or intracellular region interactions with other proteins. In particular, it is demonstrated that particular phosphorylation events control interactions with other protein which mediate other cellular functions. Various assays for this are well known to those skilled in the art, and see below.

Receptor components may be substituted by equivalent or corresponding soluble fragments from the other type polypeptide, e.g., exchanging fragments from the type A and type B polypeptide forms, or from other related polypeptides, e.g., mouse receptors, or other related receptors.

Moreover, because the type A homodimer binds all three forms of PDGF, the type A binding fragments exhibit substantial generality of ligand binding. The type A binding fragments are useful for preparing general PDGF binding reagents.

The quantities of reagents necessary for effective therapy will depend upon many different factors, including means of administration, target site, physiological state of the patient, and other medicants administered. Thus, treatment dosages should be titrated to optimize safety and efficacy.

This is the replacement sheet

Date: 7 OCT 1996

Typically, dosages used in vitro may provide useful guidance in the amounts useful for in situ administration of these reagents. Animal testing of effective doses for treatment of particular disorders will provide further predictive indication of human dosage. Various considerations are described, e.g., in Gilman et al. (eds) (1990) Goodman and Gilman's: The Pharmacological Bases of Therapeutics, 8th Ed., Pergamon Press; and Remington's Pharmaceutical Sciences, 17th ed. (1990), Mack Publishing Co., Easton, Penn.; each of which is hereby incorporated herein by reference. Methods for administration are discussed therein, e.g., for oral, intravenous, intraperitoneal, or intramuscular administration, transdermal diffusion, and others. Pharmaceutically acceptable carriers will include water, saline, buffers, and other compounds described, e.g., in the Merck Index, Merck & Co., Rahway, New Jersey. Because of the high affinity binding between PDGF and its receptors, low dosages of these reagents would be initially expected to be effective. Thus, dosage ranges would ordinarily be expected to be in amounts lower than 1 mM concentrations, typically less than about 10 μM concentrations, usually less than about 100 nM, preferably less than about 10 pM (picomolar), and most preferably less than about 1 fM (femtomolar), with an appropriate carrier. Slow release formulations, or slow release apparatus will often be utilized for continuous administration. The intracellular segments of the receptors, both the PDGF receptor and related receptors will find additional uses as described in detail below.

The pharmaceutical compositions will be administered by parenteral, topical, oral, or local administration, such as by aerosol or transdermally, for prophylactic and/or therapeutic treatment. The pharmaceutical compositions can be administered in a variety of unit dosage forms depending upon the method of administration. For example, unit dosage forms suitable for oral administration include powder, tablets, pills, capsules and dragees.

The pharmaceutical compositions will often be administered intravenously. Thus, this invention provides compositions for intravenous administration which comprise a

This is the replacement sheet

Date: 7 OCT 1996

solution of the compound dissolved or suspended in an acceptable carrier, preferably an aqueous carrier. A variety of aqueous carriers may be used, e.g., water, buffered water, 0.4% saline, and the like. These compositions will sometimes be sterilized by conventional, well known sterilization techniques, or may be sterile filtered. The resulting aqueous solutions may be packaged for use as is, or lyophilized, the lyophilized preparation being combined with a sterile aqueous solution prior to administration. The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions, such as pH adjusting and buffering agents, tonicity adjusting agents, wetting agents and the like, for example, sodium acetate, sodium lactate, sodium chloride, potassium chloride, calcium chloride, sorbitan monolaurate, triethanolamine oleate, etc.

For solid compositions, conventional nontoxic solid carriers may be used which include, for example, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharin, talcum, cellulose, glucose, sucrose, magnesium carbonate, and the like. For oral administration, a pharmaceutically acceptable nontoxic composition is formed by incorporating any of the normally employed excipients, such as those carriers previously listed, and generally 10-95% of active ingredient, preferably about 20% (see, Remington's, supra).

For aerosol administration, the compounds are preferably supplied in finely divided form along with a surfactant and propellant. The surfactant should, of course, be non-toxic, and preferably soluble in the propellant. Representative of such agents are the esters or partial esters of fatty acids containing from 6 to 22 carbon atoms, such as caproic, octanoic, lauric, palmitic, stearic, linoleic, linolenic, olesteric and oleic acids with an aliphatic polyhydric alcohol or its cyclic anhydride such as, for example, ethylene glycol, glycerol, erythritol, arabitol, mannitol, sorbitol, the hexitol anhydrides derived from sorbitol, and the polyoxyethylene and polyoxypropylene derivatives of these esters. Often mixed esters, such as

This is the replacement sheet

Date: - 7 OCT 1996

mixed or natural glycerides will be employed. The surfactant, in some embodiments, will constitute 0.1%-20% by weight of the composition, preferably 0.25-5%. The balance of the composition is ordinarily propellant. Liquefied propellants are typically gases at ambient conditions, and are condensed under pressure. Among suitable liquefied propellants are the lower alkanes containing up to 5 carbons, such as butane and propane; and preferably fluorinated or fluorochlorinated alkanes. Mixtures of the above will sometimes also be employed. In producing the aerosol, a container equipped with a suitable valve is filled with the appropriate propellant, containing the finely divided compounds and surfactant. The ingredients are thus maintained at an elevated pressure until released by action of the valve.

The compositions containing the compounds can be administered for prophylactic and/or therapeutic treatments. In therapeutic applications, compositions are administered to a patient already suffering from a disease, as described above, in an amount sufficient to cure or at least partially arrest the symptoms of the disease and its complications. An amount adequate to accomplish this is defined as "therapeutically effective dose." Amounts effective for this use will depend on the severity of the disease and the weight and general state of the patient.

In prophylactic applications, compositions containing the compounds of the invention are administered to a patient susceptible to or otherwise at risk of a particular disease. Such an amount is defined to be a "prophylactically effective dose." In this use, the precise amounts again depend on the patient's state of health and weight.

In a particularly important aspect of the present invention, it was recognized that many growth factors mediate their pleiotropic actions by binding to and activating cell surface receptors with an intrinsic protein tyrosine kinase activity. Growth factor receptors with tyrosine kinase activity, or receptor tyrosine kinases, typically possess similarities in molecular topology. For example, they possess a large extracellular ligand binding region, a hydrophobic

This is the replacement sheet

Date: - 7 OCT 1996

transmembrane region, and a cytoplasmic, i.e., intracellular, region that contains a tyrosine kinase catalytic domain. Receptor tyrosine kinases typically have a topology dictating that the ligand binding segments and protein tyrosine kinase activity are separated by the plasma membrane. Therefore, receptor activation due to extracellular ligand binding is translated across the membrane barrier into activation of intracellular region functions.

The present invention takes advantage of the discovery that phosphorylation of particular amino acid residues in a polypeptide segment is important in certain protein-protein interactions. The phosphorylation state of residues within the regions of interaction, or coupling regions, modulate the interactions. In the case of the PDGF receptor and phosphatidylinositol 3' kinase (PI3 kinase) interactions, the presence of a phosphotyrosine in the defined kinase insert sequence is important in the interacting between, e.g., binding between, the receptor and the PI3 kinase, thereby activating the PI3 kinase enzyme. An unphosphorylated KI segment fails to bind a PI3 kinase activity. These observations will be generally applicable to the interaction of phosphorylated segments with other binding proteins. With the elucidation of the nature and specificity of these interactions, analogues, e.g., organic molecules, are prepared which serve as analogues to the peptides or phosphorylated peptides.

Tyrosine kinases can be broadly classified into two groups, the transmembrane receptor kinases and the cytoplasmic non-receptor kinases, with most of the members of the second group belonging to the so-called src family of tyrosine kinases. The receptor kinases genes, including the PDGF receptor, share certain structural features, e.g., an amino-terminal signal peptide which is characteristic of membrane glycoproteins and a transmembrane segment which defines and separates the extracellular and the intracellular regions of the receptor protein. The amino-terminal portion of the receptor protein makes up the extracellular region which contains the ligand binding sites. The carboxy-terminal

This is the replacement sheet

Date: - 7 OCT 1996

portion of the protein makes up the intracellular region of the receptor, on which the kinase activity is located. Receptor kinases are responsible for transducing a signal provided by ligand binding to the extracellular region into the intracellular compartment, and through cytoplasmic second messages, ultimately to the nucleus.

Detailed comparison of established receptor tyrosine kinase (RTK) primary sequences has led to the identification of both shared and unique structural subdomains, or segments, which permit classification of the RTK family into distinct groups, see, e.g., Yarden and Ullrich (1988) Ann. Rev. Biochem. 57:443-478; and Ullrich and Schlessinger (1990) Cell 61:203-212; each of which is incorporated herein by reference.

The PDGF receptor is a member of one such subclass. These receptors, including macrophage growth factor (CSF-1-R) and the putative receptor c-kit share at least two distinct structural features. They lack cysteine-rich repeat clusters within their extracellular regions, and they possess another conserved repeat structure that includes five immunoglobulin-like repeats, suggesting a common architecture for the ligand-binding regions of the members of this receptor subclass.

Furthermore, when compared with RTKs of other subclasses, the catalytic domains of members of this group are interrupted by long, structurally unique, hydrophilic, proline-rich insertion sequences of about 77-107 amino acid residues. This "kinase insert" (KI), also referred to as a kinase insert region, divides the catalytic domain into two segments and is defined by homology to other tyrosine kinase domains. These KI sequences are highly divergent, even within each receptor subfamily, but are strongly conserved between evolutionarily distant species, such as chickens, cats and humans. Members of a related subclass, including the FGF receptor, flg, and bek, similarly have immunoglobulin-like repeats in their extracellular region, although only three in number, and kinase insert sequences of 10-19 residues located in the corresponding position of the tyrosine kinase segments.

RTK ligands induce pleiotropic cellular responses, which in many cell types culminate in cell cycle progression,

This is the replacement sheet

Date: - 7 OCT 1996

DNA synthesis, and cellular replication. Upon ligand binding, numerous responsive membrane events are initiated, including stimulation of ion transport, glucose transport, membrane kinases, pinocytosis, membrane ruffling, and other cytoskeletal and morphological changes. These events are paralleled by activation of a number of cytoplasmic pathways, including glycolysis, polyamine synthesis, and ribosomal protein S6 phosphorylation. Alterations in the pattern of specific gene transcription (e.g., c-myc and c-fos) are detectable within minutes, and increased macromolecular synthesis of protein, RNA and DNA is observed within about 3-20 hours after ligand binding. PDGF has the unique capability of stimulating both protein kinase C, through an increased turnover of phosphatidylinositol, and protein kinase A, through production of type E prostaglandin.

The importance of the correct regulation of receptor function is emphasized by the fact that a large variety of structural alterations found in receptor-derived oncogene products lead to constitutive activation and, consequently, subversion of molecular control mechanisms and alteration of receptor signals. The most common cellular lesion found in human cancers involves autocrine activation in conjunction with receptor overexpression. Many tumors and tumor cell lines have been found to coexpress growth factors and their receptors, including PDGF-A chain, PDGF-B chain, and PDGF receptor polypeptides. Autocrine receptor activation represents one scenario of subversion of normal growth control. In principle, every receptor with tyrosine kinase activity has oncogenic potential. Many more types of activating mutations, as well as specific instances of receptor tyrosine kinase overexpression, would be anticipated to be detected in animal and human tumors. The understanding and control of such defects in normal cellular metabolism and signal transduction will play an important role in the diagnosis and therapy of oncogenesis.

When activated by ligand, the PDGF β-receptor becomes phosphorylated on tyrosine residues. See, e.g., Ek and Heldin (1982) J. Biol. Chem. 257: 10486; Frackelton et al. (1984) J. Biol. Chem. 259: 7909; Kazlauskas and Cooper (1989) Cell 58:

This is the replacement sheet

Date: - 7 OCT 1996

1121; and Yarden et al. (1986) Nature 323: 226-232. The mechanism of this "autophosphorylation" reaction appears to depend on ligand-induced formation of receptor dimers and a transphosphorylation reaction in which each of the two polypeptide chains of the dimer phosphorylates the other chain. The major sites of PDGF stimulated receptor phosphorylation are located in the carboxyl terminal portion of the tyrosine kinase domain, about tyr(825) in both the human and mouse type B receptor, and in the kinase insert region, about tyr(719) in both the human and mouse type B, that splits the kinase coding sequences into two parts. It is likely that additional sites of autophosphorylation have not yet been identified.

One of the consequences of ligand-induced receptor autophosphorylation appears to be a change in conformation of the intracelluar region of the receptor. In its activated state the receptor can physically interact with several cytoplasmic molecules that are likely to be important in signal transduction.

Activation is also accompanied by complex formation with, and tyrosine phosphorylation of, a variety of proteins. The first to be described was a protein of 85 kD, which is a putative PI kinase and identical to a protein referred to as p81, see Kaplan et al. (1987) Cell 50:1021. Subsequently, a number of other proteins have been shown to complex with PDGF receptors and to become phosphorylated on tyrosine following PDGF stimulation. These include the serine/threonine kinase Raf-1, which becomes activated in the complex; see, e.g., Morrison et al. (1989) Cell 58: 649-657; and phospholipase C-γ, see, e.g., Morrison et al. (1990) Mol. Cell. Biol. 10: 2359-2366; and GAP, the GTPase activating protein involved in the control of ras activity, see, e.g., Kaplan et al. (1990) Cell 58: 1121-1133. PDGF treatment also enhances the kinase activities of some of these proteins.

Recently, transient complex formation also has been shown to occur between three src family tyrosine kinases (pp60-c-src, p59-fyn, and pp62-c-yes), p81, and PDGF receptors. In addition, PDGF treatment stimulates the tyrosine kinase activates of these proteins, suggesting that src family kinases

This is the replacement sheet

Date: - 7 OCT 1996

may play a role in the response to PDGF. Phosphorylation on tyrosine residues likely activates the mitogenic potential of the target protein either by enhancing its enzymatic activity or by altering its interactions with other cellular proteins.

The SH2 system provides possible guidance to particular mechanisms and properties which could be applicable to the PDGF receptors. Some non-receptor tyrosine kinases share a conserved noncatalytic region of approximately 100 amino acids called the src homology region 2 (SH2) domain. The SH2 domains of the Fps and Src tyrosine kinases are thought to possibly interact with and regulate the adjacent kinase domain and may also form binding sites for proteins phosphorylated by the kinase domain.

Phospholipase C-γ (PLC-γ) and p21-ras GTPase-activating protein (GAP) each contain two adjacent SH2 domains. Recent experiments have shown that SH2 domains endow proteins such as GAP, v-Crk and p60-v-src with the potential to form complexes with specific tyrosine phosphorylated ligands. The relevance of these results to the PDGF-R was, until now, unclear. In the PDGF receptor embodiments, ligand-activated PDGF receptor induces the formation of a complex of signaling molecules including PLC-γ, Raf-1, and the 85 kDa PI-3 kinase.

One of the first of the signaling molecules to be identified as a receptor-associated protein was phosphatidyl-inositol-3' kinase (PI3 kinase). This enzyme was originally found to be a kinase that co-immunoprecipitated with the complex of polyoma middle T antigen and the c-src protein, and with transforming v-src proteins. PI3 kinase activity was also found in phosphotyrosine or receptor immunoprecipitates of lysates from PDGF-stimulated cells. The specific role of PI3 kinase in mediating cell proliferation has not been determined. However, the enzyme is regulated by a number of growth factors and growth factor-treated cells contain increased levels of phosphoinositides and inositol phosphates phosphorylated on the 3 position of the inositol ring. Mutants of middle T antigen or the PDGF receptor that do not associate with PI3 kinase are defective in their mitogenic activities. The protein responsible for the PI3 kinase activity has not been well

This is the replacement shee

Date: 7 OCT 1996

characterized. However the close correlation of the enzymatic activity with the presence of an 81-85 kD protein in co-immunoprecipitation experiments with the middle T/c-src complex and with the PDGF receptor has strongly suggested that the 85 kD protein is either the PI3 kinase or an important subunit of the kinase.

Definable regions of the PDGF receptor were capable of mediating the interaction with PI3 kinase, as evidenced by an analysis of a mutated type B PDGF receptor having a deletion of the kinase insert (KI) region. This receptor mutant (ΔKI) was defective in stimulating cell proliferation and in binding PI3 kinase, even though it had tyrosine kinase activity and stimulated several other responses to PDGF binding, including phospholipase C-mediated PI hydrolysis. Recent studies have shown that the ligand-activated ΔKI mutant receptor fails to bind PI3 kinase, but binds as much PLC-γ and the c-Raf-1 proteins as the wild type receptor. However, the mutant receptor is unable to phosphorylate c-Raf-1 on tyrosine residues and the binding of PLC-γ required tyrosine phosphorylation of the receptor. A mutant of the human type B PDGF receptor that had one of the phosphorylation sites in the kinase insert domain, tyr(719) in both the human type B receptor, converted to phenylalanine was also defective in binding PI3 kinase. These experiments indicate that either the PI3 kinase binds directly to a portion of the kinase insert region containing tyr(719) or that the binding interaction involves other portions of the receptor that are conformationally dependent on sequences in the kinase insert region.

To directly study the interaction between the type B PDGF receptor and PI3 kinase, an *in vitro* system was established. With this system, it was possible to test the ability of synthetic polypeptides derived from receptor sequences in the kinase insert domain to block interaction of the PI3 kinase with the PDGF receptor. The interaction could be blocked by a tyrosine-phosphorylated peptide representing a highly conserved region of the kinase insert domain that included tyr(719). However the peptide blocked PI3 kinase

This is the replacement sheet

Date: 7 OCT 1996

binding to the PDGF receptor only when the peptide was phosphorylated on tyrosine. Scrambled versions of the peptide, even when phosphorylated on tyrosine, had no blocking activity. These studies show that phosphotyrosine in a specific sequence context serves as a recognition site for the binding of a cytoplasmic signaling molecule.

An in vitro system was used to study the interaction of PDGF receptors and PI3 kinase. The association of phosphoproteins with the PDGF receptor is demonstrated by stimulating 3T3 cells with PDGF, immunoprecipitating cell lysates with type B PDGF receptor antibodies and by examining the immunoprecipitates for the presence of receptor-associated proteins that can be phosphorylated in the immunoprecipitates. See Fig. 1a "+" lanes. These phosphoproteins include the 85 kDa protein and a 110 kDa protein that have recently been correlated with PI3 kinase activity. See Fig. 1c. In in vitro experiments, the type B PDGF receptor was expressed in an insect cell expression system, partially purified, and immobilized using anti-receptor antibodies and protein A Sepharose. The receptor was allowed to autophosphorylate *in vitro*, so that it would be in a conformation that mimicked the ligand-activated state of the receptor. Cytoplasmic cell lysates from density-arrested BALB/c 3T3 cells were mixed with the immobilized receptor and the complexes were washed extensively. PI3 kinase activity was found in the complex of proteins associated with the receptor (Fig. 1d). The receptor-associated phosphoproteins were detected by an *in vitro* kinase assay using ^{32}P-ATP. See Fig. 1b "-" lane. At least four receptor-associated proteins with apparent molecular weights of 140 kD, 120/110 kD (sometimes a doublet), 85 kD and 74 kD were radiolabeled (the bands at 160 kD and 150 kD are the baculovirus-expressed receptor and its precursor, respectively). The 140 kD band has been previously identified as PLC-γ and the band at 74 kD includes the *raf*-1 kinase and probably another molecule as well. The 85 kD protein co-migrated with the 85 kD band, previously considered to be PI3 kinase, in anti-phosphotyrosine immunoprecipitates of cell lysates prepared from PDGF-stimulated cells. The association

This is the replacement sheet

Date: - 7 OCT 1996

of 85 and 110 kD proteins with the baculovirus-expressed receptor was also observed by silver stain analysis. When cell lysate was incubated with immobilized kinase insert deletion mutant of the PDGF receptor there was no associated PI3 kinase activity, and the 110 kD and 85 kD phosphoproteins did not associate with the mutant receptor. See Fig. 2a and A2b. This in vitro result is consistent with previous indications that this receptor mutant does not associate with PI3 kinase in intact cells.

When 3T3 cells were stimulated with PDGF prior to making the lysates, there was a dramatic reduction in the amount of PI3 kinase available for association with the receptor in vitro (Fig. 1d) and a concomitant reduction in the association of the 85 kD and 110 kD phosphoproteins with the receptor (Fig. 1b "+" lane). Although the explanation of this phenomenon is not entirely clear, the observation that PDGF pretreatment of cells influences the subsequent in vitro association of cellular proteins with the receptor indicates that the associations are specific PDGF-regulated processes.

When the autophosphorylated PDGF receptor was dephosphorylated in vitro using potato acid phosphates (PAP) it lost the ability to associate with PI3 kinase activity or 84 kD protein derived from the BALB/c 3T3 cell lysates (Fig. 3b and c). This finding indicates that phosphorylation of the PDGF receptor was required for these protein interactions to occur.

To identify the site of interaction between the receptor and the PI3 kinase, phosphorylated synthetic peptides were prepared to compete for the binding of the 85 kD protein and the PI3 kinase activity to the receptor in vitro. Previous data suggested that the kinase insert region was involved in the association of the PI3 kinase activity with the PDGF receptor. In scanning sequences from the kinase insert regions of the mouse and human PDGF type B and type A receptors, a 20 amino acid region of particularly high sequence homology (8 out of 20 identities for the human and mouse PDGF type B receptor compared to 12% identity for the entire kinase insert region) was found. A peptide (Y719) containing amino acids 705 to 724

This is the replacement sheet

Date: - 7 OCT 1996

of the mouse type B PDGF receptor sequence was synthesized. This peptide sequence contained two tyrosine residues, Y708 and Y719. The tyrosine at position 719 of the mouse sequence also corresponds to tyr(719) in the human type B PDGF receptor, a known autophosphorylation site of the receptor.

To determine the ability of a series of synthetic peptides derived from this sequence to block the interaction between the receptor and the 85 kD/PI3 kinase activity, 3T3 lysates were incubated with the different peptides prior to mixing with immobilized receptor. The results of these experiments are shown in Fig. 4. The first lane of Fig. 4a shows phosphorylated proteins that associated with the wild type receptor in the absence of peptides. The arrow indicates the position of the 85 kD protein that co-purified with the PI3 kinase activity. The other lanes show the proteins that associated with the receptor in the presence of derivatives of the Y719 peptide. See Table 4. No change in the pattern of receptor-associated protein was seen when the unphosphorylated 719 peptide (Y719) was preincubated with the 3T3 lysate prior to the association with the receptor. By contrast when a derivative of this peptide that was phosphorylated at position 719 (Y719P) was added to the incubation, it blocked the binding of the 85 kD phosphoprotein (Fig. 4a) and inhibited the association of PI3 kinase activity (Fig. 4b) with the receptor. A scrambled version of this peptide that contained phosphotyrosine at a position corresponding to 719 but had a rearranged primary sequence failed to block binding of the 85 kD protein and did not prevent the association of PI3 kinase activity with the receptor.

This is the replacement sheet

Date: - 7 OCT 1996

TABLE 4

PDGF Receptor Synthetic Peptides

Peptides	Sequence
Y719	GGYMDMSKDESIDYVPMLDM
Y719P	GGYMDMSKDESIDY*VPMLDM
Y708P	GGY*MDMSKDESIDYVPMLDM
Y719P short	MDMSKDESIDY*VPMLDM
Y708P short	GGY*MDMSKDESID
Y708P/F719	GGY*MDMSKDESIDFVPMLDM
Y708/Y719P	GGFMDMSKDESIDY*VPMLDM
Y708P/Y719P	GGY*MDMSKDESIDY*VPMLDM
Y719P scrambled	MMDIKVPMDEY*MSDYSDLGG

The asterisks (*) indicate the position of a phosphate group

This is the replacement sheet

Date: - 7 OCT 1996

A shorter version of peptide Y719P that includes only 14 amino acids (Y719P short) and lacked tyrosine 708 also blocked binding of the 85 kD protein (Fig. 4a, lane 4) and inhibited the association of PI3 kinase activity with the receptor. Surprisingly, a peptide that includes tyrosine at position 719 and phosphotyrosine at position 708 blocked the interaction of PI3 kinase with the receptor (Fig. 4, lane 6). This finding suggests the possibility that tyrosine 708 is one of the autophosphorylation sites of the receptor that has not yet been mapped. A peptide that included phosphotyrosine at both positions 708 and 719 (Y708P/Y719P) also blocked binding of the 85 kD protein (Fig. 4, lane 8). Short forms of Y708P and Y719P also had blocking activity (Fig. 4 lanes 4 and 9).

To determine the amount of peptide necessary to block the association of the 85 kD and the PI3 kinase activity to the receptor, the 3T3 lysates were preincubated with a range of concentrations of phosphorylated peptide (Y719P). As shown in Fig. 5, approximately 5 μM of Y719P was sufficient to block more than 50% of the association of the 85 kD protein (Fig. 5a) and PI3 kinase activity (Fig. 6B) with the receptor. Concentrations of up to 100 μM of unphosphorylated peptide did not affect association between the receptor and the PI3 kinase. Thus the coincident blocking of 85 kD and PI3 kinase activity association with the receptor by peptide Y719P supports the hypothesis that the 85 kD protein is a subunit of the PI3 kinase enzyme.

Next, the ability of peptides to block the association of other signaling molecules with the receptor was assessed. Lysates from 3T3 cells were incubated in the absence or the presence of peptide Y719P prior to association with the receptor immunoprecipitates. The association of PLC-γ and GAP with the receptor was determined by immunoblot analysis using specific antibodies (Fig. 6), confirming previous studies that these molecules specifically associate with autophosphorylated receptors. The association of these proteins with the receptor was not affected by the addition of the phosphorylated peptide, Y719P. Thus the Y719P peptide specifically blocked association

This is the replacement sheet

Date: - 7 OCT 1996

of 85 kD/PI3 kinase activity with the receptor and did not affect the interaction of the receptor with GAP or PLC-γ.

To determine the nature of the interaction of the receptor with the 85 kD protein, a modification of Western blotting methodology was used. PDGF receptor from Sf9 cells infected with PDGF receptor baculovirus was immuno precipitated with receptor antibodies, labeled with ^{32}P in vitro, and used as a probe to bind proteins from 3T3 cell lysates that had been separated electrophoretically and transferred to nitrocellulose. The radiolabeled PDGF receptor bound directly to an 85 kDa protein in unstimulated 3T3 cell lysate (Fig. 7, lane 1). Cell lysates from PDGF-treated 3T3 cells did not show any binding when incubated with the radiolabeled PDGF receptor, (Fig. 7, lane 2) consistent with the finding that the 85 kD protein from PDGF-stimulated lysates was not available for association with the immobilized PDGF receptor (Fig. 1b). When the nitrocellulose paper that contained cell lysate proteins was pre-incubated with peptide Y719P, the radiolabeled receptor failed to associate with the 85 kD protein (Fig. 7, lane 4). The unphosphorylated peptide (Y719) or the scrambled peptide (Y719P scrambled) did not interfere with the association of the receptor with the 85 kD protein (Fig. 7, lanes 3 and 5). This experiment showed that the 85 kD protein (Fig. 7, lanes 3 and 5). This experiment showed that the 85 kD protein binds directly to the PDGF receptor and does not require the presence of other molecules. The direct binding interaction appears to involve the 20 amino acid segment in the kinase insert region containing tyrosine 719, and tyrosine phosphorylation of this segment appears to be necessary for the interaction.

These results indicate that the intracellular region of the type B PDGF receptor interacts with molecules that are likely to play a role in signal transduction. The physical association between the receptor and signaling molecules such as PI3 kinase, PLC-γ, GAP, and raf-1 only occurs when the receptor is phosphorylated on tyrosine. See Fig. 1a. A region of the receptor that is involved in the binding of the receptor to the PI3 kinase has been defined. Short peptides were used to block the in vitro association of PI3 kinase with the

This is the replacement sheet

Date: - 7 OCT 1996

phosphorylated receptor. The most likely explanation for the ability of the peptides to block the association is that they mimic the receptor and bind directly to the PI3 kinase. In this way they act as competitive antagonists. The observation that only tyrosine-phosphorylated peptides had this blocking activity suggests that phosphotyrosine is directly involved in the binding of the receptor to PI3 kinase. However phosphotyrosine alone is not sufficient for binding, since scrambled peptides containing phosphotyrosine did not interact with the PI3 kinase. Therefore the primary sequence of this portion of the receptor has essential structural information that is required for the binding of the receptor to PI3 kinase. It is somewhat surprising that peptides as short as 13 amino acids contained enough structural information to mimic the native receptor region that normally binds to the PI3 kinase (Table 4).

Tyrosine 719 of the type B PDGF receptor is one of the autophosphorylation sites of the receptor in intact cells as well as *in vitro*. Thus, it appears that the portion of the KI domain around tyrosine 719 is directly involved in the association of PI3 kinase with the receptor *in vivo*. The data presented herein indicate that peptide derivatives of Y719 containing one of the two tyrosine residues phosphorylated (Y708P or Y719P) efficiently prevented the association of the receptor with the PI3 kinase in *in vitro* assay. Similar sequences and interactions are subject to blockage by the appropriate fragment sequences. In particular, sequences known to be phosphorylation sites will be used to select for analogues which block interaction with other mediating proteins. The PI3 kinase is known to associate with the c-*fms* protein and with the middle T antigen, and both of these proteins are phosphorylated on tyrosine *in vivo*. A phosphorylation site of the middle T antigen (tyrosine 315) is in a sequence somewhat homologous (5 out of 10 amino acids are identical) to the peptide Y719P used in our experiments. There is no obvious homology of peptide Y719P to any of the tyrosine phosphorylation sites on the c-*fms* protein. However peptide Y719P blocked association of PI3 kinase with the c-*fms* protein

This is the replacement sheet

Date: - 7 OCT 1996

in vitro. Thus an autophosphorylation site of the c-*fms* protein is in a region that has a secondary structure that is similar to the corresponding domain of the PDGF receptor.

These findings indicate that signaling molecules recognize phosphotyrosine in a specific sequence context. The pattern of interactions of the kinase insert deletion mutant with GAP, PLC-γ, and c-*raf*-1 and the lack of ability of the peptides used in this report to block GAP and PLC-γ association with the receptor shows that different regions of the receptor, possibly containing different autophosphorylation sites, are involved in the binding of specific signaling molecules. The binding of these molecules to the receptor localize the signaling molecules and regulate their activities. Tyrosine phosphorylation of other cellular proteins by the receptor kinase targets them as proteins for binding signaling molecules and the present invention provides the methods and materials for generating reagents which will successfully mimic or interfere with natural signals. For example, fragments from the hPDGF-receptor, or related tyrosine kinase proteins, will be useful to block the natural interactions between them and function-mediating proteins. See, e.g., Williams (1989) *Science* 243: 1564-1570; Yarden et al. (1986) *Nature* 323: 226-232; and GenBank™, for other sequences and proteins which show homology, both structural and functional, with the phosphorylated inserts described herein.

This discovery leads the way to the production of analogues of the KI segments. For example, short peptides having non-hydrolyzable moieties will often be produced with, e.g., sulfonated moieties substituted for phosphorylated moieties. Alternatively, other organic molecules exhibiting sufficient structural homology will often be selected for their functional interaction with the PI3 kinase or other function mediating proteins. In addition, chimeric analogues, portions of which are peptide, or modified peptide, and other portions of which are organic molecules which have similar structural features which are important for interaction, will be constructed. Thus, molecules exhibiting particular interacting

This is the replacement sheet

Date: - 7 OCT 1996

features become available, including both natural or synthetically generated compounds.

The invention will better be understood by reference to the following illustrative examples. The following examples are offered by way of illustration and not by way of limitation.

This is the replacement sheet

Date: 7 OCT 1996

EXPERIMENTAL

In general, standard techniques of recombinant DNA technology are described in various publications, e.g., Sambrook et al. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory; Ausubel, et al. (1987) Current Protocols in Molecular Biology, vols. 1 and 2 and supplements; and Wu and Grossman (eds.) (1987) Methods in Enzymology Vol. 53 (Recombinant DNA Part D); each of which is incorporated herein by reference. Variations, if any, on those methods have generally been minor.

I. Screening of Human Kidney λGT11 cDNA Library and Human Placenta λGT10 cDNA Library

A full-length DNA sequence encoding the mouse PDGF receptor (mPDGF-R) protein was used as a probe to screen 250,000 plaques of a human kidney cDNA library. Nick translation was used to prepare a probe with specific activity of 12 x 10^8 cpm per μg. The filters were incubated with the probe (10^5 cpm per ml) in hybridization buffer containing 30% formamide, 1X Denhardt's solution, 5X SSC, 0.02 M sodium phosphate pH 6.5, and 500 μg per ml of salmon sperm DNA. After 14 h of hybridization at 40°C, the filters were washed four times at 55°C with 0.2X SSC and 0.1% SDS and two additional times at 65°C with 0.2X SSC. The filters were then air dried and exposed for 16 h.

Ten positive clones were obtained which were rescreened with the full-length mPDGF-R probe. Individual clones were isolated and analyzed by restriction analysis using EcoRI endonuclease. The clone containing the largest insert (2.3 kb), designated clone HK-6, was further characterized and sequenced using dideoxy terminators. Clone HK-6 contained the receptor sequence from nucleotide 3554 to nucleotide 5691 plus nine bases from the poly-A tail.

A nick-translated probe, prepared from the 2.3 kb HK-6 DNA, was used to screen 250,000 plaques of a human placenta cDNA library. This screening was performed at high hybridization stringency (50% formamide in the hybridization buffer described above). The filters were incubated with 5 x

This is the replacement sheet

Date: - 7 OCT 1996

10^5 cpm per ml of probe for 14-16 h at 42°C. The filters were than washed at 65°C in 0.1% SSC and 0.1% SDS four times.

After secondary screening with the HK-6 probe, seven clones were selected and analyzed by restriction digestion with EcoRI endonuclease. A clone (HP-7) that contained a 4.5 kb insert was selected and characterized. The sequence of that clone is described in Table 2 and encodes the type B human PDGF receptor (B-hPDGF-R).

II. Construction of Expression Vector

The 4.5 kb DNA fragment containing the complete coding sequence for the type B human PDGF receptor was isolated from the HP-7 clone by EcoRI digestion. The gel purified fragment was cloned into the EcoRI site in the polylinker region of SV40 expression vector PSV7C. The pSV7d expression vector, obtained from P. Luciw, at the University of California, Davis, was a pML derivative containing the SV40 early promoter region (SV40 nucleotides 5190-5270), a synthetic polylinker with restriction sites for EcoRI, SmaI, XbaI, and SalI followed by three translation terminator codons (TAA) and the SV40 polyadenylation signal (SV40 nucleotides 2556-2770) (Truett et al. (1984) DNA 4:333-349). The EcoRI fragment containing the cDNA sequence obtained from the HP-7 clone was inserted at the EcoRI site of the pSV7d. In the resulting expression vector, the B-hPDGF-receptor gene was under transcriptional control of the SV40 promoter.

To ensure the proper orientation of the PDGF receptor insert (4.5 kb) with respect to the SV40 promoter, the positive clones were digested with SmaI endonuclease which cuts at position 573 of the receptor sequence and in the polylinker region of the expression vector.

Clones containing the receptor in the proper transcriptional orientation released a 4.0 kb insert in addition to the 3.2 kb fragment containing the expression vector plus 573 base pairs of the 5' end of the receptor. This plasmid, PSVRH5 was used to co-transfect cells with PSV2 neo plasmid that confers resistance to the antibiotic neomycin.

This is the replacement sheet

Date: - 7 OCT 1996

III. Cell Culture and Transfection of CHO Cells

CHO cell clone KI, obtained from the U.C.S.F. Tissue Culture Facility, were grown in Ham's F-12 media supplemented with 10% FCS (UCSF Tissue Culture Facility) and penicillin and streptomycin at 37°C in 5% CO_2/95% air.

pSVRH5 plasmid DNA (10 μg) and pSV2 neo (1 μg) were used to co-transfect 1 x 10^6 CHO cells by the calcium precipitation technique of Van der Eb et al. (1980), Methods in Enzymology (1980) 65:826-839, with the addition of 10 μg chloroquinone diphosphate (CDP) to prevent degradation of the transfected DNA. After 12 h of exposure to the DNA, the cells were trypsinized and replated at 1:5 dilution. Twenty-four hours later, the antibiotic G418 (GIBCO), an analog of neomycin, was added to the cultures at a concentration of 400 μg/ml.

After two weeks under selection, independent colonies were picked and transferred to 24-well plates. Confluent cultures were assayed for the presence of PDGF receptor by immunoblot using anti-receptor antibodies. Colonies that were positive by this assay were single-cell cloned by end-limiting dilution.

Stable transfected clones were tested for the expression of the type B PDGF receptor message measured by RNA protection assays and for the presence of PDGF-stimulated receptor protein detected by anti-phosphotyrosine antibodies.

IV. Expression of B-hPDGF-R cDNA in CHO Cells

CHO cells transfected with plasmid DNA containing the human receptor cDNA under the transcriptional control of the SV40 early promoter (CHO-HR5) and CHO cells transfected with a similar plasmid containing the mouse receptor cDNA (CHO-R18) were solubilized as previously described by Escobedo et al. (1988) J. Biol. Chem. 263:1482-1487. Extracts were analyzed by Western blot analysis using an antibody that specifically recognizes sequences in the receptor carboxyl-terminal region as previously described by Escobedo et al. (1988) J. Biol. Chem.; and Keating et al. (1987) J. Biol. Chem. 262:7932-7937.

This is the replacement sheet

Date: 7 OCT 1996

The 195 kDa protein is the mature receptor and the 160 kDa protein is the receptor precursor.

The expression of the receptor protein in the transfectants was demonstrated by using antibodies that recognize an intracellular sequence in the receptor. The clone that had the highest level of human receptor expression was chosen for further study. This transfectant had receptors that were labeled with ^{125}I-PDGF as shown by the competitive binding studies described below.

V. Competitive Binding of the Different Forms of PDGF to the Type B Receptor

The ability of the human recombinant AA and BB homodimers to compete for the type B receptor sites and displace ^{125}I-labeled PDGF (prepared as described below) was studied. Each homodimer was produced selectively by a yeast expression system and was purified from yeast media that is devoid of other mesenchymal cell growth factors, thus avoiding the artifact of contamination by factors that might be present in mammalian expression systems.

BALB/c 3T3 cells and CHO transfectants (CHO-HR5) were incubated with ^{125}I-PDGF in the presence of increasing concentrations of AA or BB. Binding was carried out at 37°C for 45 min in whole cell suspension. Unbound, radiolabeled PDGF was removed by centrifugation on a Ficoll gradient Non-specific binding, determined by incubating CHO cells with ^{125}I-PDGF, accounted for 25 percent of the bound radioactivity.

The binding study demonstrated that the transfected cells can be used as a model to study the interaction of hPDGF with its receptor. In particular, this study demonstrated that the transfected type B human receptor was functionally equivalent to the native mouse receptor as indicated by the following results. Both AA and BB forms of PDGF competed for the ^{125}I-PDGF labeled sites in the human receptor transfectants. For the transfected type B human receptor as well as the native mouse receptor, the BB form was of higher affinity than the AA form. When expressed in yeast, the AA form of PDGF may be processed aberrantly, giving it a lower affinity than the BB form for both the transfected cells and mouse 3T3 cells. The

This is the replacement sheet

Date: - 7 OCT 1996

consistency of the pattern of competition shows that the AA form interacts with the transfected type B human receptor in the same way as it does with the native mouse receptor and demonstrates that these receptors are functionally equivalent.

VI. Activation of the PDGF Receptor Tyrosine Kinase

The ability of recombinant AA and BB homodimers and of human partially purified AB PDGF to activate the type B receptor tyrosine kinase was studied. The yeast-derived AA and BB homodimeric forms and the platelet-derived AB form stimulated autophosphorylation of the transfected human receptor.

BALB/c 3T3 cells and CHO cells transfected with the human PDGF receptor cDNA (CHO-HR5) were incubated with increasing amounts of the different forms of PDGF (AA, BB and AB). Following polyacrylamide-SDS electrophoresis, the phosphorylated receptor was identified by Western blot using an anti-phosphotyrosine antibody.

The receptor protein co-migrated with the 200 kDa molecular weight marker. The concentration of each form that was effective in stimulating autophosphorylation of the transfected human receptor was equivalent to the concentration that gave a similar autophosphorylation to the native mouse 3T3 receptor or the transfected mouse receptor.

These results showed for the first time that the AA form of PDGF activates the receptor tyrosine kinase of the type B receptor. Prior to use of the transfected cells, there was no demonstration that the AA form had hPDGF activity or that a single receptor, the type B receptor, was capable of recognizing all three forms of PDGF. Further, the results demonstrate that the human cDNA encodes a type B receptor that is functionally equivalent to the wild-type receptor that is responsible for PDGF-stimulated tyrosine kinase activity in mouse 3T3 cells.

Thus, the transfected cells are useful models for studying PDGF-induced mitogenic responses.

VII. Rate of DNA Synthesis in CHO Transfected Cells

This is the replacement sheet

Date: - 7 OCT 1996

BALB/c 3T3 cells and CHO cells transfected with the type B human PDGF receptor cDNA (CHO-HR5) were incubated with saturating concentrations of the three forms of PDGF. Untreated cells and cells treated with fetal calf serum (FCS) were used as negative and positive controls, respectively. The level of ^{3}H-thymidine incorporation into DNA was determined by measuring the radioactivity of the acid-precipitable material as previously described.

Transfection of CHO cells with either type B human or mouse PDGF receptor conferred a PDGF-sensitive mitogenic response. All forms of PDGF stimulated DNA synthesis in both the type B human receptor transfectant and the mouse cells bearing the native receptor.

These data showed that the A chain homodimer and the B chain homodimer, like the AB platelet-derived form, were mitogens that can act through the receptor encoded by the type B human cDNA sequence. The mitogenic action of these forms of PDGF on mouse 3T3 cells and CHO cells containing the transfected type B human receptor demonstrate that the responses were mediated by functionally equivalent receptors.

VIII. Isolation and Expression of the Type A PDGF Receptor

The type A receptor was isolated as described for the type B receptor, above, except that different probes were used and hybridization and screening were performed under low stringency conditions, as described below. In particular, a region in the type B receptor tyrosine kinase sequence having a high degree of homology to published tyrosine kinase amino acid sequences was identified and had the amino acid sequence, HRDLAARN. Oligonucleotide probes encoding the tyrosine kinase consensus sequence were prepared having the following sequences:

GTT(G/C)CGXGCXGCCAGXTC(G/C)CGXTG,

where G/C indicates either G or C was used and X indicates any of A, T, C or G was used. The human placenta λGT10 cDNA library was screened as described above but with low stringency conditions using a buffer with 6X SSC 0.1% SDS and 5X Denhardt's solution at 42°C as follows. Filters were screened

This is the replacement sheet

Date: - 7 OCT 1996

by washing at 52°C in 2X SSC. A clone encoding the type A receptor was isolated and sequenced by the procedure described for the type B receptor gene.

The DNA sequence of the gene encoding the type A receptor (A-hPDGF-R) together with the deduced amino acid sequence are shown in Table 3, above.

The clone encoding A-hPDGF-R was digested, gel purified and inserted into the SV40 expression vector, pSV7C, as described for the type B receptor clone.

That vector is used to transfect CHO cells as described above for the type B receptor. With expression of the vector coding sequence, transfected CHO cells produce a functional receptor that binds all three hPDGF forms, preferentially binding the AA homodimer.

IX. Extracellular Murine PDGF-R Fragments Construction of pSV-SRX1d Expression Vector and Transfection of DUKX-B11 Cells With a Murine Extracellular Region

To express the secreted extracellular region (XR) of PDGF type B receptor (type B PDGF-R), a cDNA clone of the murine PDGF-R was mutagenized to introduce a Stu I restriction site between codons 500 and 501, changing codon 500 from valine to arginine. The 1.7 kb EcoRI-Stu I fragment was inserted into pIBI-25 (IBI) which added an in-frame proline codon followed by a stop codon at the C-terminus. The 1.7 kb Eco RI-Xba I fragment was transferred to pSV-7DHFR in which the XR expression was driven by the SV-40 early promoter and the amplifiable marker, a dihydrofolate reductase (dhfr) transcriptional unit, was driven by an Adenovirus major late promoter. The complete plasmid encodes the signal sequence and the first 499 amino acids (the extracellular region) of the PDGF-R, followed by arginine, proline and stop codons.

The cDNA was expressed in dhfr-deficient CHO mutant cells, DUKX-B11. DUKX-B11 cells were transfected to 10-cm tissue culture plates with 5 μg of pSV-SRX1d plasmid and selected for expression with nucleoside-free MEM-α (Gibco) supplemented with 10% dialyzed calf serum, 200 μg/ml proline, 100 U/ml penicillin, and 100 μg/ml streptomycin. Colonies were

This is the replacement sheet

Date: - 7 OCT 1996

picked and screened by analyzing the conditioned media by SDS-PAGE and Western blotting with a polyclonal PDGF-R antibody directed against the extracellular region of the receptor (Ab77).

Amplification of murine extracellular region expression by methotrexate

Positive transfectants were treated sequentially with increasing concentrations of methotrexate starting at 1, 2, 5, and 10 nM. Colonies from the highest concentrations were then treated with 10 fold higher concentrations of methotrexate (20, 50, and 100 nM). Colonies from the highest concentrations were again picked and treated with increasing concentrations of methotrexate. The conditioned media from colonies at each stage were analyzed by SDS-PAGE and Western blotting with an anti-PDGF-R Ab (Ab77) for expression of the extracellular region protein. Methotrexate-resistant (5 μM) cells that secreted highest amount of the extracellular region protein were named DPXR (DUK-PDGF-R extracellular region) cells.

Enzymatic treatments of the extracellular region protein with O-glycanase (Genzyme Co.) and N-glycanase (Genzyme Co.) were performed. Briefly, extracellular region protein partially purified with wheat germ agglutinin (WGA) affinity chromatography (50 nM, in 20 mM sodium phosphate, pH 7.4) was incubated with N-glycanase (10 U/ml) overnight at 37°C and then with O-glycanase (2 mU/ml) for an additional 2 h. The sample was then boiled for 5 min and analyzed with SDS-PAGE and Western blotting with Ab77.

Conditioned media

DPXR cells at 95% confluence were washed twice with serum-free DME H21 medium. Four ml of DME H21 medium supplemented with 10% protein-free Serum Substitute (UCSF Cell Culture Facility) was added to each 10-cm culture plate. Conditioned media containing murine extracellular region polypeptide which were used for various assays were collected from DPXR cells over 48 h unless otherwise mentioned.

This is the replacement sheet

Date: - 7 OCT 1996

Preparation of ^{125}I-BB-PDGF

BB-PDGF (gift from Chiron Corporation) was iodinated. Briefly, 2.5 µg of BB-PDGF were rotary-evaporated in a silanized polypropylene tube. BB-PDGF was resuspended in 10 µl of 0.1 M sodium borate (pH 8.5). ^{125}I-mono-iodo Bolton Hunter reagent (500 µCi; Amersham) was dried under nitrogen. The BB-PDGF was added to the dry Bolton Hunter reagent and incubated for 15 min at 4°C. Fifty µl of quench solution (0.1 M sodium borate, 0.2 M glycine, pH 8.5) was added to the reaction mixture, which was incubated for an additional 10 min at 4°C. The entire reaction mixture was loaded on a PD-10 column (Pharmacia) equilibrated with 0.1 M acetic acid containing 1 mg/ml BSA (ICN Biochemicals), and eluted with the same buffer.

X. Binding Assays

Of relevance to the production of soluble fragments of PDGF receptor polypeptides, and assays for their use, are techniques and results reported in Kimball and Warren (1984) Biochim. Biophys. Acta 771:82-88; van der Schaal et al. (1984) Anal. Biochem. 140:48-55; van Driel et al. (1989) J. Biol. Chem. 264:9533-9538; Heldin et al. (1988) EMBO J. 7:1387-1393; Williams et al. (1982) Proc. Nat'l Acad. Sci. USA 79:5867-5870; Williams et al. (1984) J. Biol. Chem. 259:5287-5294; and particularly, Orchansky et al. (1988),"Phosphatidylinositol Linkage of a Truncated Form of the Platelet-derived Growth Factor Receptor" J. Biol. Chem. 263:15159-15165; each of which is hereby incorporated herein by reference. In particular, the Orchansky publication provides evidence that a whole extracellular region of a PDGF receptor, when separated from the TM and intracellular regions, still is capable of binding to PDGF ligands.

In whole cell binding assays, 2.x 10^4 R18 cells (PDGF type B receptor transfectant CHO cells) detached with PBS/EDTA (2 mM) were incubated with 10 µl of platelet-poor plasma, and PBS/Hepes (25 mM final, pH 7.4). Conditioned media, 12.5 µl to 200 µl, from DPXR cells (collected as described above), which contain the extracellular region protein, were added to the

This is the replacement sheet

Date: 7 OCT 1996

binding mixtures in a final volume of 250 µl to form 20 to 1.25 fold dilutions. The mixtures were shaken overnight at 4°C and spun through 750 µl of Ficoll gradient (28.5% Ficoll-Paque (Pharmacia) in PBS) at 4°C. The supernatants were aspirated and the radioactivity in the cell pellets was determined with a gamma-counter.

To measure its affinity for BB-PDGF, extracellular region protein was immobilized by adsorption onto a plastic surface. 50 µl of WGA affinity chromatography purified extracellular region (approximately 80 nM, estimated by silver straining) was diluted in 10 ml of 25 mM Tris Cl, 75 mM NaCl, 20 mM NH_4HCO_3, pH 7.5, and 100 µl aliquots were plated in each well of 96-well ELISA microtiter plates (Dynatech Products Co.). After overnight incubation at 4°C, the plate was washed once and blocked with 0.5% gelatin in 100 mM NaCl, 25 mM Hepes, pH 7.35, for 3 h at 4°C. The plate was then washed twice with binding buffer (0.3% gelatin, 100 mM NaCl, 25 mM Hepes, pH 7.35). ^{125}I-BB-PDGF and/or unlabeled BB-PDGF were added to the wells in a final volume of 100 µl and the plate was incubated at 4°C for 16 h for steady state binding. The plate was washed three times again with binding buffer and stripped with 200 µl of 1% SDS, 0.5% BSA for counting in a gamma-counter.

Crosslinking experiments

Extracellular region polypeptide partially purified by WGA chromatography (25 µl, ~50 nM) was incubated with 0.2 µCi of 125I-BB-PDGF (2 nM final concentration) and various concentrations of unlabeled BB-PDGF in a final volume of 100 µl at 4°C for 3 h. The extracellular region protein was then crosslinked to the ligand by 1 mM bis(sulfosuccinimidyl) suberate (BS^3) (cat. #21579, Pierce Chemicals, Rockford, Illinois) at room temperature for 30 min. The reaction was stopped by 25 mM Tris buffer, pH 7.4, for 5 min. The extracellular region protein was then immunoprecipitated with a receptor antibody (Ab77) and analyzed by SDS-PAGE.

Autophosphorylation assay

This is the replacement sheet

Date: - 7 OCT 1996

Conditioned media containing the extracellular region protein (1 ml) were incubated with BB-PDGF for 3 h at 4°C. The mixtures were then added to the 6-well Falcon tissue culture plates (1 ml/well) which contained monolayers of quiescent human PDGF A-receptor transfectant CHO cells or BALB/c 3T3 cells. The cells were incubated with the mixtures for 10 min at 37°C and then washed with serum-free DME medium and lysed with Ripand lysis buffer. Cell lysates were analyzed by SDS-PAGE and Western blotting with an anti-phosphotyrosine mAb.

Mitogenesis assay

BALB/c 3T3 cells were plated in 96-well tissue culture plates for 3 days and made quiescent with Q-media (DMEM with 1 mg/ml insulin, 2 µg/ml transferrin and 0.5 mg/ml BSA) for 24 h. Conditioned media containing the extracellular region protein was diluted in 2-fold serial dilutions with fresh DME H21 medium, preincubated with 2 nM BB-PDGF for 3 h at 4°C and then added to BALB/c 3T3 cells (200 µl/well). The cells were incubated for 18 h at 37°C. One µCi [^{3}H]-thymidine (in 50 µl) was then added to each well for 4 h. The cells were washed twice with cold PBS and fixed with cold trichloracetic acid (TCA, 5%). Precipitated cell debris were then washed extensively with cold TCA (5%) and dissolved in 0.25 N NaOH for scintillation counting.

XI. Human Extracellular Region

Equivalent techniques for construction, expression, and determination of the physiological effect of truncation or deletion analogues of the soluble extracellular receptor fragments from the human receptor may be performed using the nucleic acid, polypeptide, and other reagents provided herein.

Human Deletion and Truncation Constructs

PDGF-R CONSTRUCTS

The 3.9 kb EcoRI-Hind III cDNA fragment of the human type B hPDGF-R was subcloned into the EcoRI-Hind III site of M13 Mp18 to produce a vector Mp18PR. For techniques, see

This is the replacement sheet

Date: - 7 OCT 1996

Maniatis et al., Molecular Cloning: A Laboratory Manual (1982), Cold Spring Harbor, N.Y., which is incorporated herein by reference. Verification of subcloning was performed by restriction enzyme digestion analysis and dideoxy chain termination sequencing, as described by Sanger et al. (1977) Proc. Nat'l Acad. Sci. USA 74:5463. Oligonucleotide directed in vitro mutagenesis was performed according to the method described by Kunkel et al. (1987) Methods in Enzymol., 154:367. The strategy for oligonucleotide directed in vitro deletion mutagenesis of Mp18PR is outlined in Fig. 8. In brief, an oligonucleotide was designed to create a soluble type B hPDGF receptor extracellular region by deletion mutagenesis. A mutagenic oligonucleotide aligned with the appropriate region of the human PDGF receptor can be used to generate deletions. The antisense strand was used for mutagenesis throughout. Mutagenesis of PΔ1 utilized Mp18PR as the template. PΔ1, a 41 bp oligomer, introduced a TAG stop codon after Lysine$_{499}$ (K_{499}) of D5 and removed the transmembrane (TM) as well as the entire intracellular kinase domain (K), producing an Mp18 PΔ1. PΔ1 codes for 530_{aa} 148_{aa} precursor proteins.

The human PDGF receptor constructs were subsequently subcloned into the EcoRI-Hind III site of pBJ1 a derivation of pCDL-SRα296, as described in Takabe et al. (1988) Molec. Cell Biol. 8:466, and co-transfected with pSV2NEO, as described by Southern and Berg (1982) J. Mol. Appl. Gen., 1: 327, into Chinese hamster ovary cells (CHO).

Function of the construct was demonstrated as follows:

A sample of 0.33 nM PDGF BB ligand is preincubated for 1 hr at 4°C under the following conditions:

1. a polyclonal antibody to human PDGF (this antibody recognizes human PDGF AA, PDGF BB and PDGF AB);
2. 18 nM (60 fold molar excess to PDGF BB) human type B PDGF receptor;
3. phosphate buffered saline solution that the receptor and antibody are in; or
4. no additions but the ligand itself.

This is the replacement sheet

Date: 7 OCT 1996

In a duplicate set of experiments, 0.33 nM PDGF AA is incubated with three of the above preincubation conditions, e.g., 2, 3, and 4 above. The human type B PDGF receptor does not appreciably recognize PDGF AA but this ligand will still activate cell-associated human type A PDGF receptor from NIH3T3 cells and so is a control for human type B PDGF receptor specificity and PDGF BB-dependent activation versus non-specific general cellular effect, e.g., cytotoxicity.

The preincubated materials were in a final volume of 0.5 ml. They were placed in one well each of a six well tissue culture dish containing a confluent layer of serum starved (quiescent) NIH3T3 cells which were chilled to 4°C. The cells and incubation mixtures were agitated, e.g., rocked, at 4°C for 2 h. They were then washed twice with 4°C phosphate buffered saline. Forty µl of 125 mM Tris(hydroxymethyl)amino methane (Tris), pH 6.8, 20% (v/v) glycerol, 2% (w/v) sodium dodecyl sulfate (SDS), 2% (v/v) 2-mercaptoethanol, and 0.001% bromphenol blue, (known as SDS sample buffer), was added per microtiter well followed by 40 µl of 100 mM Tris, pH 8.0, 30 mM sodium pyrosphoshate, 50 mM sodium fluoride, 5 mM ethylenediaminetetraacetic acid (EDTA), 5 mM ethylenebis(oxyethylenenitrilio)tetraacetic acid, 1% (w/v) SDS, 100 mM dithiothreitol, 2 mM phenylmethylsulfonylfluoride (PMSF), and 200 µM sodium vanadate was added to the cells. The cells were solubilized and 40 µl additional SDS sample buffer was added to the solubilizate. This material was boiled 5 minutes and loaded onto a single gel sample well of a 7.5% sodium dodecyl sulfate polyacrylamide gel. Cellular proteins were separated by electrophoresis.

The separated proteins were transferred to nitrocellulose by electrotransfer and the resulting "Western blot" was incubated with 3 changes of 0.5% (w/v) sodium chloride, 5 mg/ml bovine serum albumin, 50 mM Tris, pH 7.5, (designated blocking buffer) for 20 minutes each at room temperature. A 1/1000 dilution of PY20 (a commercially available monoclonal antibody to phosphotyrosine [ICN]) in blocking buffer was incubated with the blot overnight at 4°C. The blot was washed 3 times for 20 minutes each at room

This is the replacement sheet

Date: 7 OCT 1996

temperature in blocking buffer. The blot was incubated with 4 μCi/40 ml of ^{125}I-Protein A [Amersham] in blocking buffer for 1 hour at room temperature and washed 3 times for 20 minutes each at room temperature in blocking buffer. The blot was exposed to X-ray film for 48 h with one intensifying screen at -70°C and developed with standard reagents.

XII. PDGF Plate Assay

Polystyrene microtiter plates (Immulon, Dynatech Laboratories) were coated with the extracellular region fragment of the type B human PDGF receptor (described above) by incubating approximately 30-100 ng of this protein per well in 100 μl of 25 mM Tris, 75 mM NaCl, pH 7.35 for 12 to 18 h at 4°C. The protein was expressed in transfected CHO cells and collected in serum-free media (Gibco MEMα) at a concentration of 0.2 - 1 μg/ml, with a total protein concentration of 150 - 300 μg/ml.

The human type B PDGF receptor extracellular region fragment was concentrated and partially purified by passing the media over wheat germ-agglutinin-sepharose at 4°C (at 48 ml/h) in the presence of 1 mM PMSF. After extensive washing, the protein was eluted in 0.3 M N-acetyl-glucosamine, 25 mM Hepes, 100 mM NaCl, 1 mM PMSF, pH 7.4. This fraction was then applied to Sephacryl S-200 HR (Pharmacia) equilibrated in 0.15 M ammonium bicarbonate pH 7.9. The fractions containing receptor (3 - 10 ng/μl) were detected by SDS-PAGE and Western blotting with a polyclonal rabbit antibody against a peptide from the receptor external region. These fractions (3 - 10 ng/μl) were used to coat the microtiter wells as described above. The wells were then drained, rinsed once with 200 μl each of 0.5% gelatin (Bio-Rad, EIA grade), 25 mM Hepes, 100 mM NaCl, pH 7.4, and incubated for 3 h at 4°C with 150 μl of this same solution. The wells were drained and rinsed twice with 0.3% gelatin, 25 mM Hepes, 100 mM NaCl, pH 7.4 (150 μl each). The plate was put on ice and 90 μl of the 0.3% gelatin solution was put in each well (wells used to test nonspecific binding received just 80 μl and then 10 μl of 0.01 mg/ml non-labeled PDGF in the 0.3% gelatin solution). PDGF BB (Amgen) was iodinated at 4°C to

This is the replacement sheet

Date: - 7 OCT 1996

52,000 CPM/ng with di-iodo Bolton-Hunter reagent (Amersham) and approximately 40,000 CPM was added per well in 10 µl, containing 0.024% BSA, 0.4% gelatin, 20 mM Hepes, 80 mM NaCl, 70 mM acetic acid, pH 7.4. The plate was incubated for 4 h at 4°C, after which wells were washed three times with 150 µl each with 0.3% gelatin, 25 mM Hepes, 100 mM NaCl, pH 7.4 at 4°C. The bound radioactivity remaining was solubilized from the wells in 200 µl 1% SDS, 0.05% BSA, and counted in a gamma-counter. The nonspecific binding was determined in the presence of a 150-fold excess of unlabeled PDGF BB (Amgen) and was about 7% of the total bound ^{125}I-PDGF.

These studies were made possible by the availability of growth factor preparations devoid of contamination with other growth factors and by the use of a receptor expression system in which all of the measured PDGF responses could be attributed to this single transfected receptor cDNA.

XIII. Intracellular Region

Cell Culture and recombinant baculovirus.

BALB c/3T3 cells clone A31 from C.D. Scher, Children's Hospital of Philadelphia, PA., were cultured in Dulbecco Modified Eagle's medium (DMEM) supplemented with 10% bovine serum and penicillin and streptomycin (50 µg/ml each). Spodoptera frugiperda (Sf9) cells (from M. Summers, Texas A&M, TX) were grown in Grace's medium supplemented with 10% fetal bovine serum, 3.3 g/l yeastolate, 3.3 g/l lactalbumin hydrolysate, and penicillin and streptomycin 50 µg/ml each. Recombinant type B PDGF receptor and PDGF type B receptor ΔKI mutant baculovirus vectors were prepared by standard procedures. Recombinant baculovirus was collected from supernatant of Sf9 cells 48-60 hours after infection at a multiplicity of infection (MOI) of 1.

Antibodies, mitogens, and peptides

A type B PDGF receptor antibody (Ab77) directed against a synthetic peptide (amino acids 425-446) located at the extracellular region was used. PLC-γ monoclonal antibody was kindly provided by S.G. Rhee, NIH, Bethesda, MD, and GAP

This is the replacement sheet

Date: - 7 OCT 1996

antibodies were provided by F. McCormick, Cetus Corp., Emeryville, California. Recombinant BB-PDGF was provided by C.G. Nascimento from Chiron Corp. Emeryville, California. Peptides used in the association experiments were prepared by conventional peptide synthesis using phosphorylated tyrosine (N-Boc-Tyr-O-[PO_3Bzl_2], Peninsula Laboratory, Belmont, California) for the synthesis of tyrosine phosphorylated peptides.

Association Assays

In vivo associations were performed according to Morrison et al. (1989) Cell 58:649-657. Serum starved cultures of BALB c/3T3 cells (10^7 cells) were incubated in the presence or absence of 2 nM PDGF. Cell lysates were immunoprecipitated by using receptor antibody or phosphotyrosine antibodies. Immune complexes were used assayed for the presence of PI3 kinase activity and to determine the protein that associated with the receptor. In vitro association experiments were performed according to Morrison et al (1989) or Morrison et al. (1990) Mol. Cell. Biol. 10:2359-2366. Typically, baculovirus-expressed type B PDGF receptor was collected from Sf9 cells 48-60 hours after infection (MOI:10) by immunoprecipitation using receptor antibodies. Infected cells were washed twice with cold PBS and lysed in 1 ml of lysis buffer (1% NP-40, 20 mM Tris (pH 8.0), 173 mM NaCl, 10% glycerol, 2 mM EDTA, 1 mM phenylmethylsulfonylfluoride (PMSF), aprotinin (0.15 U/ml), 20 μM leupeptin, 1 mM sodium orthovanadate) at 4°C for 20 min with rocking. In most experiments (except where indicated in the legend of Fig. 3) the immunoprecipitated receptor was autophosphorylated in vitro by incubating the immune complexes in a buffer containing 20 mM Tris (pH 7.5), 20 mM MnCl, 100 μM ATP for 15 min at 25°C. Lysates were cleared of insoluble material by centrifugation at 13,000 x g for 10 min. Lysates were incubated with receptor antibodies (1:500 dilution) for 4 h at 4°C. Receptor-antibody complexes were precipitated using Protein-A sepharose (Sigma) and washed consecutively with RIPA buffer (lysis buffer with 0.1% SDS), wash buffer 1 [0.5% NP-40, 0.5 M LiCl, 50 mM Tris (pH 7.4)] and with 10 mM Tris (pH

This is the replacement sheet

Date: - 7 OCT 1996

7.4). The PI3 kinase association assay was performed by incubating the immobilized receptor with BALB c/3T3 cell lysates for 3 hours at 4°C. The immune complexes were consecutively washed with cold PBS; 0.5% NP-40, 0.5 M LiCl and 50 mM Tris (pH 7.4). In the experiment using peptides the BALB c/3T3 lysates were preincubated with the peptides (50 μM) for 30 min at 4°C prior to the incubation with the PDGF receptor protein expressed in the insect cell system.

In vitro kinase and PI3 kinase assays

In vitro protein kinase assays were performed by incubation of the immunoprecipitates in protein kinase buffer (30 mM Tris (pH 7.4), 10 mM $MnCl_2$) and 10 μCi [γ-^{32}P]-ATP 3,000 Ci/mmol, at 25°C for 15 min. The reaction was terminated by adding 4x Laemmli loading gel buffer. Samples were analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography. PI3 kinase activity was assayed as described by Kaplan et al. (1986) Proc. Nat'l Acad. Sci. USA 83:362-364. Immune complexes were incubated in PI3 kinase buffer (30 mM Hepes (pH 7.4), 30 mM $MgCl_2$, 200 μM adenosine, 40 μM ATP), 0.2 mg/ml of sonicated phosphoinositol (PI), and 10 μCi [γ-^{32}P]-ATP (3,000 Ci/mmol) at 25°C for 10 minutes. Adenosine was added in the PI3 kinase assays to inhibit any contaminating PI4 kinase activity. Reactions were terminated by the addition of 100 μl of HCl acid. The products of the reaction were extracted with chloroform and separated by thin layer chromatography. The conversion of PI into PIP was determined by exposing the TLC plate to an X-ray film for 2-3 h.

Receptor dephosphorylation

Receptor immunoprecipitates were incubated for 30 minutes at 30°C with 4 μg of potato acid phosphatase in the presence or absence of 1 mM sodium orthovanadate, see Morrison et al. (1989). After RAP treatment the immunoprecipitates were washed three times with RIPA buffer containing 1 mM sodium orthovanadate prior to the incubation with the 3T3 cell lysate.

This is the replacement sheet

Date: - 7 OCT 1996

"Blotting" of 85 kD protein with PDGF receptor probe

Cell lysate proteins transferred to nitrocellulose membranes were analyzed for their ability to bind directly to the PDGF receptor by probing the nitrocellulose membrane with ^{32}P-labeled PDGF receptor. To prepare the radiolabeled receptor probe, baculovirus-expressed receptor was immunoprecipitated from 10^6 Sf9 cells. The immune complexes were washed and labeled by autophosphorylation as described above. Labeled receptor was solubilized from the immunoprecipitates by repeated incubation of the immunoprecipitate in solubilization buffer (0.4% SDS, 100 mM NaCl, 2 mM EDTA, 2 mM β-mercaptoethanol, and 50 mM triethanolamine, pH 7.4) at 100°C for 2 min. The extracts were pooled, diluted in solubilization buffer without β-mercaptoethanol and brought to 10 mM iodoacetamide and 1% Triton X-100. Analysis of this material by SDS-polyacrylamide electrophoresis followed by radioautography revealed the presence of only the receptor band. BALB c/3T3 cells lysates were prepared and run on SDS-PAGE. Proteins were transferred onto nitrocellulose filter by electroblotting in the absence of SDS. Filters were incubated with radiolabeled PDGF receptor for 12 h at 4°C, and washed in the solubilization buffer containing 2% Triton X-100 without β-mercaptoethanol. For the peptide competition experiments the filters were incubated as described above with the ^{32}P-Labeled receptor probe and 2.5 μM of the indicated peptide.

All publications and patent applications herein are incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference. The invention now being fully described, it will be apparent to one of ordinary skill in the art that many changes and modifications can be made thereto without departing from the spirit or scope of the appended claims.

This is the replacement sheet

Date: - 7 OCT 1996

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Williams, Lewis T.
Escobedo, Jaime A.

(ii) TITLE OF INVENTION: PLATELET-DERIVED GROWTH FACTOR RECEPTORS

(iii) NUMBER OF SEQUENCES: 5

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: TOWNSEND and TOWNSEND
(B) STREET: Steuart Street Tower, 20th Floor
(C) CITY: San Francisco
(D) STATE: California
(E) COUNTRY: US
(F) ZIP: 94105

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.24

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US 07/151,141
(B) FILING DATE: 02-FEB-1988

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US 07/309,322
(B) FILING DATE: 10-FEB-1989

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Ching, Edwin P.
(B) REGISTRATION NUMBER: 34,090
(C) REFERENCE/DOCKET NUMBER: 2307U-267-2

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: (415) 326-2400
(B) TELEFAX: (415) 326-2422

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5427 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA to mRNA

(iii) HYPOTHETICAL: N

(iv) ANTI-SENSE: N

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Homo sapiens
(B) STRAIN: lambda gt10

This is the replacement sheet

Date: - 7 OCT 1996

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 187..3504
(D) OTHER INFORMATION:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

```
TGTTCTCCTG AGCCTTCAGG AGCCTGCACC AGTCCTGCCT GTCCTTCTAC TCAGCTGTTA      60

CCCACTCTGG GACCAGCAGT CTTTCTGATA ACTGGGAGAG GGCAGTAAGG AGGACTTCCT     120

GGAGGGGGTG ACTGTCCAGA GCCTGGAACT GTGCCCACAC CAGAAGCCAT CAGCAGCAAG     180

GACACC ATG CGG CTT CCG GGT GCG ATG CCA GCT CTG GCC CTC AAA GGC         228
       Met Arg Leu Pro Gly Ala Met Pro Ala Leu Ala Leu Lys Gly
         1               5                  10

GAG CTG CTG TTG CTG TCT CTC CTG TTA CTT CTG GAA CCA CAG ATC TCT       276
Glu Leu Leu Leu Leu Ser Leu Leu Leu Leu Leu Glu Pro Gln Ile Ser
 15                  20                  25                  30

CAG GGC CTG GTC GTC ACA CCC CCG GGG CCA GAG CTT GTC CTC AAT GTC       324
Gln Gly Leu Val Val Thr Pro Pro Gly Pro Glu Leu Val Leu Asn Val
                 35                  40                  45

TCC AGC ACC TTC GTT CTG ACC TGC TCG GGT TCA GCT CCG GTG GTG TGG       372
Ser Ser Thr Phe Val Leu Thr Cys Ser Gly Ser Ala Pro Val Val Trp
             50                  55                  60

GAA CGG ATG TCC CAG GAG CCC CCA CAG GAA ATG GCC AAG GCC CAG GAT       420
Glu Arg Met Ser Gln Glu Pro Pro Gln Glu Met Ala Lys Ala Gln Asp
         65                  70                  75

GGC ACC TTC TCC AGC GTG CTC ACA CTG ACC AAC CTC ACT GGG CTA GAC       468
Gly Thr Phe Ser Ser Val Leu Thr Leu Thr Asn Leu Thr Gly Leu Asp
     80                  85                  90

ACG GGA GAA TAC TTT TGC ACC CAC AAT GAC TCC CGT GGA CTG GAG ACC       516
Thr Gly Glu Tyr Phe Cys Thr His Asn Asp Ser Arg Gly Leu Glu Thr
 95                 100                 105                 110

GAT GAG CGG AAA CGG CTC TAC ATC TTT GTG CCA GAT CCC ACC GTG GGC       564
Asp Glu Arg Lys Arg Leu Tyr Ile Phe Val Pro Asp Pro Thr Val Gly
                115                 120                 125

TTC CTC CCT AAT GAT GCC GAG GAA CTA TTC ATC TTT CTC ACG GAA ATA       612
Phe Leu Pro Asn Asp Ala Glu Glu Leu Phe Ile Phe Leu Thr Glu Ile
            130                 135                 140

ACT GAG ATC ACC ATT CCA TGC CGA GTA ACA GAC CCA CAG CTG GTG GTG       660
Thr Glu Ile Thr Ile Pro Cys Arg Val Thr Asp Pro Gln Leu Val Val
        145                 150                 155

ACA CTG CAC GAG AAG AAA GGG GAC GTT GCA CTG CCT GTC CCC TAT GAT       708
Thr Leu His Glu Lys Lys Gly Asp Val Ala Leu Pro Val Pro Tyr Asp
    160                 165                 170

CAC CAA CGT GGC TTT TCT GGT ATC TTT GAG GAC AGA AGC TAC ATC TGC       756
His Gln Arg Gly Phe Ser Gly Ile Phe Glu Asp Arg Ser Tyr Ile Cys
175                 180                 185                 190

AAA ACC ACC ATT GGG GAC AGG GAG GTG GAT TCT GAT GCC TAC TAT GTC       804
Lys Thr Thr Ile Gly Asp Arg Glu Val Asp Ser Asp Ala Tyr Tyr Val
                195                 200                 205
```

This is the replacement sheet

Date: 7 OCT 1996

```
TAC AGA CTC CAG GTG TCA TCC ATC AAC GTC TCT GTG AAC GCA GTG CAG        852
Tyr Arg Leu Gln Val Ser Ser Ile Asn Val Ser Val Asn Ala Val Gln
            210                 215                 220

ACT GTG GTC CGC CAG GGT GAG AAC ATC ACC CTC ATG TGC ATT GTG ATC        900
Thr Val Val Arg Gln Gly Glu Asn Ile Thr Leu Met Cys Ile Val Ile
        225                 230                 235

GGG AAT GAT GTG GTC AAC TTC GAG TGG ACA TAC CCC CGC AAA GAA AGT        948
Gly Asn Asp Val Val Asn Phe Glu Trp Thr Tyr Pro Arg Lys Glu Ser
    240                 245                 250

GGG CGG CTG GTG GAG CCG GTG ACT GAC TTC CTC TTG GAT ATG CCT TAC        996
Gly Arg Leu Val Glu Pro Val Thr Asp Phe Leu Leu Asp Met Pro Tyr
255                 260                 265                 270

CAC ATC CGC TCC ATC CTG CAC ATC CCC AGT GCC GAG TTA GAA GAC TCG       1044
His Ile Arg Ser Ile Leu His Ile Pro Ser Ala Glu Leu Glu Asp Ser
                275                 280                 285

GGG ACC TAC ACC TGC AAT GTG ACG GAG AGT GTG AAT GAC CAT CAG GAT       1092
Gly Thr Tyr Thr Cys Asn Val Thr Glu Ser Val Asn Asp His Gln Asp
            290                 295                 300

GAA AAG GCC ATC AAC ATC ACC GTG GTT GAG AGC GGC TAC GTG CGG CTC       1140
Glu Lys Ala Ile Asn Ile Thr Val Val Glu Ser Gly Tyr Val Arg Leu
        305                 310                 315

CTG GGA GAG GTG GGC ACA CTA CAA TTT GCT GAG CTG CAT CGG AGC CGG       1188
Leu Gly Glu Val Gly Thr Leu Gln Phe Ala Glu Leu His Arg Ser Arg
    320                 325                 330

ACA CTG CAG GTA GTG TTC GAG GCC TAC CCA CCG CCC ACT GTC CTG TGG       1236
Thr Leu Gln Val Val Phe Glu Ala Tyr Pro Pro Pro Thr Val Leu Trp
335                 340                 345                 350

TTC AAA GAC AAC CGC ACC CTG GGC GAC TCC AGC GCT GGC GAA ATC GCC       1284
Phe Lys Asp Asn Arg Thr Leu Gly Asp Ser Ser Ala Gly Glu Ile Ala
                355                 360                 365

CTG TCC ACG CGC AAC GTG TCG GAG ACC CGG TAT GTG TCA GAG CTG ACA       1332
Leu Ser Thr Arg Asn Val Ser Glu Thr Arg Tyr Val Ser Glu Leu Thr
            370                 375                 380

CTG GTT CGC GTG AAG GTG GCA GAG GCT GGC CAC TAC ACC ATG CGG GCC       1380
Leu Val Arg Val Lys Val Ala Glu Ala Gly His Tyr Thr Met Arg Ala
        385                 390                 395

TTC CAT GAG GAT GCT GAG GTC CAG CTC TCC TTC CAG CTA CAG ATC AAT       1428
Phe His Glu Asp Ala Glu Val Gln Leu Ser Phe Gln Leu Gln Ile Asn
    400                 405                 410

GTC CCT GTC CGA GTG CTG GAG CTA AGT GAG AGC CAC CCT GAC AGT GGG       1476
Val Pro Val Arg Val Leu Glu Leu Ser Glu Ser His Pro Asp Ser Gly
415                 420                 425                 430

GAA CAG ACA GTC CGC TGT CGT GGC CGG GGC ATG CCG CAG CCG AAC ATC       1524
Glu Gln Thr Val Arg Cys Arg Gly Arg Gly Met Pro Gln Pro Asn Ile
                435                 440                 445

ATC TGG TCT GCC TGC AGA GAC CTC AAA AGG TGT CCA CGT GAG CTG CCG       1572
Ile Trp Ser Ala Cys Arg Asp Leu Lys Arg Cys Pro Arg Glu Leu Pro
            450                 455                 460

CCC ACG CTG CTG GGG AAC AGT TCC GAA GAG GAG AGC CAG CTG GAG ACT       1620
```

This is the replacement sheet

Date: 7 OCT 1996

```
Pro Thr Leu Leu Gly Asn Ser Ser Glu Glu Glu Ser Gln Leu Glu Thr
       465                 470                 475

AAC GTG ACG TAC TGG GAG GAG GAG CAG GAG TTT GAG GTG GTG AGC ACA      1668
Asn Val Thr Tyr Trp Glu Glu Glu Gln Glu Phe Glu Val Val Ser Thr
    480                 485                 490

CTG CGT CTG CAG CAC GTG GAT CGG CCA CTG TCG GTG CGC TGC ACG CTG      1716
Leu Arg Leu Gln His Val Asp Arg Pro Leu Ser Val Arg Cys Thr Leu
495                 500                 505                 510

CGC AAC GCT GTG GGC CAG GAC ACG CAG GAG GTC ATC GTG GTG CCA CAC      1764
Arg Asn Ala Val Gly Gln Asp Thr Gln Glu Val Ile Val Val Pro His
                515                 520                 525

TCC TTG CCC TTT AAG GTG GTG GTG ATC TCA GCC ATC CTG GCC CTG GTG      1812
Ser Leu Pro Phe Lys Val Val Val Ile Ser Ala Ile Leu Ala Leu Val
            530                 535                 540

GTG CTC ACC ATC ATC TCC CTT ATC ATC CTC ATC ATG CTT TGG CAG AAG      1860
Val Leu Thr Ile Ile Ser Leu Ile Ile Leu Ile Met Leu Trp Gln Lys
        545                 550                 555

AAG CCA CGT TAC GAG ATC CGA TGG AAG GTG ATT GAG TCT GTG AGC TCT      1908
Lys Pro Arg Tyr Glu Ile Arg Trp Lys Val Ile Glu Ser Val Ser Ser
    560                 565                 570

GAC GGC CAT GAG TAC ATC TAC GTG GAC CCC ATG CAG CTG CCC TAT GAC      1956
Asp Gly His Glu Tyr Ile Tyr Val Asp Pro Met Gln Leu Pro Tyr Asp
575                 580                 585                 590

TCC ACG TGG GAG CTG CCG CGG GAC CAG CTT GTG CTG GGA CGC ACC CTC      2004
Ser Thr Trp Glu Leu Pro Arg Asp Gln Leu Val Leu Gly Arg Thr Leu
                595                 600                 605

GGC TCT GGG GCC TTT GGG CAG GTG GTG GAG GCC ACA GCT CAT GGT CTG      2052
Gly Ser Gly Ala Phe Gly Gln Val Val Glu Ala Thr Ala His Gly Leu
            610                 615                 620

AGC CAT TCT CAG GCC ACG ATG AAA GTG GCC GTC AAG ATG CTT AAA TCC      2100
Ser His Ser Gln Ala Thr Met Lys Val Ala Val Lys Met Leu Lys Ser
        625                 630                 635

ACA GCC CGC AGC AGT GAG AAG CAA GCC CTT ATG TCG GAG CTG AAG ATC      2148
Thr Ala Arg Ser Ser Glu Lys Gln Ala Leu Met Ser Glu Leu Lys Ile
    640                 645                 650

ATG AGT CAC CTT GGG CCC CAC CTG AAC GTG GTC AAC CTG TTG GGG GCC      2196
Met Ser His Leu Gly Pro His Leu Asn Val Val Asn Leu Leu Gly Ala
655                 660                 665                 670

TGC ACC AAA GGA GGA CCC ATC TAT ATC ATC ACT GAG TAC TGC CGC TAC      2244
Cys Thr Lys Gly Gly Pro Ile Tyr Ile Ile Thr Glu Tyr Cys Arg Tyr
                675                 680                 685

GGA GAC CTG GTG GAC TAC CTG CAC CGC AAC AAA CAC ACC TTC CTG CAG      2292
Gly Asp Leu Val Asp Tyr Leu His Arg Asn Lys His Thr Phe Leu Gln
            690                 695                 700

CAC CAC TCC GAC AAG CGC CGC CCG CCC AGC GCG GAG CTC TAC AGC AAT      2340
His His Ser Asp Lys Arg Arg Pro Pro Ser Ala Glu Leu Tyr Ser Asn
        705                 710                 715

GCT CTG CCC GTT GGG CTC CCC CTG CCC AGC CAT GTG TCC TTG ACC GGG      2388
Ala Leu Pro Val Gly Leu Pro Leu Pro Ser His Val Ser Leu Thr Gly
    720                 725                 730
```

This is the replacement sheet

Date: - 7 OCT 1996

```
GAG AGC GAC GGT GGC TAC ATG GAC ATG AGC AAG GAC GAG TCG GTG GAC      2436
Glu Ser Asp Gly Gly Tyr Met Asp Met Ser Lys Asp Glu Ser Val Asp
735                 740                 745                 750

TAT GTG CCC ATG CTG GAC ATG AAA GGA GAC GTC AAA TAT GCA GAC ATC      2484
Tyr Val Pro Met Leu Asp Met Lys Gly Asp Val Lys Tyr Ala Asp Ile
                755                 760                 765

GAG TCC TCC AAC TAC ATG GCC CCT TAC GAT AAC TAC GTT CCC TCT GCC      2532
Glu Ser Ser Asn Tyr Met Ala Pro Tyr Asp Asn Tyr Val Pro Ser Ala
            770                 775                 780

CCT GAG AGG ACC TGC CGA GCA ACT TTG ATC AAC GAG TCT CCA GTG CTA      2580
Pro Glu Arg Thr Cys Arg Ala Thr Leu Ile Asn Glu Ser Pro Val Leu
        785                 790                 795

AGC TAC ATG GAC CTC GTG GGC TTC AGC TAC CAG GTG GCC AAT GGC ATG      2628
Ser Tyr Met Asp Leu Val Gly Phe Ser Tyr Gln Val Ala Asn Gly Met
    800                 805                 810

GAG TTT CTG GCC TCC AAG AAC TGC GTC CAC AGA GAC CTG GCG GCT AGG      2676
Glu Phe Leu Ala Ser Lys Asn Cys Val His Arg Asp Leu Ala Ala Arg
815                 820                 825                 830

AAC GTG CTC ATC TGT GAA GGC AAG CTG GTC AAG ATC TGT GAC TTT GGC      2724
Asn Val Leu Ile Cys Glu Gly Lys Leu Val Lys Ile Cys Asp Phe Gly
                835                 840                 845

CTG GCT CGA GAC ATC ATG CGG GAC TCG AAT TAC ATC TCC AAA GGC AGC      2772
Leu Ala Arg Asp Ile Met Arg Asp Ser Asn Tyr Ile Ser Lys Gly Ser
            850                 855                 860

ACC TTT TTG CCT TTA AAG TGG ATG GCT CCG GAG AGC ATC TTC AAC AGC      2820
Thr Phe Leu Pro Leu Lys Trp Met Ala Pro Glu Ser Ile Phe Asn Ser
        865                 870                 875

CTC TAC ACC ACC CTG AGC GAC GTG TGG TCC TTC GGG ATC CTG CTC TGG      2868
Leu Tyr Thr Thr Leu Ser Asp Val Trp Ser Phe Gly Ile Leu Leu Trp
    880                 885                 890

GAG ATC TTC ACC TTG GGT GGC ACC CCT TAC CCA GAG CTG CCC ATG AAC      2916
Glu Ile Phe Thr Leu Gly Gly Thr Pro Tyr Pro Glu Leu Pro Met Asn
895                 900                 905                 910

GAG CAG TTC TAC AAT GCC ATC AAA CGG GGT TAC CGC ATG GCC CAG CCT      2964
Glu Gln Phe Tyr Asn Ala Ile Lys Arg Gly Tyr Arg Met Ala Gln Pro
                915                 920                 925

GCC CAT GCC TCC GAC GAG ATC TAT GAG ATC ATG CAG AAG TGC TGG GAA      3012
Ala His Ala Ser Asp Glu Ile Tyr Glu Ile Met Gln Lys Cys Trp Glu
            930                 935                 940

GAG AAG TTT GAG ATT CGG CCC CCC TTC TCC CAG CTG GTG CTG CTT CTC      3060
Glu Lys Phe Glu Ile Arg Pro Pro Phe Ser Gln Leu Val Leu Leu Leu
        945                 950                 955

GAG AGA CTG TTG GGC GAA GGT TAC AAA AAG AAG TAC CAG CAG GTG GAT      3108
Glu Arg Leu Leu Gly Glu Gly Tyr Lys Lys Lys Tyr Gln Gln Val Asp
    960                 965                 970

GAG GAG TTT CTG AGG AGT GAC CAC CCA GCC ATC CTT CGG TCC CAG GCC      3156
Glu Glu Phe Leu Arg Ser Asp His Pro Ala Ile Leu Arg Ser Gln Ala
975                 980                 985                 990

CGC TTG CCT GGG TTC CAT GGC CTC CGA TCT CCC CTG GAC ACC AGC TCC      3204
```

This is the replacement sheet

Date: - 7 OCT 1996

```
Arg Leu Pro Gly Phe His Gly Leu Arg Ser Pro Leu Asp Thr Ser Ser
                995                1000                1005

GTC CTC TAT ACT GCC GTG CAG CCC AAT GAG GGT GAC AAC GAC TAT ATC      3252
Val Leu Tyr Thr Ala Val Gln Pro Asn Glu Gly Asp Asn Asp Tyr Ile
           1010                1015                1020

ATC CCC CTG CCT GAC CCC AAA CCT GAG GTT GCT GAC GAG GGC CCA CTG      3300
Ile Pro Leu Pro Asp Pro Lys Pro Glu Val Ala Asp Glu Gly Pro Leu
       1025                1030                1035

GAG GGT TCC CCC AGC CTA GCC AGC TCC ACC CTG AAT GAA GTC AAC ACC      3348
Glu Gly Ser Pro Ser Leu Ala Ser Ser Thr Leu Asn Glu Val Asn Thr
   1040                1045                1050

TCC TCA ACC ATC TCC TGT GAC AGC CCC CTG GAG CCC CAG GAC GAA GCA      3396
Ser Ser Thr Ile Ser Cys Asp Ser Pro Leu Glu Pro Gln Asp Glu Pro
1055                1060                1065                1070

GAG CCA GAG CCC CAG CTT GAG CTC CAG GTG GAG CCG GAG CCG GAG CTG      3444
Glu Pro Glu Pro Gln Leu Glu Leu Gln Val Glu Pro Glu Pro Glu Leu
                1075                1080                1085

GAA CAG TTG CCG GAT TCG GGG TGC CCT GCG CCT CGG GCG GAA GCA GAG      3492
Glu Gln Leu Pro Asp Ser Gly Cys Pro Ala Pro Arg Ala Glu Ala Glu
           1090                1095                1100

GAT AGC TTC CTG TAGGGGGCTG GCCCCTACCC TGCCCTGCCT GAAGCTCCCC          3544
Asp Ser Phe Leu
       1105

CGCTGCCAGC ACCCAGCATC TCCTGGCCTG GCCTGGCCGG GCTTCCTGTC AGCCAGGCTG    3604

CCCTTATCAG CTGTCCCCTT CTGGAAGCTT TCTGCTCCTG ACGTGTTGTG CCCCAAACCC    3664

TGGGGCTGGC TTAGGAGGCA AGAAAACTGC AGGGGCCGTG ACCAGCCCTC TGCCTCCAGG    3724

GAGGCCAACT GACTCTGAGC CAGGGTTCCC CCAGGGAACT CAGTTTTCCC ATATGTAAGA    3784

TGGGAAAGTT AGGCTTGATG ACCCAGAATC TAGGATTCTC TCCCTGGCTG ACAGGTGGGG    3844

AGACCGAATC CCTCCCTGGG AAGATTCTTG GAGTTACTGA GGTGGTAAAT TAACTTTTTT    3904

CTGTTCAGCC AGCTACCCCT CAAGGAATCA TAGCTCTCTC CTCGCACTTT TATCCACCCA    3964

GGAGCTAGGG AAGAGACCCT AGCCTCCCTG GCTGCTGGCT GAGCTAGGGC CTAGCCTTGA    4024

GCAGTGTTGC CTCATCCAGA AGAAAGCCAG TCTCCTCCCT ATGATGCCAG TCCCTGCGTT    4084

CCCTGGCCCG AGCTGGTCTG GGGCCATTAG GCAGCCTAAT TAATGCTGGA GGCTGAGCCA    4144

AGTACAGGAC ACCCCCAGCC TGCAGCCCTT GCCCAGGGCA CTTGGAGCAC ACGCAGCCAT    4204

AGCAAGTGCC TGTGTCCCTG TCCTTCAGGC CCATCAGTCC TGGGGCTTTT TCTTTATCAC    4264

CCTCAGTCTT AATCCATCCA CCAGAGTCTA GAAGGCCAGA CGGGCCCCGC ATCTGTGATG    4324

AGAATGTAAA TGTGCCAGTG TGGAGTGGCC ACGTGTGTGT GCCAGATATG GCCCTGGCTC    4384

TGCATTGGAC CTGCTATGAG GCTTTGGAGG AATCCCTCAC CCTCTCTGGG CCTCAGTTTC    4444

CCCTTCAAAA AATGAATAAG TCGGACTTAT TAACTCTGAG TGCCTTGCCA GCACTAACAT    4504

TCTAGAGTAT CCAGGTGGTT GCACATTTGT CCAGATGAAG CAAGGCCATA TACCCTAAAC    4564

TTCCATCCTG GGGGTCAGCT GGGCTCCTGG GAGATTCCAG ATCACACATC ACACTCTGGG    4624
```

This is the replacement sheet

Date: - 7 OCT 1996

GACTCAGGAA CCATGCCCCT TCCCCAGGCC CCCAGCAAGT CTCAAGAACA CAGCTGCACA 4684

GGCCTTGACT TAGAGTGACA GCCGGTGTCC TGGAAAGCCC CCAGCAGCTG CCCCAGGGAC 4744

ATGGGAAGAC CACGGGACCT CTTTCACTAC CCACGATGAC CTCCGGGGGT ATCCTGGGCA 4804

AAAGGGACAA AGAGGGCAAA TGAGATCACC TCCTGCAGCC CACCACTCCA GCACCTGTGC 4864

CGAGGTCTGC GTCGAAGACA GAATGGACAG TGAGGACAGT TATGTCTTGT AAAAGACAAG 4924

AAGCTTCAGA TGGGTACCCC AAGAAGGATG TGAGAGGTGG GCGCTTTGGA GGTTTGCCCC 4984

TCACCCACCA GCTGCCCCAT CCCTGAGGCA GCGCTCCATG GGGGTATGGT TTTGTCACTG 5044

CCCAGACCTA GCAGTGACAT CTCATTGTCC CCAGCCCAGT GGGCATTGGA GGTGCCAGGG 5104

GAGTCAGGGT TGTAGCCAAG ACGCCCCCGC ACGGGGAGGG TTGGGAAGGG GGTGCAGGAA 5164

GCTCAACCCC TCTGGGCACC AACCCTGCAT TGCAGGTTGG CACCTTACTT CCCTGGGATC 5224

CCAGAGTTGG TCCAAGGAGG GAGAGTGGGT TCTCAATACG GTACCAAAGA TATAATCACC 5284

TAGGTTTACA AATATTTTTA GGACTCACGT TAACTCACAT TTATACAGCA GAAATGCTAT 5344

TTTGTATGCT GTTAAGTTTT TCTATCTGTG TACTTTTTTT TAAGGGAAAG ATTTTAATAT 5404

TAAACCTGGT GCTTCTCACT CAC 5427

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1106 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met Arg Leu Pro Gly Ala Met Pro Ala Leu Ala Leu Lys Gly Glu Leu
1 5 10 15

Leu Leu Leu Ser Leu Leu Leu Leu Leu Glu Pro Gln Ile Ser Gln Gly
20 25 30

Leu Val Val Thr Pro Pro Gly Pro Glu Leu Val Leu Asn Val Ser Ser
35 40 45

Thr Phe Val Leu Thr Cys Ser Gly Ser Ala Pro Val Val Trp Glu Arg
50 55 60

Met Ser Gln Glu Pro Pro Gln Glu Met Ala Lys Ala Gln Asp Gly Thr
65 70 75 80

Phe Ser Ser Val Leu Thr Leu Thr Asn Leu Thr Gly Leu Asp Thr Gly
85 90 95

Glu Tyr Phe Cys Thr His Asn Asp Ser Arg Gly Leu Glu Thr Asp Glu
100 105 110

Arg Lys Arg Leu Tyr Ile Phe Val Pro Asp Pro Thr Val Gly Phe Leu
115 120 125

Pro Asn Asp Ala Glu Glu Leu Phe Ile Phe Leu Thr Glu Ile Thr Glu

This is the replacement sheet

Date: - 7 OCT 1996

130 135 140

Ile Thr Ile Pro Cys Arg Val Thr Asp Pro Gln Leu Val Val Thr Leu
145 150 155 160

His Glu Lys Lys Gly Asp Val Ala Leu Pro Val Pro Tyr Asp His Gln
165 170 175

Arg Gly Phe Ser Gly Ile Phe Glu Asp Arg Ser Tyr Ile Cys Lys Thr
180 185 190

Thr Ile Gly Asp Arg Glu Val Asp Ser Asp Ala Tyr Tyr Val Tyr Arg
195 200 205

Leu Gln Val Ser Ser Ile Asn Val Ser Val Asn Ala Val Gln Thr Val
210 215 220

Val Arg Gln Gly Glu Asn Ile Thr Leu Met Cys Ile Val Ile Gly Asn
225 230 235 240

Asp Val Val Asn Phe Glu Trp Thr Tyr Pro Arg Lys Glu Ser Gly Arg
245 250 255

Leu Val Glu Pro Val Thr Asp Phe Leu Leu Asp Met Pro Tyr His Ile
260 265 270

Arg Ser Ile Leu His Ile Pro Ser Ala Glu Leu Glu Asp Ser Gly Thr
275 280 285

Tyr Thr Cys Asn Val Thr Glu Ser Val Asn Asp His Gln Asp Glu Lys
290 295 300

Ala Ile Asn Ile Thr Val Val Glu Ser Gly Tyr Val Arg Leu Leu Gly
305 310 315 320

Glu Val Gly Thr Leu Gln Phe Ala Glu Leu His Arg Ser Arg Thr Leu
325 330 335

Gln Val Val Phe Glu Ala Tyr Pro Pro Pro Thr Val Leu Trp Phe Lys
340 345 350

Asp Asn Arg Thr Leu Gly Asp Ser Ser Ala Gly Glu Ile Ala Leu Ser
355 360 365

Thr Arg Asn Val Ser Glu Thr Arg Tyr Val Ser Glu Leu Thr Leu Val
370 375 380

Arg Val Lys Val Ala Glu Ala Gly His Tyr Thr Met Arg Ala Phe His
385 390 395 400

Glu Asp Ala Glu Val Gln Leu Ser Phe Gln Leu Gln Ile Asn Val Pro
405 410 415

Val Arg Val Leu Glu Leu Ser Glu Ser His Pro Asp Ser Gly Glu Gln
420 425 430

Thr Val Arg Cys Arg Gly Arg Gly Met Pro Gln Pro Asn Ile Ile Trp
435 440 445

Ser Ala Cys Arg Asp Leu Lys Arg Cys Pro Arg Glu Leu Pro Pro Thr
450 455 460

Leu Leu Gly Asn Ser Ser Glu Glu Glu Ser Gln Leu Glu Thr Asn Val
465 470 475 480

Thr Tyr Trp Glu Glu Glu Gln Glu Phe Glu Val Val Ser Thr Leu Arg

This is the replacement sheet

Date: - 7 OCT 1998

485 490 495

Leu Gln His Val Asp Arg Pro Leu Ser Val Arg Cys Thr Leu Arg Asn
500 505 510

Ala Val Gly Gln Asp Thr Gln Glu Val Ile Val Val Pro His Ser Leu
515 520 525

Pro Phe Lys Val Val Val Ile Ser Ala Ile Leu Ala Leu Val Val Leu
530 535 540

Thr Ile Ile Ser Leu Ile Ile Leu Ile Met Leu Trp Gln Lys Lys Pro
545 550 555 560

Arg Tyr Glu Ile Arg Trp Lys Val Ile Glu Ser Val Ser Ser Asp Gly
565 570 575

His Glu Tyr Ile Tyr Val Asp Pro Met Gln Leu Pro Tyr Asp Ser Thr
580 585 590

Trp Glu Leu Pro Arg Asp Gln Leu Val Leu Gly Arg Thr Leu Gly Ser
595 600 605

Gly Ala Phe Gly Gln Val Val Glu Ala Thr Ala His Gly Leu Ser His
610 615 620

Ser Gln Ala Thr Met Lys Val Ala Val Lys Met Leu Lys Ser Thr Ala
625 630 635 640

Arg Ser Ser Glu Lys Gln Ala Leu Met Ser Glu Leu Lys Ile Met Ser
645 650 655

His Leu Gly Pro His Leu Asn Val Val Asn Leu Leu Gly Ala Cys Thr
660 665 670

Lys Gly Gly Pro Ile Tyr Ile Ile Thr Glu Tyr Cys Arg Tyr Gly Asp
675 680 685

Leu Val Asp Tyr Leu His Arg Asn Lys His Thr Phe Leu Gln His His
690 695 700

Ser Asp Lys Arg Arg Pro Pro Ser Ala Glu Leu Tyr Ser Asn Ala Leu
705 710 715 720

Pro Val Gly Leu Pro Leu Pro Ser His Val Ser Leu Thr Gly Glu Ser
725 730 735

Asp Gly Gly Tyr Met Asp Met Ser Lys Asp Glu Ser Val Asp Tyr Val
740 745 750

Pro Met Leu Asp Met Lys Gly Asp Val Lys Tyr Ala Asp Ile Glu Ser
755 760 765

Ser Asn Tyr Met Ala Pro Tyr Asp Asn Tyr Val Pro Ser Ala Pro Glu
770 775 780

Arg Thr Cys Arg Ala Thr Leu Ile Asn Glu Ser Pro Val Leu Ser Tyr
785 790 795 800

Met Asp Leu Val Gly Phe Ser Tyr Gln Val Ala Asn Gly Met Glu Phe
805 810 815

Leu Ala Ser Lys Asn Cys Val His Arg Asp Leu Ala Ala Arg Asn Val
820 825 830

Leu Ile Cys Glu Gly Lys Leu Val Lys Ile Cys Asp Phe Gly Leu Ala

This is the replacement sheet

Date: - 7 OCT 1996

```
       835                 840                 845

Arg Asp Ile Met Arg Asp Ser Asn Tyr Ile Ser Lys Gly Ser Thr Phe
    850                 855                 860

Leu Pro Leu Lys Trp Met Ala Pro Glu Ser Ile Phe Asn Ser Leu Tyr
865                 870                 875                 880

Thr Thr Leu Ser Asp Val Trp Ser Phe Gly Ile Leu Leu Trp Glu Ile
                885                 890                 895

Phe Thr Leu Gly Gly Thr Pro Tyr Pro Glu Leu Pro Met Asn Glu Gln
            900                 905                 910

Phe Tyr Asn Ala Ile Lys Arg Gly Tyr Arg Met Ala Gln Pro Ala His
        915                 920                 925

Ala Ser Asp Glu Ile Tyr Glu Ile Met Gln Lys Cys Trp Glu Glu Lys
    930                 935                 940

Phe Glu Ile Arg Pro Pro Phe Ser Gln Leu Val Leu Leu Leu Glu Arg
945                 950                 955                 960

Leu Leu Gly Glu Gly Tyr Lys Lys Lys Tyr Gln Gln Val Asp Glu Glu
                965                 970                 975

Phe Leu Arg Ser Asp His Pro Ala Ile Leu Arg Ser Gln Ala Arg Leu
            980                 985                 990

Pro Gly Phe His Gly Leu Arg Ser Pro Leu Asp Thr Ser Ser Val Leu
        995                 1000                1005

Tyr Thr Ala Val Gln Pro Asn Glu Gly Asp Asn Asp Tyr Ile Ile Pro
    1010                1015                1020

Leu Pro Asp Pro Lys Pro Glu Val Ala Asp Glu Gly Pro Leu Glu Gly
1025                1030                1035                1040

Ser Pro Ser Leu Ala Ser Ser Thr Leu Asn Glu Val Asn Thr Ser Ser
                1045                1050                1055

Thr Ile Ser Cys Asp Ser Pro Leu Glu Pro Gln Asp Glu Pro Glu Pro
            1060                1065                1070

Glu Pro Gln Leu Glu Leu Gln Val Glu Pro Glu Pro Glu Leu Glu Gln
        1075                1080                1085

Leu Pro Asp Ser Gly Cys Pro Ala Pro Arg Ala Glu Ala Glu Asp Ser
    1090                1095                1100

Phe Leu
1105
```

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 4100 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA to mRNA

(iii) HYPOTHETICAL: N

(iv) ANTI-SENSE: N

This is the replacement sheet

Date: 7 OCT 1996

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Homo Sapiens

(vii) IMMEDIATE SOURCE:
(A) LIBRARY: lambda gt10

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 129..3395
(D) OTHER INFORMATION:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

```
TTGGAGCTAC AGGGAGAGAA ACAGAGGAGG AGACTGCAAG AGATCATTGC AGGCCGTGGG      60

CACGCTCTTT ACTCCATGTG TGGGACATTC ATTGCGGAAT AACATCGGAG GAGAAGTTTC     120

CCAGAGCT ATG GGG ACT TCC CAT CCG GCG TTC CTG GTC TTA GGC TGT CTT      170
         Met Gly Thr Ser His Pro Ala Phe Leu Val Leu Gly Cys Leu
           1               5                  10

CTC ACA GGG CTG AGC CTA ATC CTC TGC CAG CTT TCA TTA CCC TCT ATC      218
Leu Thr Gly Leu Ser Leu Ile Leu Cys Gln Leu Ser Leu Pro Ser Ile
 15                  20                  25                  30

CTT CCA AAT GAA AAT GAA AAG GTT GTG CAG CTG AAT TCA TCC TTT TCT      266
Leu Pro Asn Glu Asn Glu Lys Val Val Gln Leu Asn Ser Ser Phe Ser
                 35                  40                  45

CTG AGA TGC TTT GGG GAG AGT GAA GTG AGC TGG CAG TAC CCC ATG TCT      314
Leu Arg Cys Phe Gly Glu Ser Glu Val Ser Trp Gln Tyr Pro Met Ser
             50                  55                  60

GAA GAA GAG AGC TCC GAT GTG GAA ATC AGA AAT GAA GAA AAC AAC AGC      362
Glu Glu Glu Ser Ser Asp Val Glu Ile Arg Asn Glu Glu Asn Asn Ser
         65                  70                  75

GGC CTT TTT GTG ACG GTC TTG GAA GTG AGC AGT GCC TCG GCG GCC CAC      410
Gly Leu Phe Val Thr Val Leu Glu Val Ser Ser Ala Ser Ala Ala His
     80                  85                  90

ACA GGG TTG TAC ACT TGC TAT TAC AAC CAC ACT CAG ACA GAA GAG AAT      458
Thr Gly Leu Tyr Thr Cys Tyr Tyr Asn His Thr Gln Thr Glu Glu Asn
 95                 100                 105                 110

GAG CTT GAA GGC AGG CAC ATT TAC ATC TAT GTG CCA GAC CCA GAT GTA      506
Glu Leu Glu Gly Arg His Ile Tyr Ile Tyr Val Pro Asp Pro Asp Val
                115                 120                 125

GCC TTT GTA CCT CTA GGA ATG ACG GAT TAT TTA GTC ATC GTG GAG GAT      554
Ala Phe Val Pro Leu Gly Met Thr Asp Tyr Leu Val Ile Val Glu Asp
            130                 135                 140

GAT GAT TCT GCC ATT ATA CCT TGT CGC ACA ACT GAT CCC GAG ACT CCT      602
Asp Asp Ser Ala Ile Ile Pro Cys Arg Thr Thr Asp Pro Glu Thr Pro
        145                 150                 155

GTA ACC TTA CAC AAC AGT GAG GGG GTG GTA CCT GCC TCC TAC GAC AGC      650
Val Thr Leu His Asn Ser Glu Gly Val Val Pro Ala Ser Tyr Asp Ser
    160                 165                 170

AGA CAG GGC TTT AAT GGG ACC TTC ACT GTA GGG CCG TAT ATC TGT GAG      698
Arg Gln Gly Phe Asn Gly Thr Phe Thr Val Gly Pro Tyr Ile Cys Glu
175                 180                 185                 190
```

This is the replacement sheet

Date: - 7 OCT 1996

```
GCC ACC GTC AAA GGA AAG AAG TTC CAG ACC ATC CCA TTT AAT GTT TAT        746
Ala Thr Val Lys Gly Lys Lys Phe Gln Thr Ile Pro Phe Asn Val Tyr
                195                 200                 205

GCT TTA AAA GCA ACA TCA GAG CTG GAT CTA GAA ATG GAA GCT CTT AAA        794
Ala Leu Lys Ala Thr Ser Glu Leu Asp Leu Glu Met Glu Ala Leu Lys
            210                 215                 220

ACC GTG TAT AAG TCA GGG GAA ACG ATT GTG GTC ACC TGT GCT GTT TTT        842
Thr Val Tyr Lys Ser Gly Glu Thr Ile Val Val Thr Cys Ala Val Phe
        225                 230                 235

AAC AAT GAG GTG GTT GAC CTT CAA TGG ACT TAC CCT GGA GAA GTG AAA        890
Asn Asn Glu Val Val Asp Leu Gln Trp Thr Tyr Pro Gly Glu Val Lys
    240                 245                 250

GGC AAA GGC ATC ACA ATG CTG GAA GAA ATC AAA GTC CCA TCC ATC AAA        938
Gly Lys Gly Ile Thr Met Leu Glu Glu Ile Lys Val Pro Ser Ile Lys
255                 260                 265                 270

TTG GTG TAC ACT TTG ACG GTC CCC GAG GCC ACG GTG AAA GAC AGT GGA        986
Leu Val Tyr Thr Leu Thr Val Pro Glu Ala Thr Val Lys Asp Ser Gly
                275                 280                 285

GAT TAC GAA TGT GCT GCC CGC CAG GCT ACC AGG GAG GTC AAA GAA ATG       1034
Asp Tyr Glu Cys Ala Ala Arg Gln Ala Thr Arg Glu Val Lys Glu Met
            290                 295                 300

AAG AAA GTC ACT ATT TCT GTC CAT GAG AAA GGT TTC ATT GAA ATC AAA       1082
Lys Lys Val Thr Ile Ser Val His Glu Lys Gly Phe Ile Glu Ile Lys
        305                 310                 315

CCC ACC TTC AGC CAG TTG GAA GCT GTC AAC CTG CAT GAA GTC AAA CAT       1130
Pro Thr Phe Ser Gln Leu Glu Ala Val Asn Leu His Glu Val Lys His
    320                 325                 330

TTT GTT GTA GAG GTG CGG GCC TAC CCA CCT CCC AGG ATA TCC TGG CTG       1178
Phe Val Val Glu Val Arg Ala Tyr Pro Pro Pro Arg Ile Ser Trp Leu
335                 340                 345                 350

AAA AAC AAT CTG ACT CTG ATT GAA AAT CTC ACT GAG ATC ACC ACT GAT       1226
Lys Asn Asn Leu Thr Leu Ile Glu Asn Leu Thr Glu Ile Thr Thr Asp
                355                 360                 365

GTG GAA AAG ATT CAG GAA ATA AGG TAT CGA AGC AAA TTA AAG CTG ATC       1274
Val Glu Lys Ile Gln Glu Ile Arg Tyr Arg Ser Lys Leu Lys Leu Ile
            370                 375                 380

CGT GCT AAG GAA GAA GAC AGT GGC CAT TAT ACT ATT GTA GCT CAA AAT       1322
Arg Ala Lys Glu Glu Asp Ser Gly His Tyr Thr Ile Val Ala Gln Asn
        385                 390                 395

GAA GAT GCT GTG AAG AGC TAT ACT TTT GAA CTG TTA ACT CAA GTT CCT       1370
Glu Asp Ala Val Lys Ser Tyr Thr Phe Glu Leu Leu Thr Gln Val Pro
    400                 405                 410

TCA TCC ATT CTG GAC TTG GTC GAT GAT CAC CAT GGC TCA ACT GGG GGA       1418
Ser Ser Ile Leu Asp Leu Val Asp Asp His His Gly Ser Thr Gly Gly
415                 420                 425                 430

CAG ACG GTG AGG TGC ACA GCT GAA GGC ACG CCG CTT CCT GAT ATT GAG       1466
Gln Thr Val Arg Cys Thr Ala Glu Gly Thr Pro Leu Pro Asp Ile Glu
                435                 440                 445

TGG ATG ATA TGC AAA GAT ATT AAG AAA TGT AAT AAT GAA ACT TCC TGG       1514
```

This is the replacement sheet

Date: 7 OCT 1996

```
Trp Met Ile Cys Lys Asp Ile Lys Lys Cys Asn Asn Glu Thr Ser Trp
            450                 455                 460

ACT ATT TTG GCC AAC AAT GTC TCA AAC ATC ATC ACG GAG ATC CAC TCC      1562
Thr Ile Leu Ala Asn Asn Val Ser Asn Ile Ile Thr Glu Ile His Ser
        465                 470                 475

CGA GAC AGG AGT ACC GTG GAG GGC CGT GTG ACT TTC GCC AAA GTG GAG      1610
Arg Asp Arg Ser Thr Val Glu Gly Arg Val Thr Phe Ala Lys Val Glu
    480                 485                 490

GAG ACC ATC GCC GTG CGA TGC CTG GCT AAG AAT CTC CTT GGA GCT GAG      1658
Glu Thr Ile Ala Val Arg Cys Leu Ala Lys Asn Leu Leu Gly Ala Glu
495                 500                 505                 510

AAC CGA GAG CTG AAG CTG GTG GCT CCC ACC CTG CGT TCT GAA CTC ACG      1706
Asn Arg Glu Leu Lys Leu Val Ala Pro Thr Leu Arg Ser Glu Leu Thr
                515                 520                 525

GTG GCT GCT GCA GTC CTG GTG CTG TTG GTG ATT GTG ATC ATC TCA CTT      1754
Val Ala Ala Ala Val Leu Val Leu Leu Val Ile Val Ile Ile Ser Leu
            530                 535                 540

ATT GTC CTG GTT GTC ATT TGC AAA CAG AAA CCG AGG TAT GAA ATT CGC      1802
Ile Val Leu Val Val Ile Trp Lys Gln Lys Pro Arg Tyr Glu Ile Arg
        545                 550                 555

TGG AGG GTC ATT GAA TCA ATC AGC CCA GAT GGA CAT GAA TAT ATT TAT      1850
Trp Arg Val Ile Glu Ser Ile Ser Pro Asp Gly His Glu Tyr Ile Tyr
    560                 565                 570

GTG GAC CCG ATG CAG CTG CCT TAT GAC TCA AGA TGG GAG TTT CCA AGA      1898
Val Asp Pro Met Gln Leu Pro Tyr Asp Ser Arg Trp Glu Phe Pro Arg
575                 580                 585                 590

GAT GGA CTA GTG CTT GGT CGG GTC TTG GGG TCT GGA GCG TTT GGG AAG      1946
Asp Gly Leu Val Leu Gly Arg Val Leu Gly Ser Gly Ala Phe Gly Lys
                595                 600                 605

GTG GTT GAA GGA ACA GCC TAT GGA TTA AGC CGG TCC CAA CCT GTC ATG      1994
Val Val Glu Gly Thr Ala Tyr Gly Leu Ser Arg Ser Gln Pro Val Met
            610                 615                 620

AAA GTT GCA GTG AAG ATG CTA AAA CCC ACG GCC AGA TCC AGT GAA AAA      2042
Lys Val Ala Val Lys Met Leu Lys Pro Thr Ala Arg Ser Ser Glu Lys
        625                 630                 635

CAA GCT CTC ATG TCT GAA CTG AAG ATA ATG ACT CAC CTG GGG CCA CAT      2090
Gln Ala Leu Met Ser Glu Leu Lys Ile Met Thr His Leu Gly Pro His
    640                 645                 650

TTG AAC ATT GTA AAC TTG CTG GGA GCC TGC ACC AAG TCA GGC CCC ATT      2138
Leu Asn Ile Val Asn Leu Leu Gly Ala Cys Thr Lys Ser Gly Pro Ile
655                 660                 665                 670

TAC ATC ATC ACA GAG TAT TGC TTC TAT GGA GAT TTG GTC AAC TAT TTG      2186
Tyr Ile Ile Thr Glu Tyr Cys Phe Tyr Gly Asp Leu Val Asn Tyr Leu
                675                 680                 685

CAT AAG AAT AGC GAT AGC TTC CTG AGC CAC CAC CCA GAG AAG CCA AAG      2234
His Lys Asn Arg Asp Ser Phe Leu Ser His His Pro Glu Lys Pro Lys
            690                 695                 700

AAA GAG CTG GAT ATC TTT GGA TTG AAC CCT GCT GAT GAA AGC ACA CGG      2282
Lys Glu Leu Asp Ile Phe Gly Leu Asn Pro Ala Asp Glu Ser Thr Arg
        705                 710                 715
```

This is the replacement sheet

Date: - 7 OCT 1996

```
AGC TAT GTT ATT TTA TCT TTT GAA AAC AAT GGT GAC TAC ATG GAC ATG      2330
Ser Tyr Val Ile Leu Ser Phe Glu Asn Asn Gly Asp Tyr Met Asp Met
    720                 725                 730

AAG CAG GCT GAT ACT ACA CAG TAT GTC CCC ATG CTA GAA AGG AAA GAG      2378
Lys Gln Ala Asp Thr Thr Gln Tyr Val Pro Met Leu Glu Arg Lys Glu
735                 740                 745                 750

GTT TCT AAA TAT TCC GAC ATC CAG AGA TCA CTC TAT GAT CGT CCA GCC      2426
Val Ser Lys Tyr Ser Asp Ile Gln Arg Ser Leu Tyr Asp Arg Pro Ala
                755                 760                 765

TCA TAT AAG AAG AAA TCT ATG TTA GAC TCA GAA GTC AAA AAC CTC CTT      2474
Ser Tyr Lys Lys Lys Ser Met Leu Asp Ser Glu Val Lys Asn Leu Leu
            770                 775                 780

TCA GAT GAT AAC TCA GAA GGC CTT ACT TTA TTG GAT TTG TTG AGC TTC      2522
Ser Asp Asp Asn Ser Glu Gly Leu Thr Leu Leu Asp Leu Leu Ser Phe
        785                 790                 795

ACC TAT CAA GTT GCC CGA GGA ATG GAG TTT TTG GCT TCA AAA AAT TGT      2570
Thr Tyr Gln Val Ala Arg Gly Met Glu Phe Leu Ala Ser Lys Asn Cys
    800                 805                 810

GTC CAC CGT GAT CTG GCT GCT CGC AAC GTT CTC CTG GCA CAA GGA AAA      2618
Val His Arg Asp Leu Ala Ala Arg Asn Val Leu Leu Ala Gln Gly Lys
815                 820                 825                 830

ATT GTG AAG ATC TGT GAC TTT GGC CTG GCC AGA GAC ATC ATG CAT GAT      2666
Ile Val Lys Ile Cys Asp Phe Gly Leu Ala Arg Asp Ile Met His Asp
                835                 840                 845

TCG AAC TAT GTG TCG AAA GGC AGT ACC TTT CTG CCC GTG AAG TGG ATG      2714
Ser Asn Tyr Val Ser Lys Gly Ser Thr Phe Leu Pro Val Lys Trp Met
            850                 855                 860

GCT CCT GAG AGC ATC TTT GAC AAC CTC TAC ACC ACA CTG AGT GAT GTC      2762
Ala Pro Glu Ser Ile Phe Asp Asn Leu Tyr Thr Thr Leu Ser Asp Val
        865                 870                 875

TGG TCT TAT GGC ATT CTG CTC TGG GAG ATC TTT TCC CTT GGT GGC ACC      2810
Trp Ser Tyr Gly Ile Leu Leu Trp Glu Ile Phe Ser Leu Gly Gly Thr
    880                 885                 890

CCT TAC CCC GGC ATG ATG GTG GAT TCT ACT TTC TAC AAT AAG ATC AAG      2858
Pro Tyr Pro Gly Met Met Val Asp Ser Thr Phe Tyr Asn Lys Ile Lys
895                 900                 905                 910

AGT GGG TAC CGG ATG GCC AAG CCT GAC CAC GCT ACC AGT GAA GTC TAC      2906
Ser Gly Tyr Arg Met Ala Lys Pro Asp His Ala Thr Ser Glu Val Tyr
                915                 920                 925

GAG ATC ATG GTG AAA TGC TGG AAC AGT GAG CCG GAG AAG AGA CCC TCC      2954
Glu Ile Met Val Lys Cys Trp Asn Ser Glu Pro Glu Lys Arg Pro Ser
            930                 935                 940

TTT TAC CAC CTG AGT GAG ATT GTG GAG AAT CTG CTG CCT GGA CAA TAT      3002
Phe Tyr His Leu Ser Glu Ile Val Glu Asn Leu Leu Pro Gly Gln Tyr
        945                 950                 955

AAA AAG AGT TAT GAA AAA ATT CAC CTG GAC TTC CTG AAG AGT GAC CAT      3050
Lys Lys Ser Tyr Glu Lys Ile His Leu Asp Phe Leu Lys Ser Asp His
    960                 965                 970

CCT GCT GTG GCA CGC ATG CGT GTG GAC TCA GAC AAT GCA TAC ATT GGT      3098
```

This is the replacement sheet

Date: - 7 OCT 1996

```
Pro Ala Val Ala Arg Met Arg Val Asp Ser Asp Asn Ala Tyr Ile Gly
975                 980                 985                 990

GTC ACC TAC AAA AAC GAG GAA GAC AAG CTG AAG GAC TGG GAG GGT GGT      3146
Val Thr Tyr Lys Asn Glu Glu Asp Lys Leu Lys Asp Trp Glu Gly Gly
                995                1000                1005

CTG GAT GAG CAG AGA CTG AGC GCT GAC AGT GGC TAC ATC ATT CCT CTG      3194
Leu Asp Glu Gln Arg Leu Ser Ala Asp Ser Gly Tyr Ile Ile Pro Leu
           1010                1015                1020

CCT GAC ATT GAC CCT GTC CCT GAG GAG GAG GAC CTG GGC AAG AGG AAC      3242
Pro Asp Ile Asp Pro Val Pro Glu Glu Glu Asp Leu Gly Lys Arg Asn
      1025                1030                1035

AGA CAC AGC TCG CAG ACC TCT GAA GAG AGT GCC ATT GAG ACG GGT TCC      3290
Arg His Ser Ser Gln Thr Ser Glu Glu Ser Ala Ile Glu Thr Gly Ser
 1040                1045                1050

AGC AGT TCC ACC TTC ATC AAG AGA GAG GAC GAG ACC ATT GAA GAC ATC      3338
Ser Ser Ser Thr Phe Ile Lys Arg Glu Asp Glu Thr Ile Glu Asp Ile
1055                1060                1065                1070

GAC ATG ATG GAC GAC ATC GGC ATA GAC TCT TCA GAC CTG GTG GAA GAC      3386
Asp Met Met Asp Asp Ile Gly Ile Asp Ser Ser Asp Leu Val Glu Asp
                1075                1080                1085

AGC TTC CTG TAACTGGCGG ATTCGAGGGG TTCCTTCCAC TTCTGGGGCC              3435
Ser Phe Leu

ACCTCTGGAT CCCGTTCAGA AAACCACTTT ATTGCAATGC GGAGGTTGAG AGGAGGACTT    3495

GGTTGATGTT TAAAGAGAAG TTCCCAGCCA AGGGCCTCGG GGAGCCTTTC TAAATATGAA    3555

TGAATGGGAT ATTTTGAAAT GAACTTTGTC AGTGTTGCCT CTTGCAATGC CTCAGTAGCA    3615

TCTCAGTGGT GTGTGAAGTT TGGAGATACA TGGATAAGGG AATAATAGGC CACAGAAGGT    3675

GAACTTTCTG CTTCAAGGAC ATTGGTGAGA GTCCAACAGA CACAATTTAT ACTGCGACAG    3735

AACTTCAGCA TTGTAATTAT GTAAATAACT CTAACCACGG CTGTGTTTAG ATTGTATTAA    3795

CTATCTTCTT TGGACTTCTG AAGAGACCAC TCAATCGATC CATGTACTTC CCTCTTGAAA    3855

CCTGATGTCA GCTGCTGTTG AACTTTTTAA AGAAGTGCAT GAAAAACCAT TTTTGACCTT    3915

AAAAGGTACT GGTACTATAG CATTTTGCTA TCTTTTTTAG TGTTAAAGAG ATAAAGAATA    3975

ATAATTAACC AACCTTGTTT AATASATTTG GGTCATTTAG AAGCCTGACA ACTCATTTTC    4035

ATATTGTAAT CTATGTTTAC AATACTACTA CTGTTATCAG TAATGCTAAA TGTGTAATAA    4095

TGTAA                                                                4100
```

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1069 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

This is the replacement sheet

Date: 7 OCT 1996

```
Met Gly Thr Ser His Pro Ala Phe Leu Val Leu Gly Cys Leu Leu Thr
 1               5                  10                  15

Gly Leu Ser Leu Ile Leu Cys Gln Leu Ser Leu Pro Ser Ile Leu Pro
            20                  25                  30

Asn Glu Asn Glu Lys Val Val Gln Leu Asn Ser Ser Phe Ser Leu Arg
        35                  40                  45

Cys Phe Gly Glu Ser Glu Val Ser Trp Gln Tyr Pro Met Ser Glu Glu
    50                  55                  60

Glu Ser Ser Asp Val Glu Ile Arg Asn Glu Glu Asn Asn Ser Gly Leu
65                  70                  75                  80

Phe Val Thr Val Leu Glu Val Ser Ser Ala Ser Ala Ala His Thr Gly
                85                  90                  95

Leu Tyr Thr Cys Tyr Tyr Asn His Thr Gln Thr Glu Glu Asn Glu Leu
            100                 105                 110

Glu Gly Arg His Ile Tyr Ile Tyr Val Pro Asp Pro Asp Val Ala Phe
        115                 120                 125

Val Pro Leu Gly Met Thr Asp Tyr Leu Val Ile Val Glu Asp Asp Asp
    130                 135                 140

Ser Ala Ile Ile Pro Cys Arg Thr Thr Asp Pro Glu Thr Pro Val Thr
145                 150                 155                 160

Leu His Asn Ser Glu Gly Val Val Pro Ala Ser Tyr Asp Ser Arg Gln
                165                 170                 175

Gly Phe Asn Gly Thr Phe Thr Val Gly Pro Tyr Ile Cys Glu Ala Thr
            180                 185                 190

Val Lys Gly Lys Lys Phe Gln Thr Ile Pro Phe Asn Val Tyr Ala Leu
        195                 200                 205

Lys Ala Thr Ser Glu Leu Asp Leu Glu Met Glu Ala Leu Lys Thr Val
    210                 215                 220

Tyr Lys Ser Gly Glu Thr Ile Val Val Thr Cys Ala Val Phe Asn Asn
225                 230                 235                 240

Glu Val Val Asp Leu Gln Trp Thr Tyr Pro Gly Glu Val Lys Gly Lys
                245                 250                 255

Gly Ile Thr Met Leu Glu Glu Ile Lys Val Pro Ser Ile Lys Leu Val
            260                 265                 270

Tyr Thr Leu Thr Val Pro Glu Ala Thr Val Lys Asp Ser Gly Asp Tyr
        275                 280                 285

Glu Cys Ala Ala Arg Gln Ala Thr Arg Glu Val Lys Glu Met Lys Lys
    290                 295                 300

Val Thr Ile Ser Val His Glu Lys Gly Phe Ile Glu Ile Lys Pro Thr
305                 310                 315                 320

Phe Ser Gln Leu Glu Ala Val Asn Leu His Glu Val Lys His Phe Val
                325                 330                 335

Val Glu Val Arg Ala Tyr Pro Pro Pro Arg Ile Ser Trp Leu Lys Asn
            340                 345                 350
```

This is the replacement sheet

Date: - 7 OCT 1996

```
Asn Leu Thr Leu Ile Glu Asn Leu Thr Glu Ile Thr Thr Asp Val Glu
          355                 360                 365

Lys Ile Gln Glu Ile Arg Tyr Arg Ser Lys Leu Lys Leu Ile Arg Ala
    370                 375                 380

Lys Glu Glu Asp Ser Gly His Tyr Thr Ile Val Ala Gln Asn Glu Asp
385                 390                 395                 400

Ala Val Lys Ser Tyr Thr Phe Glu Leu Leu Thr Gln Val Pro Ser Ser
                405                 410                 415

Ile Leu Asp Leu Val Asp Asp His His Gly Ser Thr Gly Gly Gln Thr
            420                 425                 430

Val Arg Cys Thr Ala Glu Gly Thr Pro Leu Pro Asp Ile Glu Trp Met
        435                 440                 445

Ile Cys Lys Asp Ile Lys Lys Cys Asn Asn Glu Thr Ser Trp Thr Ile
    450                 455                 460

Leu Ala Asn Asn Val Ser Asn Ile Ile Thr Glu Ile His Ser Arg Asp
465                 470                 475                 480

Arg Ser Thr Val Glu Gly Arg Val Thr Phe Ala Lys Val Glu Glu Thr
                485                 490                 495

Ile Ala Val Arg Cys Leu Ala Lys Asn Leu Leu Gly Ala Glu Asn Arg
            500                 505                 510

Glu Leu Lys Leu Val Ala Pro Thr Leu Arg Ser Glu Leu Thr Val Ala
        515                 520                 525

Ala Ala Val Leu Val Leu Leu Val Ile Val Ile Ile Ser Leu Ile Val
    530                 535                 540

Leu Val Val Ile Trp Lys Gln Lys Pro Arg Tyr Glu Ile Arg Trp Arg
545                 550                 555                 560

Val Ile Glu Ser Ile Ser Pro Asp Gly His Glu Tyr Ile Tyr Val Asp
                565                 570                 575

Pro Met Gln Leu Pro Tyr Asp Ser Arg Trp Glu Phe Pro Arg Asp Gly
            580                 585                 590

Leu Val Leu Gly Arg Val Leu Gly Ser Gly Ala Phe Gly Lys Val Val
        595                 600                 605

Glu Gly Thr Ala Tyr Gly Leu Ser Arg Ser Gln Pro Val Met Lys Val
    610                 615                 620

Ala Val Lys Met Leu Lys Pro Thr Ala Arg Ser Ser Glu Lys Gln Ala
625                 630                 635                 640

Leu Met Ser Glu Leu Lys Ile Met Thr His Leu Gly Pro His Leu Asn
                645                 650                 655

Ile Val Asn Leu Leu Gly Ala Cys Thr Lys Ser Gly Pro Ile Tyr Ile
            660                 665                 670

Ile Thr Glu Tyr Cys Phe Tyr Gly Asp Leu Val Asn Tyr Leu His Lys
        675                 680                 685

Asn Arg Asp Ser Phe Leu Ser His His Pro Glu Lys Pro Lys Lys Glu
    690                 695                 700
```

This is the replacement sheet

Date: - 7 OCT 1996

Leu Asp Ile Phe Gly Leu Asn Pro Ala Asp Glu Ser Thr Arg Ser Tyr
705 710 715 720

Val Ile Leu Ser Phe Glu Asn Asn Gly Asp Tyr Met Asp Met Lys Gln
725 730 735

Ala Asp Thr Thr Gln Tyr Val Pro Met Leu Glu Arg Lys Glu Val Ser
740 745 750

Lys Tyr Ser Asp Ile Gln Arg Ser Leu Tyr Asp Arg Pro Ala Ser Tyr
755 760 765

Lys Lys Lys Ser Met Leu Asp Ser Glu Val Lys Asn Leu Leu Ser Asp
770 775 780

Asp Asn Ser Glu Gly Leu Thr Leu Leu Asp Leu Leu Ser Phe Thr Tyr
785 790 795 800

Gln Val Ala Arg Gly Met Glu Phe Leu Ala Ser Lys Asn Cys Val His
805 810 815

Arg Asp Leu Ala Ala Arg Asn Val Leu Leu Ala Gln Gly Lys Ile Val
820 825 830

Lys Ile Cys Asp Phe Gly Leu Ala Arg Asp Ile Met His Asp Ser Asn
835 840 845

Tyr Val Ser Lys Gly Ser Thr Phe Leu Pro Val Lys Trp Met Ala Pro
850 855 860

Glu Ser Ile Phe Asp Asn Leu Tyr Thr Thr Leu Ser Asp Val Trp Ser
865 870 875 880

Tyr Gly Ile Leu Leu Trp Glu Ile Phe Ser Leu Gly Gly Thr Pro Tyr
885 890 895

Pro Gly Met Met Val Asp Ser Thr Phe Tyr Asn Lys Ile Lys Ser Gly
900 905 910

Tyr Arg Met Ala Lys Pro Asp His Ala Thr Ser Glu Val Tyr Glu Ile
915 920 925

Met Val Lys Cys Trp Asn Ser Glu Pro Glu Lys Arg Pro Ser Phe Tyr
930 935 940

His Leu Ser Glu Ile Val Glu Asn Leu Leu Pro Gly Gln Tyr Lys Lys
945 950 955 960

Ser Tyr Glu Lys Ile His Leu Asp Phe Leu Lys Ser Asp His Pro Ala
965 970 975

Val Ala Arg Met Arg Val Asp Ser Asp Asn Ala Tyr Ile Gly Val Thr
980 985 990

Tyr Lys Asn Glu Glu Asp Lys Leu Lys Asp Trp Glu Gly Gly Leu Asp
995 1000 1005

Glu Gln Arg Leu Ser Ala Asp Ser Gly Tyr Ile Ile Pro Leu Pro Asp
1010 1015 1020

Ile Asp Pro Val Pro Glu Glu Glu Asp Leu Gly Lys Arg Asn Arg His
1025 1030 1035 1040

Ser Ser Gln Thr Ser Glu Glu Ser Ala Ile Glu Thr Gly Ser Ser Ser
1045 1050 1055

This is the replacement sheet

Date: - 7 OCT 1996

Ser Thr Phe Ile Lys Arg Glu Asp Glu Thr Ile Glu Asp Ile Asp Met
1060 1065 1070

Met Asp Asp Ile Gly Ile Asp Ser Ser Asp Leu Val Glu Asp Ser Phe
1075 1080 1085

Leu

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6375 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA to mRNA

(iii) HYPOTHETICAL: N

(iv) ANTI-SENSE: N

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Homo Sapiens

(vii) IMMEDIATE SOURCE:
(A) LIBRARY: lambda gt10

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 129..3395
(D) OTHER INFORMATION: /note= "nucleotide number 1 of this sequence is identical to the nucleotide number 1 of the previous 4100 long sequence."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

TTGGAGCTAC AGGGAGAGAA ACAGAGGAGG AGACTGCAAG AGATCATTGG AGGCCGTGGG 60

CACGCTCTTT ACTCCATGTG TGGGACATTC ATTGCGGAAT AACATCGGAG GAGAAGTTTC 120

CCAGAGCTAT GGGGACTTCC CATCCGGCGT TCCTGGTCTT AGGCTGTCTT CTCACAGGGC 180

TGAGCCTAAT CCTCTGCCAG CTTTCATTAC CCTCTATCCT TCCAAATGAA AATGAAAAGG 240

TTGTGCAGCT GAATTCATCC TTTTCTCTGA GATGCTTTGG GGAGAGTGAA GTGAGCTGGC 300

AGTACCCCAT GTCTGAAGAA GAGAGCTCCG ATGTGGAAAT CAGAAATGAA GAAAACAACA 360

GCGGCCTTTT TGTGACGGTC TTGGAAGTGA GCAGTGCCTC GGCGGCCCAC ACAGGGTTGT 420

ACACTTGCTA TTACAACCAC ACTCAGACAG AAGAGAATGA GCTTGAAGGC AGGCACATTT 480

ACATCTATGT GCCAGACCCA GATGTAGCCT TTGTACCTCT AGGAATGACG GATTATTTAG 540

TCATCGTGGA GGATGATGAT TCTGCCATTA TACCTTGTCG CACAACTGAT CCCGAGACTC 600

CTGTAACCTT ACACAACAGT GAGGGGGTGG TACCTGCCTC CTACGACAGC AGACAGGGCT 660

TTAATGGGAC CTTCACTGTA GGGCCCTATA TCTGTGAGGC CACCGTCAAA GGAAAGAAGT 720

TCCAGACCAT CCCATTTAAT GTTTATGCTT TAAAAGCAAC ATCAGAGCTG GATCTAGAAA 780

This is the replacement sheet

Date: - 7 OCT 1996

```
TGGAAGCTGT TAAAACCGTG TATAAGTCAG GGGAAACGAT TGTGGTCACC TGTGCTGTTT    840
TTAACAATGA GGTGGTTGAC CTTCAATGGA CTTACCCTGG AGAAGTGAAA GGCAAAGGCA    900
TCACAATGCT GGAAGAAATC AAAGTCCCAT CCATCAAATT GGTGTACACT TTGACGGTCC    960
CCGAGGCCAC GGTGAAAGAC AGTGGAGATT ACGAATGTGC TGCCCGCCAG GCTACCAGGG   1020
AGGTCAAAGA AATGAAGAAA GTCACTATTT CTGTCCATGA GAAAGGTTTC ATTGAAATCA   1080
AACCCACCTT CAGCCAGTTG GAAGCTGTCA ACCTGCATGA AGTCAAACAT TTTGTTGTAG   1140
AGGTGCGGGC CTACCCACCT CCCAGGATAT CCTGGCTGAA AAACAATCTG ACTCTGATTG   1200
AAAATCTCAC TGAGATCACC ACTGATGTGG AAAAGATTCA GGAAATAAGG TATCGAAGCA   1260
AATTAAAGCT GATCCGTGCT AAGGAAGAAG ACAGTGGCCA TTATACTATT GTAGCTCAAA   1320
ATGAAGATGC TGTGAAGAGC TATACTTTTG AACTGTTAAC TCAAGTTCCT TCATCCATTC   1380
TGGACTTGGT CGATGATCAC CATGGCTCAA CTGGGGGACA GACGGTGAGG TGCACAGCTG   1440
AAGGCACGCC GCTTCCTGAT ATTGAGTGGA TGATATGCAA AGATATTAAG AAATGTAATA   1500
ATGAAACTTC CTGGACTATT TTGGCCAACA ATGTCTCAAA CATCATCACG GAGATCCACT   1560
CCCGAGACAG GAGTACCGTG GAGGGCCGTG TGACTTTCGC CAAAGTGGAG GAGACCATCG   1620
CCGTGCGATG CCTGGCTAAG AATCTCCTTG GAGCTGAGAA CCGAGAGCTG AAGCTGGTGG   1680
CTCCCACCCT GCGTTCTGAA CTCACGGTGG CTGCTGCAGT CCTGGTGCTG TTGGTGATTG   1740
TGATCATCTC ACTTATTGTC CTGGTTGTCA TTTGGAAACA GAAACCGAGG TATGAAATTC   1800
GCTGGAGGGT CATTGAATCA ATCAGCCCAG ATGGACATGA ATATATTTAT GTGGACCCGA   1860
TGCAGCTGCC TTATGACTCA AGATGGGAGT TTCCAAGAGA TGGACTAGTG CTTGGTCGGG   1920
TCTTGGGGTC TGGAGCGTTT GGGAAGGTGG TTGAAGGAAC AGCCTATGGA TTAAGCCGGT   1980
CCCAACCTGT CATGAAAGTT GCAGTGAAGA TGCTAAAACC CACGGCCAGA TCCAGTGAAA   2040
AACAAGCTCT CATGTCTGAA CTGAAGATAA TGACTCACCT GGGGCCACAT TTGAACATTG   2100
TAAACTTGCT GGGAGCCTGC ACCAAGTCAG GCCCCATTTA CATCATCACA GAGTATTGCT   2160
TCTATGGAGA TTTGGTCAAC TATTTGCATA AGAATAGGGA TAGCTTCCTG AGCCACCACC   2220
CAGAGAAGCC AAAGAAAGAG CTGGATATCT TTGGATTGAA CCCTGCTGAT GAAAGCACAC   2280
GGAGCTATGT TATTTTATCT TTTGAAAACA ATGGTGACTA CATGGACATG AAGCAGGCTG   2340
ATACTACACA GTATGTCCCC ATGCTAGAAA GGAAAGAGGT TTCTAAATAT TCCGACATCC   2400
AGAGATCACT CTATGATCGT CCAGCCTCAT ATAAGAAGAA ATCTATGTTA GACTCAGAAG   2460
TCAAAAACCT CCTTTCAGAT GATAACTCAG AAGGCCTTAC TTTATTGGAT TTGTTGAGCT   2520
TCACCTATCA AGTTGCCCGA GGAATGGAGT TTTTGGCTTC AAAAAATTGT GTCCACCGTG   2580
ATCTGGCTGC TCGCAACGTT CTCCTGGCAC AAGGAAAAAT TGTGAAGATC TGTGACTTTG   2640
GCCTGGCCAG AGACATCATG CATGATTCGA ACTATGTGTC GAAAGGCAGT ACCTTTCTGC   2700
CCGTGAAGTG GATGGCTCCT GAGAGCATCT TTGACAACCT CTACACCACA CTGAGTGATG   2760
```

This is the replacement sheet

Date: - 7 OCT 1996

```
TCTGGTCTTA TGGCATTCTG CTCTGGGAGA TCTTTTCCCT TGGTGGCACC CCTTACCCCG   2820
GCATGATGGT GGATTCTACT TTCTACAATA AGATCAAGAG TGGGTACCGG ATGGCCAAGC   2880
CTGACCACGC TACCAGTGAA GTCTACGAGA TCATGGTCAA ATGCTGGAAC AGTGAGCCGG   2940
AGAAGAGACC CTCCTTTTAC CACCTGAGTG AGATTGTGGA GAATCTGCTG CCTGGACAAT   3000
ATAAAAAGAG TTATGAAAAA ATTCACCTGG ACTTCCTGAA GAGTGACCAT CCTGCTGTGG   3060
CACGCATGCG TGTGGACTCA GACAATGCAT ACATTGGTGT CACCTACAAA AACGAGGAAG   3120
ACAAGCTGAA GGACTGGGAG GGTGGTCTGG ATGAGCAGAG ACTGAGCGCT GACAGTGGCT   3180
ACATCATTCC TCTGCCTGAC ATTGACCCTG TCCCTGAGGA GGAGGACCTG GGCAAGAGGA   3240
ACAGACACAG CTCGCAGACC TCTGAAGAGA GTGCCATTGA GACGGGTTCC AGCAGTTCCA   3300
CCTTCATCAA GAGAGAGGAC GAGACCATTG AAGACATCGA CATGATGGAC GACATCGGCA   3360
TAGACTCTTC AGACCTGGTG GAAGACAGCT TCCTGTAACT GGCGGATTCG AGGGGTTCCT   3420
TCCACTTCTG GGGCCACCTC TGGATCCCGT TCAGAAAACC ACTTTATTGC AATGCGGAGG   3480
TTGAGAGGAG GACTTGGTTG ATGTTTAAAG AGAAGTTCCC AGCCAAGGGC CTCGGGGAGC   3540
CTTTCTAAAT ATGAATGAAT GGGATATTTT GAAATGAACT TTGTCAGTGT TGCCTCTTGC   3600
AATGCCTCAG TAGCATCTCA GTGGTGTGTG AAGTTTGGAG ATAGATGGAT AAGGGAATAA   3660
TAGGCCACAG AAGGTGAACT TTCTGCTTCA AGGACATTGG TGAGAGTCCA ACAGACACAA   3720
TTTATACTGC GACAGAACTT CAGCATTGTA ATTATGTAAA TAACTCTAAC CACGGCTGTG   3780
TTTAGATTGT ATTAACTATC TTCTTTGGAC TTCTGAAGAG ACCACTCAAT CCATCCATGT   3840
ACTTCCCTCT TGAAACCTGA TGTCAGCTGC TGTTGAACTT TTTAAAGAAG TGCATGAAAA   3900
ACCATTTTTG ACCTTAAAAG GTACTGGTAC TATAGCATTT TGCTATCTTT TTTAGTGTTA   3960
AAGAGATAAA GAATAATAAT TAACCAACCT TGTTTAATAG ATTTGGGTCA TTTAGAAGCC   4020
TGACAACTCA TTTTCATATT GTAATCTATG TTTATAATAC TACTACTGTT ATCAGTAATG   4080
CTAAATGTGT AATAATGTAA CATGATTTCC CTCCACACAA AGCACAATTT AAAAACAATC   4140
CTTACTAAGT AGGTGATGAG TTTGACAGTT TTTGACATTT ATATTAAATA ACATGTTTCT   4200
CTATAAAGTA TGGTAATAGC TTTAGTGAAT TAAATTTAGT TGAGCATAGA GAACAAAGTA   4260
AAAGTAGTGT TGTCCAGGAA GTCAGAATTT TTAACTGTAC TGAATAGGTT CCCCAATCCA   4320
TCGTATTAAA AAACAATTAA CTGCCCTCTG AAATAATGGG ATTAGAAACA AACAAAACTC   4380
TTAAGTCCTA AAAGTTCTCA ATGTAGAGGC ATAAACCTGT GCTGAACATA ACTTCTCATG   4440
TATATTACCC AATGGAAAAT ATAATGATCA GCGCANAAAG ACTGGATTTG CAGAAGTTNT   4500
TTTTTTTTTT TCTTCTTGCC TGATGAAAGC TTTGGCGACC CCAATATATG TATTTTTTGA   4560
ATCTATGAAC CTGAAAAGGG TCACAAAGGA TGCCCAGACA TCAGCCTCCT TCTTTCACCC   4620
CTTACCCCAA AGAGAAAGAG TTTGAAACTC GAGACCATAA AGATATTCTT TAGTGGAGGC   4680
TGGAAGTGCA TTAGCCTGAT CCTCAGTTCT CAAATGTGTG TGGCAGCCAG GTAGACTAGT   4740
```

This is the replacement sheet

Date: - 7 OCT 1996

```
ACCTGGGTTT CCATCCTTGA GATTCTGAAG TATGAAGTCT GAGGGAAACC AGAGTCTGTA   4800
TTTTTCTAAA CTCCCTGGCT GTTCTGATCG GCCAGGTTTC GGAAACACTG ACTTAGGTTT   4860
CAGGAAGTTG CCATGGGAAA CAAATAATTT GAACTTTGGA ACAGGGTTCT TAAGTTGGTC   4920
CGTCCTTCGG ATGATAAATT TAGGAACCGA AGTCCAATCA CTGTAAATTA CGGTAGATCG   4980
ATCGTTAACG CTGGAATTAA ATTGAAAGGT CAGAATCGAC TCCGACTCTT TCGATTTCAA   5040
ACCAAAACTG TCCAAAAGGT TTTCATTTCT ACGATGAAGG GTGACATACC CCCTCTAACT   5100
TGAAAGGGGC AGAGGGCAGA AGAGCGGAGG GTGAGGTATG GGGCGGTTCC TTTCCGTACA   5160
TGTTTTTAAT ACGTTAAGTC ACAAGGTTCA GAGACACATT GGTCGAGTCA CAAAACCACC   5220
TTTTTTGTAA AATTCAAAAT GACTATTAAA CTCCAATCTA CCCTCCTACT TAACAGTGTA   5280
GATAGGTGTG ACAGTTTGTC CAACCACACC CAAGTAACCG TAAGAAACGT TATGACGAAT   5340
TAACGACTAT GGTATACTTA CTTTGTACCC GACACTAATG ACGTTAGTGA CACGATAGCC   5400
GTCTACTACG AAACCTTCTA CGTCTTCGTT ATTATTTCAT GAACTGATGG ATGACCACAT   5460
TAGAGTTACG TTCGGGGTTC AAAGAATAGG TTGAAAAAGT ATCATTCACG CTTCTGACTC   5520
GGTCTAACCG GTTAATTTTT CTTTTGGACT GATCCAAGAC ATCTCGGTTA ATCTGAACTT   5580
TATGCAAACA CAAAGATCTT AGTGTCGAGT TCGTAAGACA AATAGCGAGT GAGAGGGAAC   5640
ATGTCGGAAT AAAACAACCA CGAAACGTAA AACTATAACG ACACTCGGAA CGTACTGTAG   5700
TACTCCGGCC TACTTTGAAG AGTCAGGTCG TCAAAGGTCA GGATTGTTTA CGAGGGTGGA   5760
CTTAAACATA TACTGACGTA AACACCCACA CACACACAAA AGTCGTTTAA GGTCTAAACA   5820
AAGGAAAACC GGAGGACGTT TCAGAGGTCT TCTTTTAAAC GGTTAGAAAG GATGAAAGAT   5880
AAAAATACTA CTGTTAGTTT CGGCCGGACT CTTTGTGATA AACACTGAAA AATTTGCTAA   5940
TCACTACAGG AATTTTACAC CAGACGGTTA GACATGTTTT ACCAGGATAA AAACACTTCT   6000
CCCTGTATTC TATTTTACTA CAATATGTAG TTATACATAT ATACATAAAG ATATATCTGA   6060
ACCTCTTATG ACGGTTTTGT AAATACTGTT CGACATAGTG ACGGAAGCAA ATATAAAAAA   6120
ATTGACACTA TTAGGGGTGT CCGTGTAATT GACAACGTGA AAACTTACAG GTTTTAAATA   6180
TAAAATCTTT ATTATTTTTC TTTCTATGAA TGTACAAGGG TTTTGTTACC ACACCACTTA   6240
CACACTCTTT TTGATTGAAC TATCCCAGAT GGTTATGTTT TACATAATGC TTACGGGGAC   6300
AAGTACAAAA ACAAAATTTT GCACATTTAC TTCTAGAAAT ATAAAGTTAT TTACTATATA   6360
TTAAATTTCC TTAAG                                                    6375
```

This is the replacement sheet

Date: - 7 OCT 1996

* C L M S *

WHAT IS CLAIMED IS:

1. A purified and isolated recombinant nucleic acid of less than about 50 kbp comprising at least about 24 contiguous nucleotides which encode a human platelet-derived growth factor receptor (hPDGF-R) polypeptide segment.

2. A nucleic acid of Claim 1, wherein said segment is a soluble polypeptide.

3. A nucleic acid of Claim 1, wherein said segment consists essentially of a full length extracellular region of a B type or an A type hPDGF receptor, and further has a sequence of a polypeptide in Table 2 or Table 3.

4. A nucleic acid of Claim 1, wherein said segment comprises a phosphorylation site.

5. A nucleic acid of Claim 1, wherein the segment is less than about 300 amino acids.

6. A nucleic acid of Claim 1, wherein said segment is capable of binding to PDGF.

7. A nucleic acid of Claim 1, wherein said segment is a substrate for phosphorylation.

8. A nucleic acid of Claim 1, wherein said segment is capable of binding to a PI3 kinase.

9. A cell transformed with a nucleic acid of Claim X1, and wherein said cell is a mammalian cell.

10. A cell of Claim 9, further comprising a glycosylation enzyme originating from a non-fungal species.

This Sheet Has Been Replaced

This is the replacement sheet

Date: - 7 OCT 1996

* C L M S *

11. A nucleic acid of Claim 1, wherein said nucleotides encoding said segment are operably linked to a promoter.

12. A nucleic acid of Claim 1, further encoding a heterologous polypeptide which is fused to said hPDGF-R segment.

13. A method for evaluating the ability of a compound to function as a hPDGF-R agonist or antagonist comprising the step of comparing the amount of a PDGF-induced response in a cell of Claim 9 with the response from a control cell, and wherein said PDGF-induced response is compared by measuring synthesis of DNA in a cell after contacting said cell with PDGF.

14. A substantially pure hPDGF-R polypeptide fragment of at least about twenty amino acids having platelet-derived growth factor (PDGF) binding activity or tyrosine kinase activity.

15. A substantially pure polypeptide fragment of Claim 14, wherein said polypeptide fragment is soluble.

16. A hPDGF-R fragment having hPDGF-R binding activity consisting essentially of amino acids beginning at about 1 and ending at about 499 of a type B hPDGF-R, and is further derived from Table 2.

17. A hPDGF-R fragment having hPDGF-R binding activity consisting essentially of amino acids beginning about 1 and ending at about 501 of a type A hPDGF-R, and is further derived from Table 3.

18. A composition comprising an unglycosylated hPDGF-R fragment.

This Sheet Has Been Replaced

This is the replacement sheet

Date: - 7 OCT 1996

* C L M S *

19. A composition of Claim 18, wherein said fragment is substantially pure.

20. A composition comprising a hPDGF-R fragment, which exhibits a glycosylation pattern which is non-fungal and non-human.

21. A composition of claim 20, wherein said fragment is essentially the extracellular region of a type B or a type A hPDGF-R.

22. A composition of Claim 20 having a sequence from Table 2, or from Table 3.

23. A composition comprising a combination of:

a) a recombinant nucleic acid encoding a human platelet-derived growth factor receptor polypeptide (hPDGF-R) fragment; and

b) a non-fungal glycosylation enzyme capable of glycosylating said fragment when expressed.

24. A method for introducing a hPDGF-R activity to a cell, said method comprising the step of introducing a hPDGF-R protein fragment of at least about five hundred daltons to said cell.

25. A method for assaying the presence of a ligand for a PDGF receptor in a sample, comprising the steps of:

combining said sample with a hPDGF receptor ligand binding site; and

detecting binding between said ligand and said hPDGF receptor ligand binding site.

26. An isolated polypeptide of less than about 200 amino acids comprising a receptor kinase insert region.

This Sheet Has Been Replaced

This is the replacement sheet

Date: - 7 OCT 1996

* C L M S *

27. An isolated polypeptide of claim 26, wherein said polypeptide has a phosphorylated amino acid residue.

28. An isolated polypeptide of claim 26, wherein said polypeptide comprises a sequence substantially homologous to a kinase insert segment of a PDGF receptor, and further has a sequence from Table 2 or Table 3.

29. An isolated polypeptide of Claim 26, with a pharmaceutically acceptable carrier.

30. A method for modulating the biological activity of a first protein which binds to a phosphorylated region of a second protein, said method comprising a step of:

adding to said first protein a peptide analogue of said phosphorylated region, wherein said analogue is capable of inhibiting the binding of said first protein to said second protein.

31. A method of selecting a molecule capable of inhibiting binding of a protein which binds to a target phosphorylated polypeptide, comprising the steps of:

contacting said protein with said target phosphorylated polypeptide in the presence of said molecule in a first analysis;

contacting said protein with said target phosphorylated polypeptide in the absence of said molecule in a second analysis; and

comparing said analyses to determine the effect of said molecule on said binding.

32. A method of Claim 31, wherein said contacting steps are performed in succession.

33. A method for modulating a PI3 kinase activity comprising the step of:

This Sheet Has Been Replaced

This is the replacement sheet

Date: - 7 OCT 1996

* C L M S *

adding a phosphorylated PDGF receptor kinase insert region polypeptide to said PI3 kinase, thereby allowing binding between said polypeptide and said PI3 kinase.

34. A method of purifying, from a sample, a protein capable of binding to a PDGF receptor kinase insert segment, comprising the step of:

contacting said sample with an analogue of a phosphorylated polypeptide substantially homologous to a PDGF receptor kinase insert region polypeptide, thereby allowing said protein to bind specifically to said phosphorylated polypeptide.

35. A method of isolating a nucleic acid encoding a protein capable of binding to a PDGF receptor, comprising the steps of:

combining a labeled and phosphorylated PDGF receptor kinase insert region polypeptide with cells expressing various proteins, thereby labeling those cells which express said nucleic acid to produce a protein which binds said phosphorylated polypeptide, and

isolating those cells which have been labeled.

36. A method of Claim 35, wherein said protein capable of binding a PDGF receptor is PI3 kinase or c-fms.

This Sheet Has Been Replaced



This is the replacement sheet

Date: - 7 OCT 1996

ABSTRACT

HUMAN PLATELET-DERIVED GROWTH FACTOR RECEPTORS

DNA sequences encoding human platelet-derived growth factor receptors (hPDGF-R), and expression constructs comprising sequences which encode a receptor that can be secreted or incorporated into the membrane of a mammalian cell, Peptide fragments with functions equivalent to the wild-type receptor, conferring a PDGF-sensitive mitogenic response on cells lacking that receptor are provided. The constructs can be used for enhancing PDGF response of cells, determining the regions involved transducing the signal in response to PDGF binding, providing mutated analogs and evaluating drugs for their physiologic activity. Soluble fragments comprising PDGF receptor sequences are also provided, including important intracellular kinase insert sequences which interact with intracellular proteins.

This is the replacement sheet

Date: 7 OCT 1996

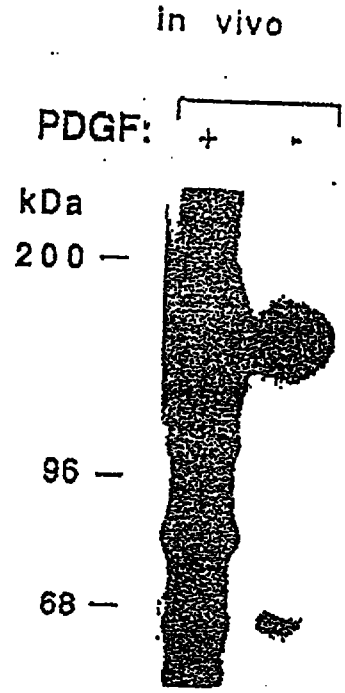


FIG. 1A.

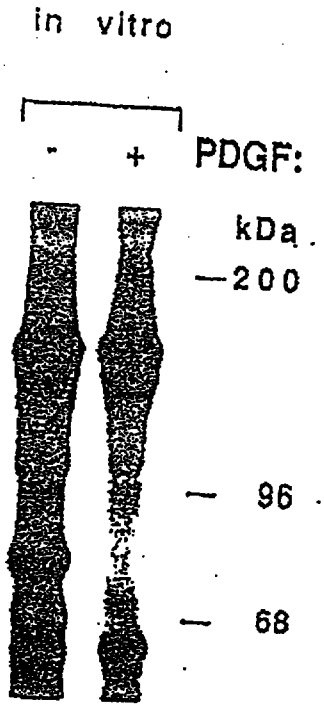


FIG. 1B.

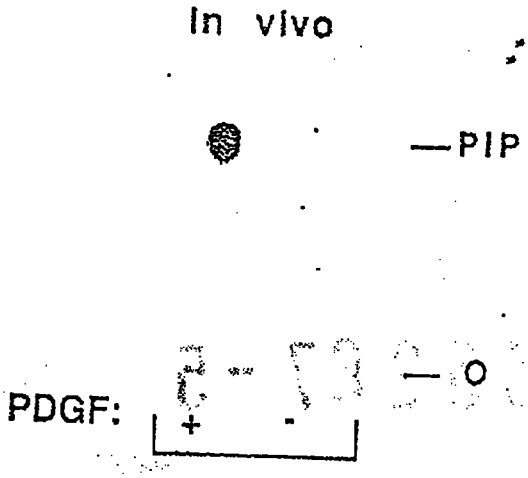


FIG. 1C.

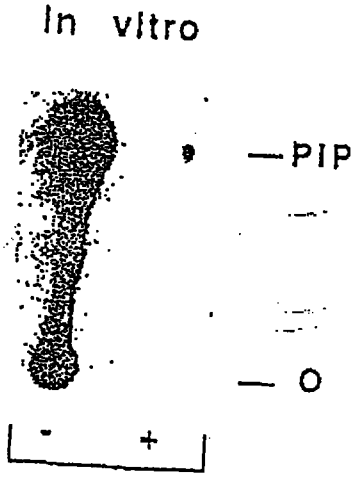
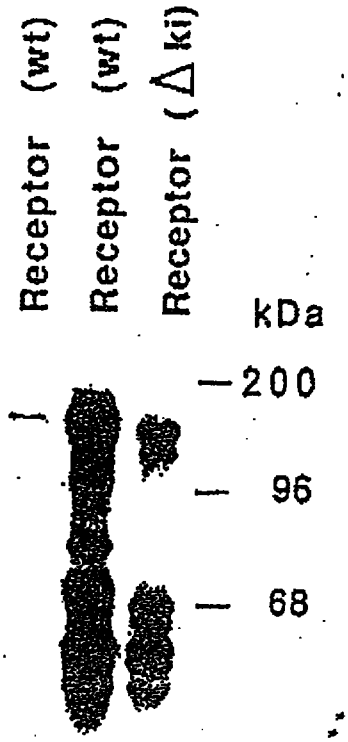


FIG. 1D.

This is the replacement sheet

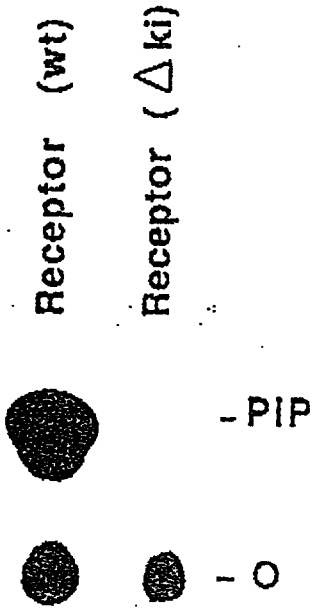
Date: - 7 OCT 1996

FIG. 2A.



Unstimulated - + +
3T3 lysate

FIG. 2B.



This is the replacement sheet

Date: 7 OCT 1996

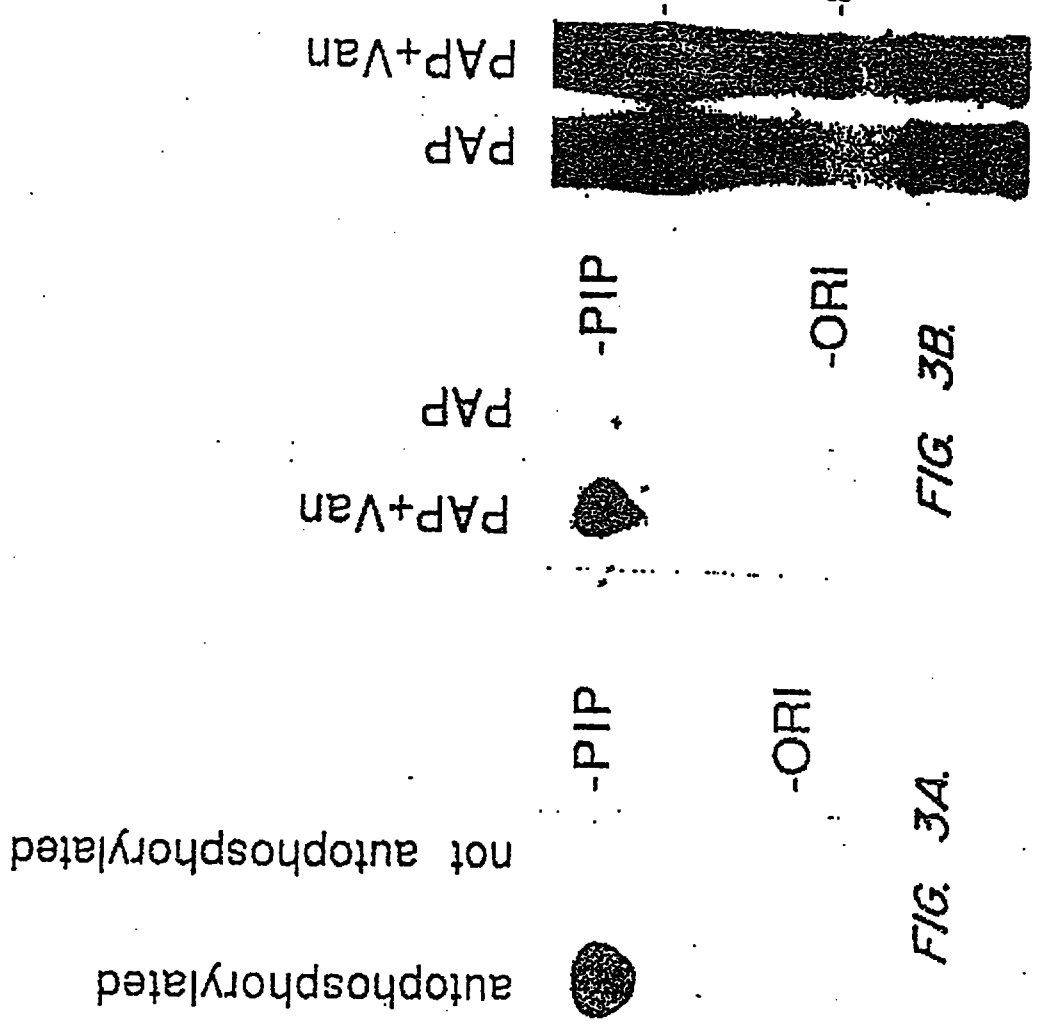


FIG. 3C.

FIG. 3B.

FIG. 3A.

This is the replacement sheet

Date: - 7 OCT 1996

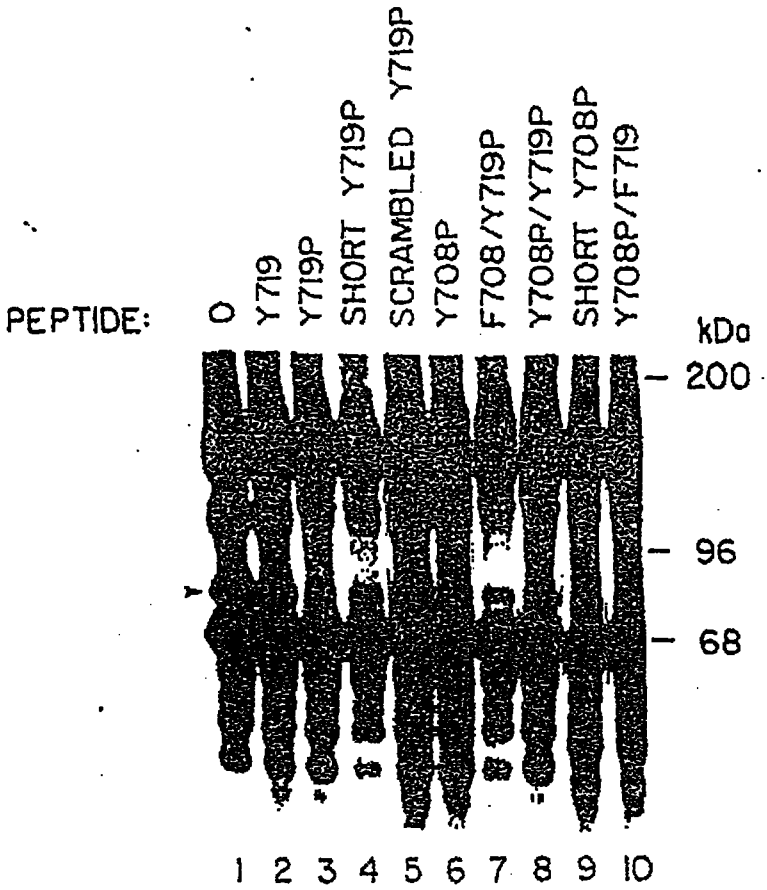
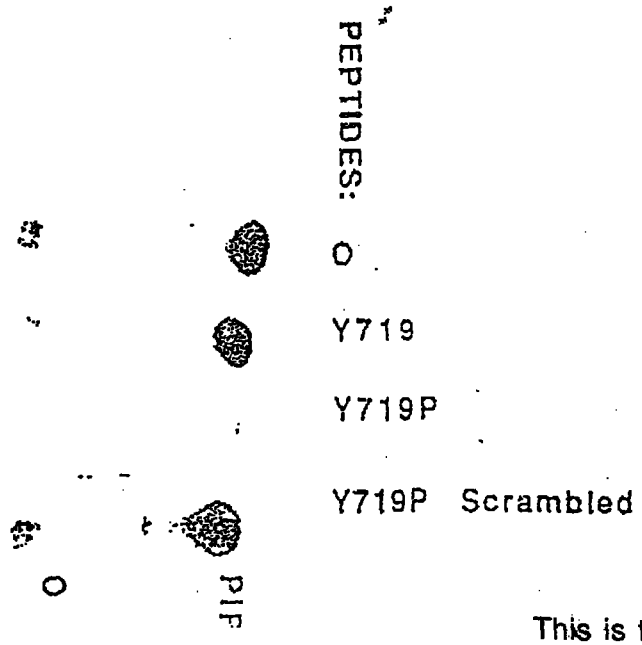


FIG. 4A.



This is the replacement sheet

Date: - 7 OCT 1996

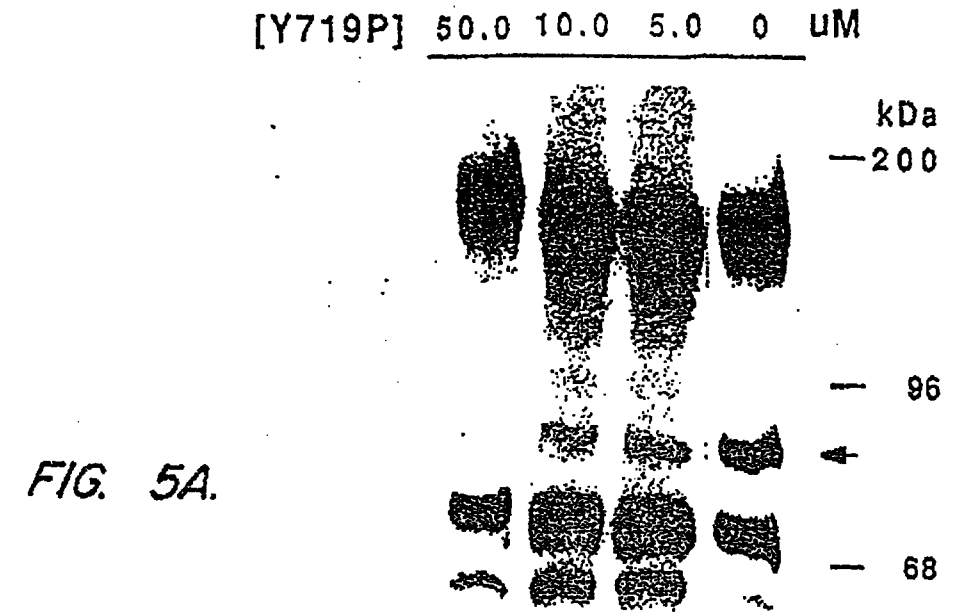


FIG. 5A.

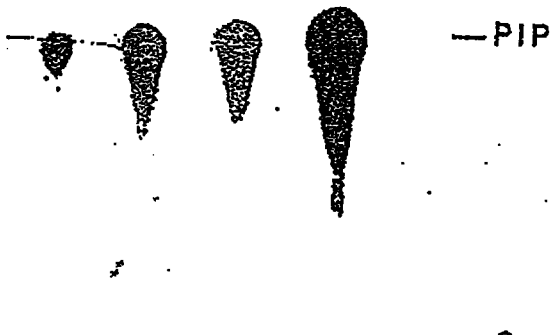


FIG. 5B.

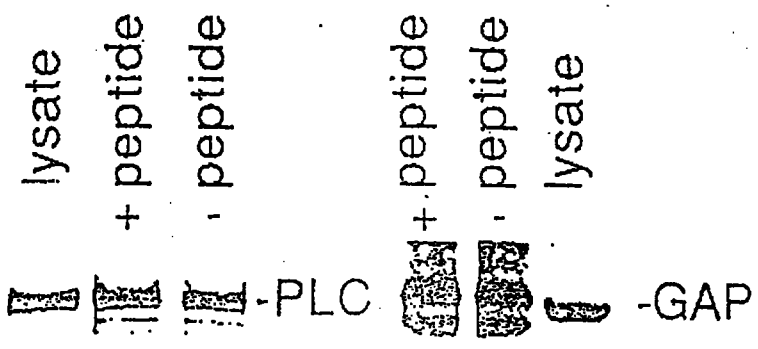


FIG. 6.

This is the replacement sheet

Date: - 7 OCT 1996

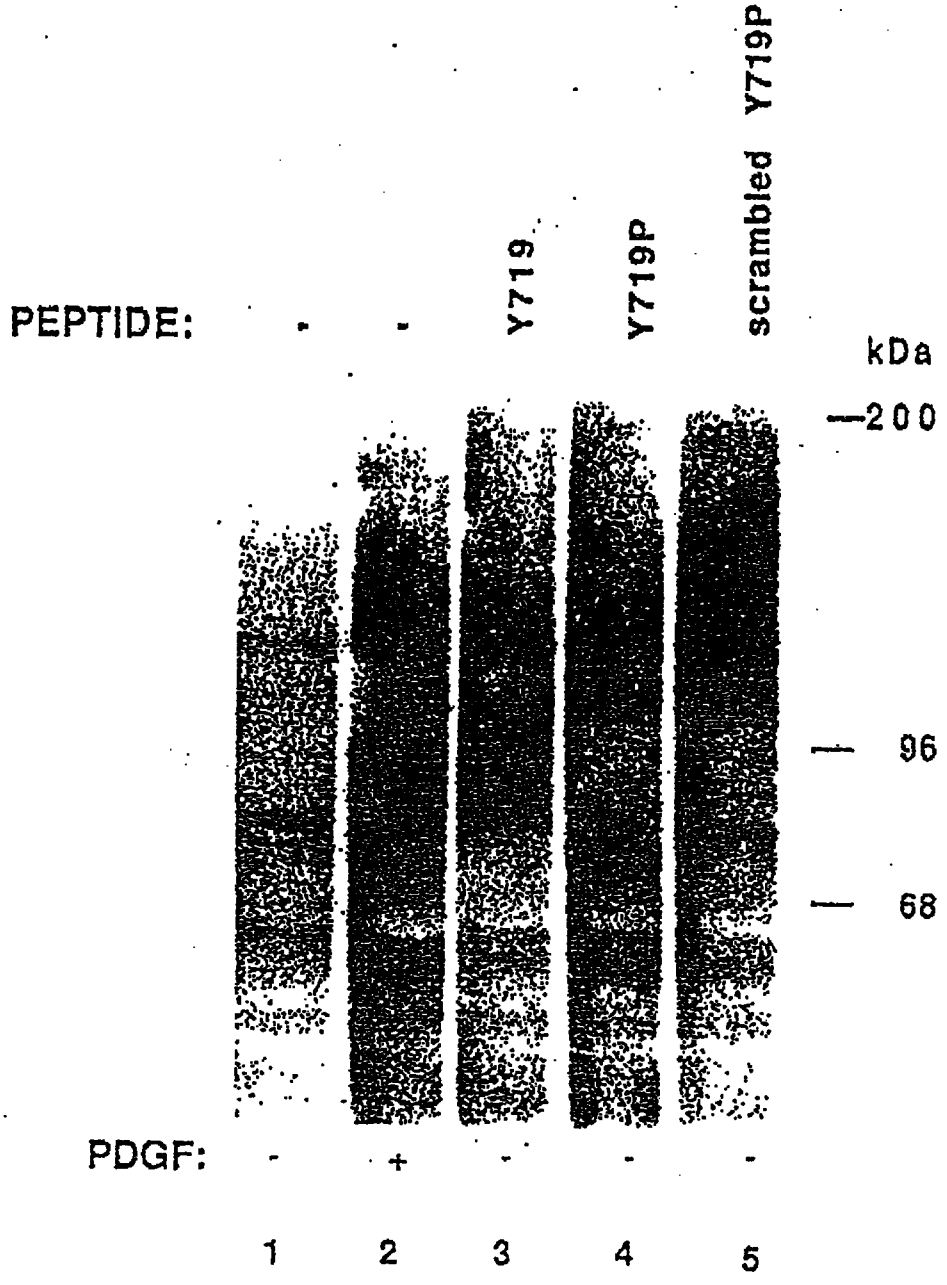


FIG. 7.

This is the replacement sheet

Date: - 7 OCT 1996

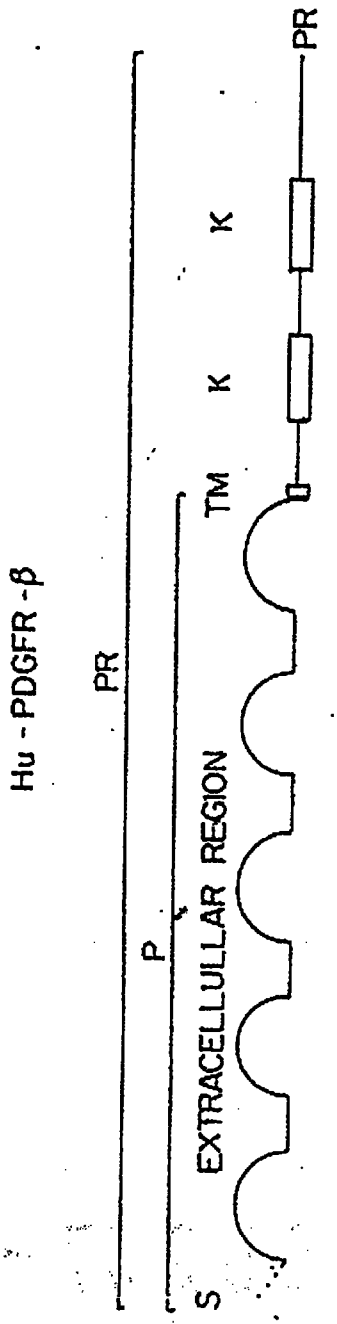


FIG. 8.

This is the replacement sheet

Date: - 7 OCT 1996

REPLACEMENT SHEETS 90
Description : page(s) .... to page(s) ....
✓ Claim(s) : page(s) 108 to page(s) 111
Drawing(s) : page(s) .... to page(s) ....
Abstract : page(s) .... to page(s) ....
DATE OF RECEIPT 25/2/99

CLAIMS

1. A purified and isolated recombinant nucleic acid of less than about 50 kbp comprising at least about 24 contiguous nucleotides which encode a human platelet-derived growth factor receptor (hPDGF-R) polypeptide segment consisting essentially of extracellular region of hPDGF-R.

2. A nucleic acid as claimed in claim 1, characterised in that said segment is a soluble polypeptide.

3. A nucleic acid as claimed in claim 1 or 2, characterised in that said segment consists essentially of a full length extracellular region of a B-type or an A-type hPDGF receptor, and further has a sequence of a polypeptide in Table 2 or Table 3.

4. A nucleic acid as claimed in claim 1, 2 or 3, characterised in that the segment is less than about 300 amino acids.

5. A nucleic acid as claimed in any preceding claim, characterised in that said segment is capable of binding to PDGF.

6. A nucleic acid as claimed in any preceding claim, characterised in that said nucleotides encoding said segment are operably linked to a promoter.

7. A nucleic acid as claimed in any preceding claim, further encoding a heterologous polypeptide which is fused to said hPDGF-R segment.

8. A cell transformed with a nucleic acid of any

This is the replacement sheet
Date: 25 FEB 1999
* C L M S *

preceding claim, and wherein said cell is a mammalian cell.

9. A cell as claimed in claim 8, further comprising a glycosylation enzyme originating from a non-fungal species.

10. A substantially pure extracellular hPDGF-R polypeptide fragment of at least about twenty amino acids having platelet-derived growth factor (PDGF) binding activity.

11. A substantially pure polypeptide fragment as claimed in claim 10, characterised in that said polypeptide fragment is soluble.

12. An extracellular hPDGF-R fragment having hPDGF-R binding activity consisting essentially of amino acids beginning at about 1 and ending at about 499 of a type B hPDGF-R, and is further derived from Table 2.

13. An extracellular hPDGF-R fragment having hPDGF-R binding activity consisting essentially of amino acids beginning about 1 and ending at about 501 of a type A hPDGF-R, and is further derived from Table 3.

14. A composition comprising an unglycosylated extracellular hPDGF-R fragment.

15. A composition as claimed in claim 14, characterised in that said fragment is substantially pure.

16. A composition comprising an extracelluclar hPDGF-R fragment, which exhibits a glycosylation pattern which is non-fungal and non-human.

17. A composition as claimed in claim 16, wherein said fragment is essentially the extracellular region of a type B or a type A hPDGF-R.

This is the replacement sheet

* C L M S *

Date: 25 FEB 1999

18. A composition as claimed in claim 17, having a sequence from Table 2, or from Table 3.

19. A composition comprising a combination of:

a) a recombinant nucleic acid encoding a human platelet-derived growth factor receptor polypeptide (hPDGF-R) fragment as claimed in any one of claims 1 to 7; and

b) a non-fungal glycosylation enzyme capable of glycosylating said fragment when expressed.

20. A method for introducing a hPDGF-R activity to a cell, said method comprising the step of introducing an extracellular hPDGF-R protein fragment of at least about five hundred daltons to said cell.

21. A method for assaying the presence of a ligand for a PDGF receptor in a sample, comprising the steps of:

combining said sample with a hPDGF receptor ligand binding site; and

detecting binding between said ligand and said hPDGF receptor ligand binding site.

22. An isolated polypeptide of less than about 200 amino acids comprising a receptor kinase insert region.

23. An isolated polypeptide of claim 22, wherein said polypeptide has a phosphorated amino acid residue.

24. An isolated polypeptide of claim 22, wherein said polypeptide comprises a sequence substantially homologous to a kinase insert segment of a PDGF receptor, and further has a sequence from Table 2 or Table 3.

25. An isolated polypeptide of claim 22, with a pharmaceutically acceptable carrier.

This is the replacement sheet

Date: 25 FEB 1999

* C L M S *

26. A method of purifying, from a sample, a protein capable of binding to a PDGF receptor kinase insert segment, comprising the step of:

contacting said sample with an analogue of a phosphorated polypeptide substantially homologous to a PDGF receptor kinase insert region polypeptide, thereby allowing said protein to bind specifically to said phosphorated polypeptide.

27. A method of isolating a nucleic acid encoding a protein capable of binding to a PDGF receptor, comprising the steps of:

combining a labeled and phosphorated PDGF receptor kinase insert region polypeptide with cells expressing various proteins, thereby labeling those cells which express said nucleic acid to produce a protein which binds said phosphorated polypeptide, and

isolating those cells which have been labeled.

28. A method of claim 27, wherein said protein capable of binding a PDGH receptor is PI3 kinase or c-fms.