

HYBRID PROTEINS WHICH FORM HETERODIMERS

FIELD OF THE INVENTION

5 The present invention relates to a hybrid protein comprising two coexpressed amino acid sequences forming a dimer, each comprising:

10 a) at least one amino acid sequence selected from a homomeric receptor, a chain of a heteromeric receptor, a ligand, and fragments thereof; and

15 b) a subunit of a heterodimeric proteinaceous hormone or fragments thereof; in which (a) and (b) are bonded directly or through a peptide linker, and, in each couple, the two subunits (b) are different and capable of aggregating to form a dimer complex.

BACKGROUND OF THE INVENTION

20 Protein-protein interactions are essential to the normal physiological functions of cells and multicellular organisms. Many proteins in nature exhibit novel or optimal functions when complexed with one or more other protein chains. This is illustrated by various ligand-receptor combinations that contribute to regulation of cellular activity. Certain ligands, such as tumor necrosis factor α (TNF α), TNF β , or
25 human chorionic gonadotropin (hCG), occur as multi-subunit complexes. Some of these complexes contain multiple copies of the same subunit. TNF α and TNF β (collectively referred to hereafter as TNF) are homotrimers formed by three identical subunits (1-4). Other ligands are composed of non-identical
30 subunits. For example, hCG is a heterodimer (5-7). Receptors may also occur or function as multi-chain complexes. For example, receptors for TNF transduce a signal after being aggregated to form dimers (8,9). Ligands to these receptors promote aggregation of two or three receptor chains, thereby
35 affording a mechanism of receptor activation. For example, TNF-mediated aggregation activates TNF receptors (10-12).

 The modulation of protein-protein interactions can be a useful mechanism for therapeutic intervention in various diseases and pathologies. Soluble binding proteins, that can

interact with ligands, can potentially sequester the ligand away from the receptor, thereby reducing the activation of that particular receptor pathway. Alternatively, sequestration of the ligand may delay its elimination or degradation, thereby increasing its duration of effect, and perhaps its apparent activity in vivo. In the case of TNF, soluble TNF receptors have been primarily associated with inhibition of TNF activity (13-17).

Soluble binding proteins may be useful for treating human diseases. For example, soluble TNF receptors have been shown to have efficacy in animal models of arthritis (18,19).

Since TNF has three binding sites for its receptor (10-12), and dimerization of the cell surface receptor is sufficient for bioactivity (8,9), it is likely that binding of a single soluble receptor to TNF will leave open the possibility that this 1:3 complex of soluble receptor:TNF (trimer) can still bind and activate a pair of cell surface TNF receptors. To achieve an inhibitory effect, it would be expected that two of the receptor binding sites on the TNF trimer must be occupied or blocked by the soluble binding protein. Alternatively, the binding protein could block proper orientation of TNF at the cell surface.

Generally speaking, the need was felt of synthesizing proteins that contain two receptor (or ligands) chains, as dimeric hybrid protein. See Wallach et al., U.S. patent 5,478,925.

The primary strategy employed for generating dimeric or multimeric hybrid proteins, containing binding domains from extracellular receptors, has been to fuse these proteins to the constant regions of an antibody heavy chain.

This strategy led, for example, to the construction of CD4 immunoadhesins (20). These are hybrid molecules consisting of the first two (or all four) immunoglobulin-like domains of CD4 fused to the constant region of antibody heavy and light chains. This strategy for creating hybrid molecules was adapted to the receptors for TNF (10,16,21) and led to the generation of constructs with higher *in vitro* activity than the monomeric soluble binding proteins.

It is widely held that the higher *in vitro* potency of the dimeric fusion proteins should translate into higher *in vivo* activity. One study does support this, revealing an at least 50-fold higher activity for a p75(TBP2)-Ig fusion protein in protecting mice from the consequences of intravenous LPS injection (16).

However, despite the widespread utilization of immunoglobulin fusion proteins, this strategy has several drawbacks. One is that certain immunoglobulin Fc domains participate in effector functions of the immune system. These functions may be undesirable in a particular therapeutic setting (22).

A second limitation pertains to the special cases where it is desirable to produce heteromeric fusion proteins, for example soluble analogs of the heteromeric IL-6 or type I interferon receptors. Although there are numerous methods for producing bifunctional antibodies (e.g., by co-transfection or hybridoma fusions), the efficiency of synthesis is greatly compromised by the mixture of homodimers and heterodimers that typically results (23). Recently there have been several reports describing the use of leucine zipper motifs to guide assembly of heterodimers (24-26). This appears to be a promising approach for research purposes, but the non-native or intracellular sequences employed may not be suitable for chronic applications in the clinic due to antigenicity. The efficiency of assembly and stability post assembly may also be limitations.

On the other hand, in the particular case of TNF receptors, certain modifications to the p55 TNF receptor have been found to facilitate homodimerization and signaling in the absence of ligand (27,28). It has been found that a cytoplasmic region of the receptor, termed the "death domain," can act as a homodimerization motif (28,30). As an alternative to an immunoglobulin hybrid protein, fusion of the extracellular domain of the TNF receptor to its cytoplasmic death domain could conceivably result in a secreted protein which can dimerize in the absence of TNF. Such fusion proteins

have been disclosed and claimed in the International Patent Application WO 95/31544.

A third further strategy employed for generating dimers of soluble TNF receptors has been to chemically cross-link the monomeric proteins with polyethylene glycol (31).

SUMMARY OF THE INVENTION

An alternative for obtaining such dimeric proteins, offering some important advantages, is the one of the present invention and consists in using a natural heterodimeric scaffold corresponding to a circulating non-immunoglobulin protein with a long half-life. A preferred example is hCG, a protein that is secreted well, has good stability, and has a long half-life (32-33). Given hCG's prominent role as a marker of pregnancy, many reagents have been developed to quantitate and study the protein *in vitro* and *in vivo*. In addition, hCG has been extensively studied using mutagenesis, and it is known that small deletions to the protein, such as removal of five residues at the extreme carboxyl-terminus of the α subunit, can effectively eliminate its biological activity while preserving its capability to form heterodimer (34,35). Small insertions, of up to 30 amino acids, have been shown to be tolerated at the amino- and carboxyl-termini of the α subunit (36), while fusion of the α subunit to the carboxyl terminus of the β subunit also had little effect on heterodimer formation (37).

An analog of hCG in which an immunoglobulin Fc domain was fused to the C-terminus of hCG β subunit has also been reported; however, this construct was not secreted and no effort was made to combine it with an α subunit (38).

Therefore, the main object of the present invention is a hybrid protein comprising two coexpressed amino acid sequences forming a dimer, each comprising:

a) at least one amino acid sequence selected among a homomeric receptor, a chain of a heteromeric receptor, a ligand, and fragments thereof; and

b) a subunit of a heterodimeric proteinaceous hormone, or fragments thereof; in which (a) and (b) are bonded

directly or through a peptide linker, and in each couple the two subunits (b) are different and capable of aggregating forming a dimer complex.

5 According to the present invention, the linker may be enzymatically cleavable.

Sequence (a) is preferably selected among: the extracellular domain of the TNF Receptor 1 (55 kDa, also called TBP1), the extracellular domain of the TNF Receptor 2
10 (75 kDa, also called TBP2), or fragments thereof still containing the ligand binding domain; the extracellular domains of the IL-6 receptors (also called gp80 and gp130); the extracellular domain of the IFN α/β receptor or IFN γ receptor; a gonadotropin receptor or its extracellular
15 fragments; antibody light chains, or fragments thereof, optionally associated with the respective heavy chains; antibody heavy chains, or fragments thereof, optionally associated with the respective light chains; antibody Fab domains; or ligand proteins, such as cytokines, growth factors
20 or hormones other than gonadotropins, specific examples of which include IL-6, IFN- β , TPO, or fragments thereof.

Sequence (b) is preferably selected among a hCG, FSH, LH, TSH, inhibin subunit, or fragments thereof.

Modifications to the proteins, such as chemical or
25 protease cleavage of the protein backbone, or chemical or enzymatic modification of certain amino acid side chains, can be used to render the components of the hybrid protein of the invention inactive. This restriction of activity may also be accomplished through the use of recombinant DNA techniques to
30 alter the coding sequence for the hybrid protein in a way that results directly in the restriction of activity to one component, or that renders the protein more amenable to subsequent chemical or enzymatic modification.

The above hybrid proteins will result in
35 monofunctional, bifunctional or multifunctional molecules, depending on the amino acid sequences (a) that are combined with (b). In each couple, (a) can be linked to the amino termini or to the carboxy termini of (b), or to both.

A monoclonal hybrid protein of the present invention can, for instance, comprise the extracellular domain of a gonadotropin receptor linked to one of the corresponding
5 receptor-binding gonadotropin subunits. According to such an embodiment, the hybrid protein of the invention can be a molecule in which, for example, the FSH receptor extracellular domain is linked to FSH to increase plasma half-life and improve biological activity.

10 This preparation can be employed to induce follicular maturation in assisted reproduction methods, such as ovulation induction or in vitro fertilisation, and to serve as a means to dramatically amplify the biological activity of the hormone essential for the success of the process, thus reducing the
15 requirement for both the hormone itself and the number of injections to achieve ovulation.

The FSH receptor and the production of the extracellular domain of the human FSH receptor have been described respectively in WO 92/16620 and WO 96/38575.

20 According to a particular embodiment, the extracellular domain of the FSH receptor (ECD) can be fused in frame with a peptide linker that contains the thrombin recognition/cleavage site (29) and represents a "tethered" arm. The peptide linker links the extracellular domain of FSH with a
25 FSH subunit. This will allow for removal of the extracellular domain of the FSH receptor by cleavage at the thrombin cleavage site as the molecule comes in contact with thrombin in the systemic circulation.

In another embodiment, instead of the thrombin
30 cleavage site, an enzyme recognition site for an enzyme that is found in greatest abundance in the ovary is used. In this way, as the ECD-FSH molecule travels to the ovary, it will be exposed to enzymes found in the highest concentrations in that tissue and the ECD will be removed so that the FSH can interact
35 with the membrane bound receptor.

In yet another embodiment, instead of an enzyme recognition site, a flexible hinge region is cloned between ECD and FSH so that the ECD will not be enzymatically removed from the hormone. In this way, when the ECD-FSH molecule arrives at

the ovary, a competition will be established between the hinge-attached ECD and the ECD of the FSH receptor found on the ovarian cell membrane.

5 In a further preferred embodiment of the invention, the hybrid protein consists of the aggregation between a couple of aa sequences, one of which contains TBP1 (or the fragments from aa 20 to aa 161 or to aa 190) as (a) and the α subunit of hCG as (b), and the other contains always TBP1 (or the same
10 fragments as above) as (a) and the β subunit of hCG, or fragments thereof, as (b). According to this embodiment, depending on the particular sequence that is chosen as (b) (the entire β subunit of hCG, or fragments or modifications thereof), the resulting hybrid protein will have one activity
15 (only that of TBP1) or a combination of activities (that of TBP1 with that of hCG). In this latter case the hybrid protein can be used, for example, in the combined treatment of Kaposi's sarcoma and metabolic wasting in AIDS.

In a further embodiment of the invention, one or more
20 covalent bonds between the two subunits (b) are added to enhance the stability of the resulting hybrid protein. This can be done, e.g., by adding one or more non-native interchain disulfide bonds. The sites for these cross-links can be deduced from the known structures of the heterodimeric
25 hormones. For example, a suitable site in hCG could be to place cysteine residues at α subunit residue Lys45 and β subunit residue Glu21, replacing a salt bridge (non-covalent bond) with a disulfide bond (covalent bond). Another object of the present invention are PEGylated or other chemically
30 modified forms of the hybrid proteins.

A further object of the present invention is a DNA molecule comprising the DNA sequence coding for the above hybrid protein, as well as nucleotide sequences substantially the same. "Nucleotide sequences substantially the same"
35 includes all other nucleic acid sequences which, by virtue of the degeneracy of the genetic code, also code for the given amino acid sequence.

For the production of the hybrid protein of the invention, the DNA sequence (a) is obtained from existing

clones, as is (b). The DNA sequence coding for the desired sequence (a) is ligated with the DNA sequence coding for the desired sequence (b). Two of these fused products are inserted and ligated into a suitable plasmid or each into a different plasmid. Once formed, the expression vector, or the two expression vectors, is introduced into a suitable host cell, which then expresses the vector(s) to yield the hybrid protein of the invention as defined above.

The preferred method for preparing the hybrid of the invention is by way of PCR technology using oligonucleotides specific for the desired sequences to be copied from the clones encoding sequences (a) and (b).

Expression of any of the recombinant proteins of the invention as mentioned herein can be effected in eukaryotic cells (e.g., yeasts, insect or mammalian cells) or prokaryotic cells, using the appropriate expression vectors. Any method known in the art can be employed.

For example the DNA molecules coding for the proteins obtained by any of the above methods are inserted into appropriately constructed expression vectors by techniques well known in the art (see Sambrook et al, 1989). Double stranded cDNA is linked to plasmid vectors by homopolymeric tailing or by restriction linking involving the use of synthetic DNA linkers or blunt-ended ligation techniques: DNA ligases are used to ligate the DNA molecules and undesirable joining is avoided by treatment with alkaline phosphatase.

In order to be capable of expressing the desired protein, an expression vector should comprise also specific nucleotide sequences containing transcriptional and translational regulatory information linked to the DNA coding the desired protein in such a way as to permit gene expression and production of the protein. First in order for the gene to be transcribed, it must be preceded by a promoter recognizable by RNA polymerase, to which the polymerase binds and thus initiates the transcription process. There are a variety of such promoters in use, which work with different efficiencies (strong and weak promoters).

For eukaryotic hosts, different transcriptional and translational regulatory sequences may be employed, depending on the nature of the host. They may be derived from viral sources, such as adenovirus, bovine papilloma virus, Simian virus or the like, where the regulatory signals are associated with a particular gene which has a high level of expression. Examples are the TK promoter of the Herpes virus, the SV40 early promoter, the yeast gal4 gene promoter, etc.

Transcriptional initiation regulatory signals may be selected which allow for repression and activation, so that expression of the genes can be modulated.

The DNA molecule comprising the nucleotide sequence coding for the hybrid protein of the invention is inserted into a vector(s), having the operably linked transcriptional and translational regulatory signals, which is capable of integrating the desired gene sequences into the host cell. The cells which have been stably transformed by the introduced DNA can be selected by also introducing one or more markers which allow for selection of host cells which contain the expression vector. The marker may also provide for phototrophy to a auxotrophic host, biocide resistance, e.g., antibiotics, or heavy metals such as copper, or the like. The selectable marker gene can either be directly linked to the DNA gene sequences to be expressed, or introduced into the same cell by co-transfection. Additional elements may also be needed for optimal synthesis of proteins of the invention.

Factors of importance in selecting a particular plasmid or viral vector include: the ease with which recipient cells that contain the vector may be recognized and selected from those recipient cells which do not contain the vector; the number of copies of the vector which are desired in a particular host; and whether it is desirable to be able to "shuttle" the vector between host cells of different species.

Once the vector(s) or DNA sequence containing the construct(s) has been prepared for expression, the DNA construct(s) may be introduced into an appropriate host cell by any of a variety of suitable means: transformation,

transfection, conjugation, protoplast fusion, electroporation, calcium phosphate-precipitation, direct microinjection, etc.

Host cells may be either prokaryotic or eukaryotic.

5 Preferred are eukaryotic hosts, e.g., mammalian cells, such as human, monkey, mouse, and Chinese hamster ovary (CHO) cells, because they provide post-translational modifications to protein molecules, including correct folding or glycosylation at correct sites. Also, yeast cells can carry out post-
10 translational peptide modifications including glycosylation. A number of recombinant DNA strategies exist which utilize strong promoter sequences and high copy number of plasmids which can be utilized for production of the desired proteins in yeast. Yeast recognizes leader sequences on cloned mammalian gene
15 products and secretes peptides bearing leader sequences (i.e., pre-peptides).

After the introduction of the vector(s), the host cells are grown in a selective medium, which selects for the growth of vector-containing cells. Expression of the cloned
20 gene sequence(s) results in the production of the desired proteins.

Purification of the recombinant proteins is carried out by any one of the methods known for this purpose, i.e., any conventional procedure involving extraction, precipitation,
25 chromatography, electrophoresis, or the like. A further purification procedure that may be used in preference for purifying the protein of the invention is affinity chromatography using monoclonal antibodies which bind the target protein and which are produced and immobilized on a gel
30 matrix contained within a column. Impure preparations containing the recombinant protein are passed through the column. The protein will be bound to the column by the specific antibody while the impurities will pass through. After washing, the protein is eluted from the gel by a change
35 in pH or ionic strength.

The term "hybrid protein", as used herein, generically refers to a protein which contains two or more different proteins or fragments thereof.

As used herein, "fusion protein" refers to a hybrid protein, which consists of two or more proteins, or fragments thereof, linked together covalently.

5 The term "aggregation", as used herein, means the formation of strong specific non-covalent interactions between two polypeptide chains forming a complex, such as those existing between the α and β subunit of a heterodimeric hormone (such as FSH, LH, hCG or TSH).

10 The terms "ligand" or "ligand protein", as used herein, refer to a molecule, other than an antibody or an immunoglobulin, capable of being bound by the ligand-binding domain of a receptor; such molecule may occur in nature, or may be chemically modified or chemically synthesised.

15 The term "ligand-binding domain", as used herein, refers to a portion of the receptor that is involved in binding a ligand and is generally a portion or essentially all of the extracellular domain.

20 The term "receptor", as used herein, refers to a membrane protein, whose binding with the respective ligand triggers secondary cellular responses that result in the activation or inhibition of intracellular process.

25 In a further aspect, the present invention provides the use of the hybrid protein as a medicament. The medicament is preferably presented in the form of a pharmaceutical composition comprising the protein of the invention together with one or more pharmaceutically acceptable carriers and/or excipients. Such pharmaceutical compositions represent yet a further aspect of the invention.

30 BRIEF DESCRIPTION OF THE DRAWINGS

The invention will be better understood by reference to the appended drawings, in which:

35 Figures 1(a) and 1(b) show the TBP(20-161)-hCG α and TBP(20-161)-hCG β constructs, respectively, and the corresponding sequences (SEQ ID NOS:1-4).

 Figures 2(a) and 2(b) show the TBP(20-190)-hCG α and TBP(20-190)-hCG β constructs, respectively, and the corresponding sequences (SEQ ID NOS:5-8).



Figure 3 is a schematic summary of the constructs of Figures 1 and 2 showing p55 TNFR1, TBP1 and TBP1 fusion constructs. The linker sequences shown on the last two lines are SEQ ID NO:9 (Ala-Gly-Ala-Ala-Pro-Gly) and SEQ ID NO:10 (Ala-Gly-Ala-Gly).

Figure 4 is a graph illustrating the dose dependent protective effect of CHO cell expressed TBP-hCG(20-190) on TNF α -induced cytotoxicity on BT-20 cells and various controls.

Figure 5 is a graph illustrating the dose dependent protective effect of COS cell expressed TBP-hCG(20-190) on TNF α -induced cytotoxicity on BT-20 cells and various controls.

Figure 6 is a graph illustrating the dose dependent protective effect of affinity purified CHO cell expressed TBP-hCG(20-161) on TNF α -induced cytotoxicity on BT-20 cells and various controls.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The invention will now be described by means of the following Examples, which should not be construed as in any way limiting the present invention.

EXAMPLES

Materials and Methods

Cell lines used in this study were obtained from the American Type Culture Collection (ATCC), Rockville, Maryland, unless otherwise specified. The CHO-DUKX cell line was obtained from L. Chasin at Columbia University through D. Houseman at MIT (39). The CHO-DUKX cells, which lack a functional gene for dihydrofolate reductase, were routinely maintained in complete α -plus Modified Eagles Medium (α (+)MEM) supplemented with 10% fetal bovine serum (FBS). The COS-7 cells were routinely maintained in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% FBS. Unless specified otherwise, cells were split to maintain them in log phase of growth, and culture reagents were obtained from GIBCO (Grand Island, New York).

This Sheet Has Been Replaced



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REPLACED

1. Assembly of the genetic constructs encoding the hybrid proteins

The numbering assignments for the p55 TNF receptor are based on the cloning paper from Wallach (40), while the numbering assignments for the hCG subunits are based on the numbering assignments from the Fiddes cloning papers (41,42). The designation TBP, or TNF binding protein, refers to the extracellular domain portions of the TNF receptors capable of binding TNF. In these Examples, the DNA constructs will be named as TBP-hybrid proteins, with the partner and region of TBP indicated in the construct nomenclature. All of the TBP-hCG constructs contain the human growth hormone (hGH) signal peptide in place of the native p55 signal sequence. In addition, the hGH signal peptide has been placed so that it immediately precedes TBP residue Asp20, which is anticipated to make this the first residue in the mature, secreted protein. These modifications are not essential to the basic concept of using hCG as a partner of the hybrid protein.

The DNAs encoding the hybrid proteins were constructed using PCR methodology (43).

a. TBP1(20-161)-hCG

The initial TBP-hCG construct was engineered to contain the ligand binding domain from the extracellular region of the p55 TNF receptor (from Asp20 inclusive of residue Cys161) fused though a short linker to the hCG α and β subunits (starting at residues α Cys7 or β Pro7, respectively). This construct, hereafter referred to as TBP1(20-161)-hCG, is a heterodimer of two modified hCG subunits, TBP1(20-161)-hCG α and TBP1(20-161)-hCG β .

The oligodeoxynucleotide primers used for the TBP1(20-161)-hCG α construct were:

primer 1($\alpha\beta$) TTT TCT CGA GAT GGC TAC AGG TAA GCG
CCC (SEQ ID NO:11)

primer 2(α) ACC TGG GGC AGC ACC GGC ACA GGA GAC ACA
CTC GTT TTC (SEQ ID NO:12)

primer 3(α) TGT GCC GGT GCT GCC CCA GGT TGC CCA GAA
TGC ACG CTA CAG (SEQ ID NO:13)

primer 4(α) TTT TGG ATC CTT AAG ATT TGT GAT AAT AAC
AAG TAC (SEQ ID NO:14)

These and all of the other primers described in these
5 Examples were synthesized on an Applied Biosystems Model 392
DNA synthesis machine (ABI, Foster City, California), using
phosphoramidite chemistry.

Since both of the TBP-hCG subunit constructs have the
same 5'-end (i.e., the 5'-end of the hGH/TBP construct), primer
10 1($\alpha\beta$) was used for both TBP-hCG subunit constructs. The
other primers used for the TBP1(20-161)-hCG β construct were:

primer 2(β) CCG TGG ACC AGC ACC AGC ACA GGA GAC
ACA CTC GTT TTC (SEQ ID NO:15)

primer 3(β) TGT GCT GGT GCT GGT CCA CGG TGC CGC
15 CCC ATC AAT (SEQ ID NO:16)

primer 4(β) TTT TGG ATC CTT ATT GTG GGA GGA TCG
GGG TG (SEQ ID NO:17)

Primers 2(α) and 3(α) are reverse complements, and
cover both the 3'-end of the coding region for the p55
20 extracellular domain, and the 5'-end of the hCG α subunit.
Similarly, primers 2(β) and 3(β) are also reverse
complements, and cover both the 3'-end of the coding region for
the p55 extracellular domain, and the 5'-end of the hCG β
subunit.

25 Two PCR reactions were run for each of the two TBP-
hCG subunit constructs. The first used primers 1($\alpha\beta$) and 2
(α or β), and used as the template a plasmid encoding soluble
p55 residues 20-180 preceded by the hGH signal peptide (plasmid
pCMVhGHspcDNA.pA4). The second used primers 3 (α or β) and 4
30 (α or β), and used as the template either plasmid pSVL-hCG α
or pSVL-hCG β (44). The PCR was performed using Vent (TM)
polymerase from New England Biolabs (Beverly, Massachusetts) in
accordance with the manufacturer's recommendations, using for
each reaction 25 cycles and the following conditions:

35 100 μ g of template DNA

1 μ g of each primer

2U of Vent(TM) polymerase (New England Biolabs)

denaturation at 99°C for 30 seconds

annealing at: 59°C for 30 seconds for primers 1($\alpha\beta$) and 2(α)

59°C for 30 seconds for primers 3(α) and 4(α)
57°C for 30 seconds for primers 1($\alpha\beta$) and 2(β)
63°C for 30 seconds for primers 3(β) and 4(β)
extension at 75°C for 75 seconds.

The PCR products were confirmed to be the expected size by electrophoresis in a 2% agarose gel and ethidium bromide staining. The fragments were then purified by passage over a Wizard column (Promega) in accordance with the column manufacturer's recommendations.

The final coding sequence for TBP1(20-161)-hCG α was assembled by fusion PCR using primer 1($\alpha\beta$) and primer 4(α), and using as template the purified products from the p55 and hCG α fragments obtained from the first PCR reactions. First the two templates, which due to the overlap between primers 2(α) and 3(α) could be denatured and annealed together, were passed through 10 cycles of PCR in the absence of any added primers. The conditions for these cycles were essentially the same as those used earlier, except that the annealing was done at 67°C and the extension was performed for 2 minutes. At the end of these 10 cycles, primers 1($\alpha\beta$) and 4(α) were added, and another 10 cycles were performed. The conditions for this final set of reactions was the same as used earlier, except that an annealing temperature of 59°C was used, and the extension was performed for 75 seconds.

Analysis of the products of this reaction by electrophoresis in a 1% agarose gel confirmed that the expected fragment of about 1100bp was obtained. The reaction was passed over a Wizard column to purify the fragment, which was then digested with XbaI and BamHI and re-purified in a 0.7% low-melting point agarose gel. The purified fragment was subcloned into plasmid pSVL (Pharmacia), which had first been digested with XbaI and BamHI and gel purified on a 0.8% low-melting point agarose gel. Following ligation with T4 ligase, the mixture was used to transform AG1 E. coli and then plated onto LB/ampicillin plates for overnight culture at 37°C. Plasmid DNAs from ampicillin-resistant colonies were analyzed by digestion with XhoI and BamHI to confirm the presence of the insert (which is excised in this digest). Six clones were

found to contain inserts, and one (clone 7) was selected for further advancement and designated pSVLTBPhCG α (containing TBP1(20-161)-hCG α). Dideoxy DNA sequencing (using SequenaseTM, U.S. Biochemicals, Cleveland, Ohio) of the insert in this vector confirmed that the construct was correct, and that no undesired changes had been introduced.

The final coding sequence for TBP1(20-161)-hCG β was assembled in a manner similar to that described for TBP1(20-161)-hCG α using fusion PCR and primers 1($\alpha\beta$) and 4(β), and using as template the purified products from the p55 and hCG β fragments obtained from the first PCR reactions. The resulting pSVL plasmid containing the insert of interest was designated pSVLTBPhCG β .

b. TBP(20-190)-hCG

A second set of TBP-hCG proteins was prepared by modification of the TBP(20-161)-hCG constructs to produce an analog containing TBP spanning from Asp20 to Thr190, in place of the 20-161 region in the initial analog. This was done by replacing the fragment between the BglIII and XbaI sites in plasmid pSVLTBPhCG α with a PCR fragment containing the change. This PCR fragment was generated using fusion PCR. The primers were:

primer 1 TTT TAG ATC TCT TCT TGC ACA GTG GAC
(SEQ ID NO:18)

primer 2 TGT GGT GCC TGA GTC CTC AGT (SEQ ID
NO:19)

primer 3 ACT GAG GAC TCA GGC ACC ACA GCC GGT GCT
GCC CCA GGT TG (SEQ ID NO:20)

primer 4 TTT TTC TAG AGA AGC AGC AGC AGC CCA TG
(SEQ ID NO:21)

Primers 1 and 2 were used to generate the sequence coding the additional p55 residues from 161-190. The PCR reaction was performed essentially as described earlier, using 1 μ g of each primer and pUC-p55 as template. Similarly, primers 3 and 4 were used to generate by PCR the linker between the 3'-end of the TBP-coding region, and the 5'-end of the hCG α subunit coding region, using as a template plasmid pSVLTBPhCG α . Products from these PCR reactions were confirmed

to be the correct size (about 296 bp and 121 bp respectively) by polyacrylamide gel electrophoresis (PAGE) on an 8% gel, and were then purified using a Wizard column. The design of primers 2 and 3 was such that they contained a region of overlap, so that the two PCR products (from primers 1 and 2, and from primers 3 and 4) could be annealed for fusion PCR with primers 1 and 4. Subsequent to the fusion reaction, the desired product of about 400 bp was confirmed and purified using a 1.5% agarose gel and a Wizard column. This DNA was then digested with BglII and XbaI, and ligated with BglII/XbaI-digested pSVLTBPhCG α . The presence of an insert in plasmids isolated from transformed AG1 *E. coli* was confirmed by digestion with BglII and XbaI. The new construct was designated pSVLTBP(20-190)-hCG α .

Similarly, plasmid pSVLTBPhCG β was modified by substitution of the BglII-XcmI fragment. However, this was done by subcloning of a single PCR product, rather than with a fusion PCR product. Primers 1 and 2b (see below) were used with pUC-p55 as the template.

primer 2b TTT TCC ACA GCC AGG GTG GCA TTG ATG GGG
 CGG CAC CGT GGA CCA GCA CCA GCT GTG GTG
 CCT GAG TCC TCA GTG (SEQ ID NO:22)

The resulting PCR product (about 337bp) was confirmed and purified as described above, digested with BglII and XcmI, and then ligated into BglII/XbaI-digested pSVLTBPhCG β . The presence of an insert in plasmids isolated from transformed AG1 *E. coli* was confirmed by digestion with BglII and XcmI. The new construct was designated pSVLTBP(20-190)-hCG β .

The new constructs were subsequently confirmed by DNA sequencing.

In addition to producing these new pSVL-based plasmids, these constructs were also subcloned into other expression vectors likely to be more suitable for stable expression in CHO, particularly vector D α , previously described as plasmid CLH3AXSV2DHFR (45). This was accomplished by converting a BamHI site flanking the inserts in the pSVL-based vectors to an XhoI site, and then excising the insert with XhoI and cloning it into XhoI digested D α .

2. Transient and stable expression of the hybrid proteins

Transfections of COS-7 cells (ATCC CRL 1651, ref. 46) for transient expression of the TBP-hCG hybrid proteins were performed using electroporation (47). Exponentially growing COS-7 cells were removed by trypsinization, collected by gentle centrifugation (800 rpm, 4 minutes), washed with cold phosphate buffered saline (PBS), pH 7.3-7.4, and then repelleted by centrifugation. Cells were resuspended at a concentration of 5×10^6 cells per 400 μ l cold PBS and mixed with 10 μ g of plasmid DNA in a prechilled 2 mm gap electroporation cuvette. For cotransfections, 5 μ g of each plasmid were used. The cuvette and cells were chilled on ice for a further 10 minutes, and then subjected to electroporation using a BTX Model 600 instrument and conditions of 125 V, 950 μ F and R=8. Afterward the cells were set to cool on ice for 10 minutes, transferred to a 15 ml conical tube containing 9.5 ml complete medium (Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% L-glutamine) at room temperature, and left at room temperature for 5 minutes. After gentle mixing in the 15 ml tube, the entire contents was seeded onto two P100 plates and placed into a 37°C, 5% CO₂ incubator. After 18 hours the media was changed, and in some cases the new media contained only 1% or 0% FBS. After another 72 hours, the conditioned media was harvested, centrifuged to remove cells, and then stored frozen at -70°C.

Transfections of CHO-DUKX (CHO) cells for transient or stable expression were performed using calcium phosphate precipitation of DNA. Twenty-four hours prior to the transfection, exponentially growing CHO cells were plated onto 100 mm culture plates at a density of 7.5×10^5 cells per plate. On the day of the transfection, 10 μ g of plasmid DNA was brought to 0.5 ml in transfection buffer (see below), 31 μ l of 2 M CaCl₂ were added, the DNA-CaCl₂ solution was mixed by vortexing, and left to stand at room temperature for 45 minutes. After this the media was aspirated from the plates, the DNA was added to the cells using a sterile plastic pipette, and the cells were left at room temperature for 20 minutes. At

the end of this period, 5 ml of complete α (+)MEM containing 10% FBS was added to the plates, which were incubated at 37°C for 4-6 hours. The media was then aspirated off the plates, and the cells were subjected to a glycerol shock by incubating them with a solution of 15% glycerol in transfection buffer at 37°C for 3.5 minutes. After removal of the glycerol solution, the cells were washed twice with PBS, refed with 10 ml complete α (+)MEM, 10% FBS, and returned to the 37°C incubator. For stable transfections, after 48 hours the cells were split 1:10 and fed with selection medium (complete α -minus MEM (lacking nucleosides), 10% dialyzed FBS, and 0.02 μ M methotrexate). Non-transfected (non-resistant) cells were typically eliminated in 3-4 weeks, leaving a population of transfected, methotrexate-resistant cells.

3. Quantitation of expression

Secretion of the hybrid proteins by transfected cells was assessed using a commercial assay kit for soluble p55 (R&D Systems; Minneapolis, Minnesota) in accordance with the manufacturer's instructions. This assay also provides an estimate of the hybrid protein levels in conditioned and processed media, which served as the basis for selecting doses to be used in the bioassay.

4. Assessment of heterodimer formation

To assess the ability of the TBP-hCG subunit fusions to combine and form heterodimers, a sandwich immunoassay using antibodies to the hCG subunits was performed. In this assay, a monoclonal antibody to the hCG β subunit is coated onto microtiter plates and used for analyte capture. The primary detection antibody is a goat polyclonal raised against the human TSH α subunit (#082422G - Biodesign International; Kennebunkport, Maine), which is in turn detected using a horse radish peroxidase conjugated rabbit anti-goat polyclonal antibody (Cappel; Durham, North Carolina).

Several different anti-hCG β subunit antibodies were used in this work, all of which show no detectable cross-reactivity with the free α subunit. One of these antibodies (3/6) is used in the commercially available MAIAclone hCG assay kit (Biodata; Rome, Italy).

High-protein binding microtiter plates (Costar #3590) were coated with capture antibody by incubation (2 hours at 37°C) with 100 μ l/well of a 5 μ g/ml solution of antibody in coating buffer (PBS, pH 7.4, 0.1 mM Ca^{++} , 0.1 mM Mg^{++}). After washing once with wash solution (PBS, pH 7.4 + 0.1% Tween 20) the plate is blocked by completely filling the wells (~400 μ l/well) with blocking solution (3% bovine serum albumin (BSA; fraction V - A-4503 Sigma) in PBS, pH 7.4) and incubating for one hour at 37°C or overnight at 4°C. The plate is then washed twice with wash solution, and the reference and experimental samples, diluted in diluent (5 mg/ml BSA in PBS, pH 7.4) to yield a 100 μ l volume, are added. After incubating the samples and the plate for two hours at 37°C, the plate is again twice washed with wash solution. The primary detection antibody, diluted 1:5000 in diluent, is added (100 μ l/well) and incubated for one hour at 37°C. The secondary detection antibody (HRP conjugated rabbit anti-goat Ig), diluted 1:5000 in diluent, is added (100 μ l/well) and after incubation for one hour at 37°C, the plate is washed three times with wash solution. One hundred μ l of TMB substrate solution (Kirkegaard and Perry Laboratories) is added, the plate is incubated 20 minutes in the dark at room temperature, and then the enzymatic reaction is stopped by addition of 50 μ l/well 0.3M H_2SO_4 . The plate is then analyzed using a microtiter plate reader set for a wavelength of 450 nm.

5. Partial purification

To better quantitate the activities of these hybrid proteins, TBP-hCG hybrid proteins were partially purified by immunoaffinity chromatography. The antibody used was a monoclonal commercially available from R&D Systems (MAB #225). The column was CNBr-activated sepharose, charged with the antibody by following the manufacturer's (Pharmacia) instructions.

Conditioned media was collected from confluent T-175 flasks of each line using daily harvests of 50 ml SFMII media (GIBCO), five harvests for each line. The collections were subjected to centrifugation (1000 RPM) to remove cellular debris. The material was then assayed for TBP content using

the commercial immunoassay and concentrated (Centricon units by Amicon; Beverly, Massachusetts) so that the apparent TBP concentration was about 50 ng/ml.

5 Ten ml of the concentrated TBP-hCG (sample #18873) was brought to approximately 1 M NaCl by addition of NaCl and adjustment of the solution to a conductivity of approximately 85 mS/cm. This was passed through a 0.5 ml anti-TBP immunoaffinity column. The flow-through was collected and run
10 through the column a second time. After this the column was washed with 1 M NaCl in PBS. The bound TBP(20-161)-hCG was collected after elution with 50 mM citric acid (pH 2.5). The eluate (approximately 7 ml) was concentrated by filtration using Amicon Centricon-10's in accordance with the
15 manufacturer's (Amicon) instructions, to a volume of approximately 200 μ l. Approximately 800 μ l of PBS was added to bring the sample volume to 1 ml, which was stored at 4°C until tested by bioassay.

6. Assessment of anti-TNF activity

20 Numerous *in vitro* TNF-induced cytotoxicity assays have been described for evaluating analogs of soluble TNF receptors. We utilized an assay employing a human breast carcinoma cell line, BT-20 cells (ATCC HTB 19). The use of
25 these cells as the basis for a TNF bioassay has been described previously (48). These cells are cultured at 37°C in RPMI 1640 media supplemented with 10% heat-inactivated FBS. The cells were grown to a maximum 80-90% confluence, which entailed splitting every 3-4 days with a seeding density of about 3×10^6 cells per T175cm² flask.

30 The BT-20 assay uses the inclusion of a cellular stain, crystal violet, as a detection method to assess survival of cells after treatment with TNF. Dead cells are unable to take up and retain the dye.

35 In brief, the protocol used for the assay of anti-TNF activity is the following. Recombinant human TNF α (R&D Systems) and the experimental samples are constituted in media (RPMI 1640 with 5% heat-inactivated FBS) and added to the wells of 96-well culture plates. The cells are then plated into these wells at a density of 1×10^5 cells/well. The quantity of

TNF α added was determined earlier in titration studies, and represents a dose at which about 50% of the cells are killed.

After addition of the samples, the cells are cultured for 48 hours at 39°C, after which the proportion of live cells is determined using crystal violet staining and a microtiter plate reader (570 nm).

RESULTS

1. Constructs under study

The designs of the hybrid proteins studied are briefly summarized below; two control proteins, a monomeric soluble p55 (r-hTBP-1) and a dimeric TBP-immunoglobulin fusion protein (TBP-IgG3) (prepared essentially as described in (10)), were studied for comparative purposes.

<u>Construct</u>	<u>TBP N-term</u>	<u>TBP C-term</u>	<u>Fusion partner</u>
r-hTBP-1	mix of 9 and 20	180	none
TBP-IgG3	mix of 9 and 20	190	IgG3 heavy chain constant region
TBP(20-161)-hCG	20	161	hCG α and hCG β (heterodimer)
TBP(20-190)-hCG	20	190	hCG α and hCG β (heterodimer)

The sequences of the DNAs encoding, TBP(20-190)-hCG and TBP(20-161)-hCG are provided in Figures 1 and 2, respectively. A schematic summary of the constructs is provided in Figure 3.

2. Secretion of TBP-hCG proteins

All of the constructs tested were found to be produced and secreted into culture media by transfected mammalian cells. Data illustrating this are shown in Tables 1 and 2.

3. TBP-hCG(α/β) fusion proteins assemble into heterodimers

The combination of TBP-hCG α and TBP-hCG β was confirmed using the sandwich assay for the hCG heterodimer.



Only the combined transfection of α and β subunit fusions resulted in heterodimer detection (Table 3).

4. TBP-hCG hybrid proteins exhibit increased activity over TBP monomer

Hybrid proteins produced in either COS-7 or CHO cells were found to be potent inhibitors of TNF α in the BT-20 bioassay. Some of the samples tested are summarized in Table 4.

Negative controls (conditioned media from mock transfections) were included for the 1x media samples.

As illustrated in Figures 4-6 (points on y-axis), addition of TNF (2.5 ng/ml) results in a clear reduction in live cell number (as assessed by OD 570). In every case, active samples have as a maximal protective effect the restoration of cell viability to the level seen in the absence of added TNF (i.e., the control labeled "cells alone").

The positive controls, r-hTBP-1 and TBP-IgG3, are both protective, showing a clear dose-dependence and ED50s of approximately 100 ng/ml for the r-hTBP-1 (Figs. 4-6) and about 1.5 ng/ml for TBP-IgG3 (Fig. 4) respectively.

The TBP-hCG constructs from 1x media (CHO or COS) or from the immunopurification show dose-dependent protection, with approximate ED50s ranging from 2-11 ng/ml (Figs. 4-6).

The results from the *in vitro* bioassay are reported in Table 5. The data indicate that the hybrid proteins inhibit TNF cytotoxicity, and that they are substantially more potent than the TBP monomer. The negative controls were devoid of protective activity.

In addition to the possibility that dimerization of TBP may increase potency, it is also possible that the activity of the hybrid proteins are not related to dimeric interaction with TBP, but rather to steric inhibition due to the partner of the hybrid interfering with soluble TBP/TNF binding to cell-surface TNF receptors.

All references cited herein, including journal articles or abstracts, published or corresponding U.S. or foreign patent applications, issued U.S. or foreign patents, or any other references, are entirely incorporated by reference

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- 23 -

THIS SHEET HAS BEEN
REPLACED

herein, including all data, tables, figures, and text presented in the cited references. Additionally, the entire contents of the references cited within the references cited herein are also entirely incorporated by reference.

Reference to known method steps, conventional method steps, known methods or conventional methods is not in any way an admission that any aspect, description or embodiment of the present invention is disclosed, taught or suggested in the relevant art.

The foregoing description of the specific embodiments will so fully reveal the general nature of the invention that others can, by applying knowledge within the skill of the art (including the contents of the references cited herein), readily modify and/or adapt for various applications such specific embodiments, without undue experimentation, without departing from the general concept of the present invention. Therefore, such adaptations and modifications are intended to be within the meaning and range of equivalents of the disclosed embodiments, based on the teaching and guidance presented herein. It is to be understood that the phraseology or terminology herein is for the purpose of description and not of limitation, such that the terminology or phraseology of the present specification is to be interpreted by the skilled artisan in light of the teachings and guidance presented herein, in combination with the knowledge of one of ordinary skill in the art.

TABLES

Table 1: COS-7 transient expression (TBP ELISA)	
Hybrid Protein	Concentration (pg/ml)
TBP1	66
TBP-hCG α (20-161)	5.1
TBP-hCG β (20-161)	0.5
TBP-hCG(20-161)	2.7
control	<0.25

Constructs were expressed using pSVL (Pharmacia)

Table 2: COS-7 transient expression (TBP ELISA)	
Hybrid protein	Concentration (ng/ml)
TBP1	131
TBP-hCG α (20-190)	81
TBP-hCG β (20-190)	9
TBP-hCG(20-190)	62
control	<1

Constructs were expressed using a mouse metallothionein promoter-containing vector - pD α

Table 3: COS-7 transient expression (hCG heterodimer assay)	
Hybrid Protein	Concentration (ng/ml)
TBP1	<0.2
TBP-hCG α (20-190)	<0.2
TBP-hCG β (20-190)	<0.2
TBP-hCG(20-190)	38
control	<0.2

Constructs were expressed using a mouse
metallothionein promoter-containing vector - pD α

Table 4: Samples tested for anti-TNF activity		
<i>Construct</i>	<i>Cell source</i>	<i>Nature of sample</i>
r-hTBP-1	CHO	purified
TBP-IgG3	CHO	1x conditioned media
TBP(20-161)-hCG	CHO	immunopurified (anti-TBP)
TBP(20-190)-hCG	CHO	1x conditioned media
TBP(20-190)-hCG	COS	1x conditioned media

Table 5 :Preliminary Assessment of the hybrid proteins in TNF Cytotoxicity Assay		
Construct	Fusion partner	Anti-TNF activity (ED50) in BT-20 bioassay^{**}
r-hTBP-1	none	100 ng/ml
TBP-IgG3	IgG3 heavy chain constant region	1.5 ng/ml
TBP(20-161)-hCG	hCG α and hCG β (heterodimer)	2 ng/ml
TBP(20-190)-hCG	hCG α and hCG β (heterodimer)	8-11 ng/ml

^{**}The quantitation of material for dosing and estimation of ED50 was made using the TBP ELISA.

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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1049 base pairs
(B) TYPE: nucleic acid

- (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear
 (ii) MOLECULE TYPE: cDNA
 (ix) FEATURE:
 (A) NAME/KEY: CDS
 (B) LOCATION: 278..1047

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(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 256 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

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

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Tyr	Leu	Tyr	Asn	Asp	Cys	Pro	Gly	Pro	Gly	Gln	Asp	Thr	Asp	Cys	Arg	
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Glu	Cys	Glu	Ser	Gly	Ser	Phe	Thr	Ala	Ser	Glu	Asn	His	Leu	Arg	His	
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Ser	Ser	Cys	Thr	Val	Asp	Arg	Asp	Thr	Val	Cys	Gly	Cys	Arg	Lys	Asn	
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Gln Glu Asn Pro Phe Phe Ser Gln Pro Gly Ala Pro Ile Leu Gln Cys
 180 185 190

Met Gly Cys Cys Phe Ser Arg Ala Tyr Pro Thr Pro Leu Arg Ser Lys
 195 200 205

Lys Thr Met Leu Val Gln Lys Asn Val Thr Ser Glu Ser Thr Cys Cys
 210 215 220

Val Ala Lys Ser Tyr Asn Arg Val Thr Val Met Gly Gly Phe Lys Val
 225 230 235 240

Glu Asn His Thr Gly Cys His Cys Ser Thr Cys Tyr Tyr His Lys Ser
 245 250 255

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1202 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

- (A) NAME/KEY: CDS
 (B) LOCATION: 279..1199

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

CTCGAGATGG CTACAGGTAA GCGCCCCTAA AATCCCTTTG GGCACAATGT GTCCTGAGGG 60

GAGAGGTAGC GACCTGTAGA TGGGACGGGG GCACTAACCC TGAGGTTTGG GGCTTCTGAA 120

TGTGAGTATC GCCATGTAAG CCCAGTATTT GGCCAATGTC AGAAAGCTCC TGGTCCCTGG 180

AGGGATGGAG AGAGAAAAAC AAACAGCTCC TGGAGCAGGG AGAGTGCTGG CCTCTTGCTC 240

TCCGGCTCCC TCTGTTGCC TGTGGTTTCT CCCCAGGC TCC CGG ACG TCC CTG 293

Ser Arg Thr Ser Leu
 260

CTC CTG GCT TTT GGC CTG CTC TGC CTG CCC TGG CTT CAA GAG GGC AGT 341

Leu Leu Ala Phe Gly Leu Leu Cys Leu Pro Trp Leu Gln Glu Gly Ser
 265 270 275

GCC GAT AGT GTG TGT CCC CAA GGA AAA TAT ATC CAC CCT CAA AAT AAT 389

Ala Asp Ser Val Cys Pro Gln Gly Lys Tyr Ile His Pro Gln Asn Asn
 280 285 290

TCG ATT TGC TGT ACC AAG TGC CAC AAA GGA ACC TAC TTG TAC AAT GAC 437

Ser Ile Cys Cys Thr Lys Cys His Lys Gly Thr Tyr Leu Tyr Asn Asp
 295 300 305

TGT CCA GGC CCG GGG CAG GAT ACG GAC TGC AGG GAG TGT GAG AGC GGC 485

Cys Pro Gly Pro Gly Gln Asp Thr Asp Cys Arg Glu Cys Glu Ser Gly
 310 315 320 325

TCT TTC ACC GCT TCA GAA AAC CAC CTC AGA CAC TGC CTC AGC TGC TCC 533

Ser Phe Thr Ala Ser Glu Asn His Leu Arg His Cys Leu Ser Cys Ser
 330 335 340

AAA TGC CGA AAG GAA ATG GGT CAG GTG GAG ATC TCT TCT TGC ACA GTG 581

Lys Cys Arg Lys Glu Met Gly Gln Val Glu Ile Ser Ser Cys Thr Val
 345 350 355

GAC	CGG	GAC	ACC	GTG	TGT	GGC	TGC	AGG	AAG	AAC	CAG	TAC	CGG	CAT	TAT	629
Asp	Arg	Asp	Thr	Val	Cys	Gly	Cys	Arg	Lys	Asn	Gln	Tyr	Arg	His	Tyr	
		360					365					370				
TGG	AGT	GAA	AAC	CTT	TTC	CAG	TGC	TTC	AAT	TGC	AGC	CTC	TGC	CTC	AAT	677
Trp	Ser	Glu	Asn	Leu	Phe	Gln	Cys	Phe	Asn	Cys	Ser	Leu	Cys	Leu	Asn	
	375					380					385					
GGG	ACC	GTG	CAC	CTC	TCC	TGC	CAG	GAG	AAA	CAG	AAC	ACC	GTG	TGC	ACC	725
Gly	Thr	Val	His	Leu	Ser	Cys	Gln	Glu	Lys	Gln	Asn	Thr	Val	Cys	Thr	
	390				395					400					405	
TGC	CAT	GCA	GGT	TTC	TTT	CTA	AGA	GAA	AAC	GAG	TGT	GTC	TCC	TGT	GCT	773
Cys	His	Ala	Gly	Phe	Phe	Leu	Arg	Glu	Asn	Glu	Cys	Val	Ser	Cys	Ala	
			410						415					420		
GGT	GCT	GGT	CCA	CGG	TGC	CGC	CCC	ATC	AAT	GCC	ACC	CTG	GCT	GTG	GAG	821
Gly	Ala	Gly	Pro	Arg	Cys	Arg	Pro	Ile	Asn	Ala	Thr	Leu	Ala	Val	Glu	
			425					430					435			
AAG	GAG	GGC	TGC	CCC	GTG	TGC	ATC	ACC	GTC	AAC	ACC	ACC	ATC	TGT	GCC	869
Lys	Glu	Gly	Cys	Pro	Val	Cys	Ile	Thr	Val	Asn	Thr	Thr	Ile	Cys	Ala	
		440					445					450				
GGC	TAC	TGC	CCC	ACC	ATG	ACC	CGC	GTG	CTG	CAG	GGG	GTC	CTC	CCC	GCC	917
Gly	Tyr	Cys	Pro	Thr	Met	Thr	Arg	Val	Leu	Gln	Gly	Val	Leu	Pro	Ala	
	455					460					465					
CTG	CCT	CAG	GTG	GTG	TGC	AAC	TAC	CGC	GAT	GTG	CGC	TTC	GAG	TCC	ATC	965
Leu	Pro	Gln	Val	Val	Cys	Asn	Tyr	Arg	Asp	Val	Arg	Phe	Glu	Ser	Ile	
	470				475				480						485	
CGG	CTC	CCT	GGC	TGC	CCG	CGC	GGC	GTG	AAC	CCC	GTG	GTC	TCC	TAC	GCT	1013
Arg	Leu	Pro	Gly	Cys	Pro	Arg	Gly	Val	Asn	Pro	Val	Val	Ser	Tyr	Ala	
			490					495						500		
GTG	GCT	CTC	AGC	TGT	CAA	TGT	GCA	CTC	TGC	CGC	CGC	AGC	ACC	ACT	GAC	1061
Val	Ala	Leu	Ser	Cys	Gln	Cys	Ala	Leu	Cys	Arg	Arg	Ser	Thr	Thr	Asp	
			505				510						515			
TGC	GGG	GGT	CCC	AAG	GAC	CAC	CCC	TTG	ACC	TGT	GAT	GAC	CCC	CGC	TTC	1109
Cys	Gly	Gly	Pro	Lys	Asp	His	Pro	Leu	Thr	Cys	Asp	Asp	Pro	Arg	Phe	
		520					525					530				
CAG	GAC	TCC	TCT	TCC	TCA	AAG	GCC	CCT	CCC	CCC	AGC	CTT	CCA	AGC	CCA	1157
Gln	Asp	Ser	Ser	Ser	Ser	Lys	Ala	Pro	Pro	Pro	Ser	Leu	Pro	Ser	Pro	
	535					540					545					
TCC	CGA	CTC	CCG	GGG	CCC	TCG	GAC	ACC	CCG	ATC	CTC	CCA	CAA	TAA		1202
Ser	Arg	Leu	Pro	Gly	Pro	Ser	Asp	Thr	Pro	Ile	Leu	Pro	Gln			
	550				555					560						

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 307 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

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

Ser Arg Thr Ser Leu Leu Leu Ala Phe Gly Leu Leu Cys Leu Pro Trp
1 5 10 15

Leu Gln Glu Gly Ser Ala Asp Ser Val Cys Pro Gln Gly Lys Tyr Ile
 20 25 30
 His Pro Gln Asn Asn Ser Ile Cys Cys Thr Lys Cys His Lys Gly Thr
 35 40 45
 Tyr Leu Tyr Asn Asp Cys Pro Gly Pro Gly Gln Asp Thr Asp Cys Arg
 50 55 60
 Glu Cys Glu Ser Gly Ser Phe Thr Ala Ser Glu Asn His Leu Arg His
 65 70 75 80
 Cys Leu Ser Cys Ser Lys Cys Arg Lys Glu Met Gly Gln Val Glu Ile
 85 90 95
 Ser Ser Cys Thr Val Asp Arg Asp Thr Val Cys Gly Cys Arg Lys Asn
 100 105 110
 Gln Tyr Arg His Tyr Trp Ser Glu Asn Leu Phe Gln Cys Phe Asn Cys
 115 120 125
 Ser Leu Cys Leu Asn Gly Thr Val His Leu Ser Cys Gln Glu Lys Gln
 130 135 140
 Asn Thr Val Cys Thr Cys His Ala Gly Phe Phe Leu Arg Glu Asn Glu
 145 150 155 160
 Cys Val Ser Cys Ala Gly Ala Gly Pro Arg Cys Arg Pro Ile Asn Ala
 165 170 175
 Thr Leu Ala Val Glu Lys Glu Gly Cys Pro Val Cys Ile Thr Val Asn
 180 185 190
 Thr Thr Ile Cys Ala Gly Tyr Cys Pro Thr Met Thr Arg Val Leu Gln
 195 200 205
 Gly Val Leu Pro Ala Leu Pro Gln Val Val Cys Asn Tyr Arg Asp Val
 210 215 220
 Arg Phe Glu Ser Ile Arg Leu Pro Gly Cys Pro Arg Gly Val Asn Pro
 225 230 235 240
 Val Val Ser Tyr Ala Val Ala Leu Ser Cys Gln Cys Ala Leu Cys Arg
 245 250 255
 Arg Ser Thr Thr Asp Cys Gly Gly Pro Lys Asp His Pro Leu Thr Cys
 260 265 270
 Asp Asp Pro Arg Phe Gln Asp Ser Ser Ser Ser Lys Ala Pro Pro Pro
 275 280 285
 Ser Leu Pro Ser Pro Ser Arg Leu Pro Gly Pro Ser Asp Thr Pro Ile
 290 295 300
 Leu Pro Gln
 305

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1147 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION: 278..1132

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

TCGAGATGGC TACAGGTAAG CGCCCCTAAA ATCCCTTTGG GCACAATGTG TCCTGAGGGG	60
AGAGGCAGCG ACCTGTAGAT GGGACGGGGG CACTAACCCT CAGGTTTGGG GCTTTTGAAT	120
GTGAGTATGG CCATGTAAGC CCAGTATTTG CCCAATCTCA GAAAGCTCCT GGTCCTGGA	180
GGGATGGAGA GAGAAAAACA AACAGCTCCT GGAGCAGGGA CACTCCTGGC CTCTTGCTCT	240
GCGGCTCCGT GTGTTGCCCT GTGGTTTCTC CCCACGC TCC CGG ACG TCC CTG CTC	295
Ser Arg Thr Ser Leu Leu	310
CTG GCT TTT GGC CTG CTC TGC CTG CCC TGG CTT CAA GAG GGC AGT GCC	343
Leu Ala Phe Gly Leu Leu Cys Leu Pro Trp Leu Gln Glu Gly Ser Ala	315 320 325
GAT AGT GTG TGT CCC CAA GGA AAA TAT ATC CAC CCT CAA AAT AAT TCG	391
Asp Ser Val Cys Pro Gln Gly Lys Tyr Ile His Pro Gln Asn Asn Ser	330 335 340 345
ATT TGC TGT ACC AAG TGC CAC AAA GGA ACC TAC TTG TAC AAT GAC TGT	439
Ile Cys Cys Thr Lys Cys His Lys Gly Thr Tyr Leu Tyr Asn Asp Cys	350 355 360
CCA GGC CCG GGG CAG GAT ACC GAC TGC AGG GAG TGT GAG AGC GGC TCC	487
Pro Gly Pro Gly Gln Asp Thr Asp Cys Arg Glu Cys Glu Ser Gly Ser	365 370 375
TTC ACC GCT TCA GAA AAC CAC CTC AGA CAC TGC CTC AGC TGC TCC AAA	535
Phe Thr Ala Ser Glu Asn His Leu Arg His Cys Leu Ser Cys Ser Lys	380 385 390
TGC CGA AAG GAA ATG GGT CAG GTG GAG ATC TCT TCT TGC ACA GTG GAC	583
Cys Arg Lys Glu Met Gly Gln Val Glu Ile Ser Ser Cys Thr Val Asp	395 400 405
CGG GAC ACC GTG TGT GGC TGC AGG AAG AAC CAG TAC CGG CAT TAT TGG	631
Arg Asp Thr Val Cys Gly Cys Arg Lys Asn Gln Tyr Arg His Tyr Trp	410 415 420 425
AGT GAA AAC CTT TTC CAG TGC TTC AAT TGC ACC CTC TGC CTC AAT GGG	679
Ser Glu Asn Leu Phe Gln Cys Phe Asn Cys Thr Leu Cys Leu Asn Gly	430 435 440
ACC GTG CAC CTC TCC TGT CAG GAG AAA CAG AAC ACC GTC TGC ACC TGC	727
Thr Val His Leu Ser Cys Gln Glu Lys Gln Asn Thr Val Cys Thr Cys	445 450 455
CAT GCA GGT TTC TTT CTA AGA GAA AAC GAG TGT GTC TCC TGT AGT AAC	775
His Ala Gly Phe Phe Leu Arg Glu Asn Glu Cys Val Ser Cys Ser Asn	460 465 470
TGT AAG AAA AGC CTG GAG TGC ACG AAG TTG TCC CTA CCC CAG ATT GAG	823
Cys Lys Lys Ser Leu Glu Cys Thr Lys Leu Ser Leu Pro Gln Ile Glu	475 480 485
AAT GTT AAG GGC ACT GAG GAC TCA GGC ACC ACA GCC GGT GCT GCC CCA	871
Asn Val Lys Gly Thr Glu Asp Ser Gly Thr Thr Ala Gly Ala Ala Pro	490 495 500 505

GGT TGC CCA GAA TGC ACG CTA CAG GAA AAC CCA TTC TTC TCC CAG CCG	919
Gly Cys Pro Glu Cys Thr Leu Gln Glu Asn Pro Phe Phe Ser Gln Pro	
510 515 520	
GGT GCC CCA ATA CTT CAG TGC ATG GGC TGC TGC TTC TCT AGA GCA TAT	967
Gly Ala Pro Ile Leu Gln Cys Met Gly Cys Cys Phe Ser Arg Ala Tyr	
525 530 535	
CCC ACT CCA CTA AGG TCC AAG AAG ACG ATG TTG GTC CAA AAG AAC GTC	1015
Pro Thr Pro Leu Arg Ser Lys Lys Thr Met Leu Val Gln Lys Asn Val	
540 545 550	
ACC TCA GAG TCC ACT TGC TGT GTA GCT AAA TCA TAT AAC AGG GTC ACA	1063
Thr Ser Glu Ser Thr Cys Cys Val Ala Lys Ser Tyr Asn Arg Val Thr	
555 560 565	
GTA ATG GGG GGT TTC AAA GTG GAG AAC CAC ACG GCG TGC CAC TGC AGT	1111
Val Met Gly Gly Phe Lys Val Glu Asn His Thr Ala Cys His Cys Ser	
570 575 580 585	
ACT TGT TAT TAT CAC AAA TCT TAAGGATCCC TCGAG	1147
Thr Cys Tyr Tyr His Lys Ser	
590	

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 285 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Ser Arg Thr Ser Leu Leu Leu Ala Phe Gly Leu Leu Cys Leu Pro Trp	
1 5 10 15	
Leu Gln Glu Gly Ser Ala Asp Ser Val Cys Pro Gln Gly Lys Tyr Ile	
20 25 30	
His Pro Gln Asn Asn Ser Ile Cys Cys Thr Lys Cys His Lys Gly Thr	
35 40 45	
Tyr Leu Tyr Asn Asp Cys Pro Gly Pro Gly Gln Asp Thr Asp Cys Arg	
50 55 60	
Glu Cys Glu Ser Gly Ser Phe Thr Ala Ser Glu Asn His Leu Arg His	
65 70 75 80	
Cys Leu Ser Cys Ser Lys Cys Arg Lys Glu Met Gly Gln Val Glu Ile	
85 90 95	
Ser Ser Cys Thr Val Asp Arg Asp Thr Val Cys Gly Cys Arg Lys Asn	
100 105 110	
Gln Tyr Arg His Tyr Trp Ser Glu Asn Leu Phe Gln Cys Phe Asn Cys	
115 120 125	
Thr Leu Cys Leu Asn Gly Thr Val His Leu Ser Cys Gln Glu Lys Gln	
130 135 140	
Asn Thr Val Cys Thr Cys His Ala Gly Phe Phe Leu Arg Glu Asn Glu	
145 150 155 160	
Cys Val Ser Cys Ser Asn Cys Lys Lys Ser Leu Glu Cys Thr Lys Leu	
165 170 175	

Ser Leu Pro Gln Ile Glu Asn Val Lys Gly Thr Glu Asp Ser Gly Thr
 180 185 190

Thr Ala Gly Ala Ala Pro Gly Cys Pro Glu Cys Thr Leu Gln Glu Asn
 195 200 205

Pro Phe Phe Ser Gln Pro Gly Ala Pro Ile Leu Gln Cys Met Gly Cys
 210 215 220

Cys Phe Ser Arg Ala Tyr Pro Thr Pro Leu Arg Ser Lys Lys Thr Met
 225 230 235 240

Leu Val Gln Lys Asn Val Thr Ser Glu Ser Thr Cys Cys Val Ala Lys
 245 250 255

Ser Tyr Asn Arg Val Thr Val Met Gly Gly Phe Lys Val Glu Asn His
 260 265 270

Thr Ala Cys His Cys Ser Thr Cys Tyr Tyr His Lys Ser
 275 280 285

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1301 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 279..1287

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

CTCGAGATGG CTACAGGTAA GCGCCCCTAA AATCCCTTTG GGCACAATGT GTCCTGAGGG 60

GAGAGGCAGC GACCTGTAGA TGGGACGGGG GCACTAACCC TCAGGTTTGG GGCTTCTGAA 120

TGTGAGTATC GCCATGTAAG CCCAGTATTT GGCCAATGTC AGAAAGCTCC TGGTCCCTGG 180

AGGGATGGAG AGAGAAAAAC AAACACCTCC TGGAGCAGGG AGAGTGCTGC CCTCTTGCTC 240

TCCGGCTCCC TCTGTTGCCC TCTGGTTTCT CCCCAGGC TCC CGG ACG TCC CTG 293

Ser Arg Thr Ser Leu
290

CTC CTG GCT TTT GGC CTG CTC TGC CTG CCC TGG CTT CAA GAG GGC AGT 341

Leu Leu Ala Phe Gly Leu Leu Cys Leu Pro Trp Leu Gln Glu Gly Ser 305

295 300

GCC GAT AGT GTG TGT CCC CAA GGA AAA TAT ATC CAC CCT CAA AAT AAT 389

Ala Asp Ser Val Cys Pro Gln Gly Lys Tyr Ile His Pro Gln Asn Asn 320

310 315

TCG ATT TGC TGT ACC AAG TGC CAC AAA GGA ACC TAC TTG TAC AAT GAC 437

Ser Ile Cys Cys Thr Lys Cys His Lys Gly Thr Tyr Leu Tyr Asn Asp 335

325 330

TGT CCA GGC CCG GGG CAG GAT ACG GAC TGC AGG GAG TGT GAG AGC GGC 485

Cys Pro Gly Pro Gly Gln Asp Thr Asp Cys Arg Glu Cys Glu Ser Gly 350

340 345

TCC	TTC	ACC	GCT	TCA	GAA	AAC	CAC	CTC	AGA	CAC	TGC	CTC	AGC	TGC	TCC	533
Ser	Phe	Thr	Ala	Ser	Glu	Asn	His	Leu	Arg	His	Cys	Leu	Ser	Cys	Ser	
355					360					365					370	
AAA	TGC	CGA	AAG	GAA	ATG	GGT	CAG	GTG	GAG	ATC	TCT	TCT	TGC	ACA	GTG	581
Lys	Cys	Arg	Lys	Glu	Met	Gly	Gln	Val	Glu	Ile	Ser	Ser	Cys	Thr	Val	
				375					380					385		
GAC	CGG	GAC	ACC	GTG	TGT	GGC	TGC	AGG	AAG	AAC	CAG	TAC	CGG	CAT	TAT	629
Asp	Arg	Asp	Thr	Val	Cys	Gly	Cys	Arg	Lys	Asn	Gln	Tyr	Arg	His	Tyr	
			390					395					400			
TGG	AGT	GAA	AAC	CTT	TTC	CAG	TGC	TTC	AAT	TGC	AGC	CTC	TGC	CTC	AAT	677
Trp	Ser	Glu	Asn	Leu	Phe	Gln	Cys	Phe	Asn	Cys	Ser	Leu	Cys	Leu	Asn	
		405					410					415				
GGG	ACC	GTG	CAC	CTC	TCC	TGC	CAG	GAG	AAA	CAG	AAC	ACC	GTG	TGC	ACC	725
Gly	Thr	Val	His	Leu	Ser	Cys	Gln	Glu	Lys	Gln	Asn	Thr	Val	Cys	Thr	
		420				425					430					
TGC	CAT	GCA	GGT	TTC	TTT	CTA	AGA	GAA	AAC	GAG	TGT	GTC	TCC	TGT	AGT	773
Cys	His	Ala	Gly	Phe	Phe	Leu	Arg	Glu	Asn	Glu	Cys	Val	Ser	Cys	Ser	
435					440					445					450	
AAC	TGT	AAG	AAA	AGC	CTG	GAG	TGC	ACG	AAG	TTG	TGC	CTA	CCC	CAG	ATT	821
Asn	Cys	Lys	Lys	Ser	Leu	Glu	Cys	Thr	Lys	Leu	Cys	Leu	Pro	Gln	Ile	
				455					460					465		
GAG	AAT	GTT	AAG	GGC	ACT	GAG	GAC	TCA	GGC	ACC	ACA	GCT	GGT	GCT	GGT	869
Glu	Asn	Val	Lys	Gly	Thr	Glu	Asp	Ser	Gly	Thr	Thr	Ala	Gly	Ala	Gly	
			470					475					480			
CCA	CGG	TGC	CGC	CCC	ATC	AAT	GCC	ACC	CTG	GCT	GTG	GAG	AAG	GAG	GGC	917
Pro	Arg	Cys	Arg	Pro	Ile	Asn	Ala	Thr	Leu	Ala	Val	Glu	Lys	Glu	Gly	
		485					490					495				
TGC	CCC	GTG	TGC	ATC	ACC	GTC	AAC	ACC	ACC	ATC	TGT	GCC	GGC	TAC	TGC	965
Cys	Pro	Val	Cys	Ile	Thr	Val	Asn	Thr	Thr	Ile	Cys	Ala	Gly	Tyr	Cys	
		500				505					510					
CCC	ACC	ATG	ACC	CGC	GTG	CTG	CAG	GGG	GTC	CTG	CCG	GCC	CTG	CCT	CAG	1013
Pro	Thr	Met	Thr	Arg	Val	Leu	Gln	Gly	Val	Leu	Pro	Ala	Leu	Pro	Gln	
515					520					525					530	
GTG	GTG	TGC	AAC	TAC	CGC	GAT	GTG	CGC	TTC	GAG	TCC	ATC	CGG	CTC	CCT	1061
Val	Val	Cys	Asn	Tyr	Arg	Asp	Val	Arg	Phe	Glu	Ser	Ile	Arg	Leu	Pro	
				535					540					545		
GGC	TGC	CCG	CGC	GGC	GTG	AAC	CCC	GTG	GTC	TCC	TAC	GCC	GTG	GCT	CTC	1109
Gly	Cys	Pro	Arg	Gly	Val	Asn	Pro	Val	Val	Ser	Tyr	Ala	Val	Ala	Leu	
			550					555					560			
AGC	TGT	CAA	TGT	GCA	CTC	TGC	CGC	CGC	AGC	ACC	ACT	GAC	TGC	GGG	GGT	1157
Ser	Cys	Gln	Cys	Ala	Leu	Cys	Arg	Arg	Ser	Thr	Thr	Asp	Cys	Gly	Gly	
		565					570					575				
CCC	AAG	GAC	CAC	CCC	TTG	ACC	TGT	GAT	GAC	CCC	CGC	TTC	CAG	GAC	TCC	1205
Pro	Lys	Asp	His	Pro	Leu	Thr	Cys	Asp	Asp	Pro	Arg	Phe	Gln	Asp	Ser	
		580				585					590					
TCT	TCC	TCA	AAG	GCC	CCT	CCC	CCC	AGC	CTT	CCA	AGC	CCA	TCC	CGA	CTC	1253
Ser	Ser	Ser	Lys	Ala	Pro	Pro	Pro	Ser	Leu	Pro	Ser	Pro	Ser	Arg	Leu	
595					600					605					610	
CCG	GGG	CCC	TCG	GAC	ACC	CCG	ATC	CTC	CCA	CAA	T	AAGGATCCCT	CGAG			1301
Pro	Gly	Pro	Ser	Asp	Thr	Pro	Ile	Leu	Pro	Gln						
				615						620						

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 336 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

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Ser Arg Thr Ser Leu Leu Leu Ala Phe Gly Leu Leu Cys Leu Pro Trp
 1           5           10           15
Leu Gln Glu Gly Ser Ala Asp Ser Val Cys Pro Gln Gly Lys Tyr Ile
          20           25           30
His Pro Gln Asn Asn Ser Ile Cys Cys Thr Lys Cys His Lys Gly Thr
          35           40           45
Tyr Leu Tyr Asn Asp Cys Pro Gly Pro Gly Gln Asp Thr Asp Cys Arg
 50           55           60
Glu Cys Glu Ser Gly Ser Phe Thr Ala Ser Glu Asn His Leu Arg His
 65           70           75           80
Cys Leu Ser Cys Ser Lys Cys Arg Lys Glu Met Gly Gln Val Glu Ile
          85           90           95
Ser Ser Cys Thr Val Asp Arg Asp Thr Val Cys Gly Cys Arg Lys Asn
          100          105          110
Gln Tyr Arg His Tyr Trp Ser Glu Asn Leu Phe Gln Cys Phe Asn Cys
          115          120          125
Ser Leu Cys Leu Asn Gly Thr Val His Leu Ser Cys Gln Glu Lys Gln
          130          135          140
Asn Thr Val Cys Thr Cys His Ala Gly Phe Phe Leu Arg Glu Asn Glu
          145          150          155          160
Cys Val Ser Cys Ser Asn Cys Lys Lys Ser Leu Glu Cys Thr Lys Leu
          165          170          175
Cys Leu Pro Gln Ile Glu Asn Val Lys Gly Thr Glu Asp Ser Gly Thr
          180          185          190
Thr Ala Gly Ala Gly Pro Arg Cys Arg Pro Ile Asn Ala Thr Leu Ala
          195          200          205
Val Glu Lys Glu Gly Cys Pro Val Cys Ile Thr Val Asn Thr Thr Ile
          210          215          220
Cys Ala Gly Tyr Cys Pro Thr Met Thr Arg Val Leu Gln Gly Val Leu
          225          230          235          240
Pro Ala Leu Pro Gln Val Val Cys Asn Tyr Arg Asp Val Arg Phe Glu
          245          250          255
Ser Ile Arg Leu Pro Gly Cys Pro Arg Gly Val Asn Pro Val Val Ser
          260          265          270
Tyr Ala Val Ala Leu Ser Cys Gln Cys Ala Leu Cys Arg Arg Ser Thr
          275          280          285

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Thr	Asp	Cys	Gly	Gly	Pro	Lys	Asp	His	Pro	Leu	Thr	Cys	Asp	Asp	Pro
290						295					300				
Arg	Phe	Gln	Asp	Ser	Ser	Ser	Ser	Lys	Ala	Pro	Pro	Pro	Ser	Leu	Pro
305					310					315					320
Ser	Pro	Ser	Arg	Leu	Pro	Gly	Pro	Ser	Asp	Thr	Pro	Ile	Leu	Pro	Gln
				325					330					335	

(2) INFORMATION FOR SEQ ID NO:9:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 6 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Ala Gly Ala Ala Pro Gly
 1 5

(2) INFORMATION FOR SEQ ID NO:10:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 4 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

Ala Gly Ala Gly
 1

(2) INFORMATION FOR SEQ ID NO:11:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 30 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

TTTCTCGAG ATGGCTACAG GTAAGCGCCC

30

(2) INFORMATION FOR SEQ ID NO:12:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 39 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

ACCTGGGGCA GCACCGGCAC AGGAGACACA CTCGTTTTC

39

(2) INFORMATION FOR SEQ ID NO:13:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 42 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

TGTGCCGGTG CTGCCCCAGG TTGCCCAGAA TGCACGCTAC AG

42

(2) INFORMATION FOR SEQ ID NO:14:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 36 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

TTTTGGATCC TTAAGATTG TGATAATAAC AAGTAC

36

(2) INFORMATION FOR SEQ ID NO:15:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 39 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

CCGTGGACCA GCACCAGCAC AGGAGACACA CTCGTTTTC

39

(2) INFORMATION FOR SEQ ID NO:16:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 36 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

TGTGCTGGTG CTGGTCCACG GTGCCGCCCC ATCAAT

36

(2) INFORMATION FOR SEQ ID NO:17:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 32 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

TTTTGGATCC TTATTGTGGG AGGATCGGGG TG

32

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 27 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

TTTTAGATCT CTTCTTGCAC AGTGGAC

27

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

TGTGGTGCCT GAGTCCTCAG T

21

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 41 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

ACTGAGGACT CAGGCACCAC AGCCGGTGCT GCCCCAGGTT G

41

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 29 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

TTTTTCTAGA GAAGCAGCAG CAGCCCATG

29

(2) INFORMATION FOR SEQ ID NO:22:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 75 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

TTTTCCACAG CCAGGGTGGC ATTGATGGGG CGGCACCGTG GACCAGCACC AGCTGTGGTG	60
CCTGAGTCCT CAGTG	75

**CLAIMS**

1. A hybrid protein comprising two coexpressed amino acid sequences forming a dimer, each comprising:
 - a) at least one amino acid sequence selected from the group consisting of a homomeric receptor, a chain of a heteromeric receptor, a ligand, and fragments thereof which retain the ligand-receptor binding capability; and
 - b) a subunit of a heterodimeric proteinaceous hormone, or fragments thereof which retain the ability of the subunit to form a heterodimer with other subunits thereof;wherein sequences (a) and (b) are bonded directly or through a peptide linker, and in which the sequence (b) in each of said two coexpressed sequences are capable of aggregating to form a dimer complex.
2. A hybrid protein in accordance with claim 1, wherein said sequence (a) is selected from the group consisting of TBP1, TBP2 or fragments thereof still containing the ligand binding domain; the extracellular domain of the IFN α / β receptor or the IFN γ receptor; a gonadotropin receptor or extracellular fragments thereof; antibody light chains or fragments thereof, optionally associated with the respective heavy chains; antibody heavy chains or fragments thereof; antibody Fab domains; and IL-6, IFN- β , TPO or fragments thereof.
3. A hybrid protein in accordance with claim 1, wherein said sequence (b) is selected from the group consisting of subunits of hCG, FSH, LH, TSH or inhibin, and fragments thereof.
4. A hybrid protein in accordance with claim 1, wherein sequence (a) is linked to the amino terminus of sequence (b).



5. A hybrid protein in accordance with claim 1, wherein sequence (a) is linked to the carboxy terminus of sequence (b).

6. A hybrid protein in accordance with claim 1, wherein said two coexpressed amino acid sequences each include the sequence for TBP1 or the fragment thereof corresponding to amino acid residues 20-161 or 20-190 of TBP1, as sequence (a) and the respective α and β subunits of hCG or fragments thereof, as sequence (b).

7. A hybrid protein in accordance with claim 1, wherein said two coexpressed amino acid sequences each include the extracellular domain of a gonadotropin receptor as sequence (a) and the respective α and β subunits of a gonadotropin as sequence (b).

8. A hybrid protein in accordance with claim 7, wherein said sequence (a) is the FSH receptor extracellular domain and sequence (b) is a subunit of FSH.

9. A hybrid protein in accordance with claim 7, wherein said sequences (a) and (b) are linked with a peptide linker.

10. A hybrid protein in accordance with claim 9, wherein said peptide linker has an enzyme cleavage site.

11. A hybrid protein in accordance with claim 10, wherein said enzyme cleavage site is a thrombin cleavage site.

12. A hybrid protein in accordance with claim 10, wherein said enzyme cleavage site is recognized and cleaved by an enzyme which is found in the ovary.

13. A hybrid protein in accordance with claim 9, wherein said peptide linker serves as a flexible hinge.



14. A hybrid protein in accordance with claim 1, wherein one or more covalent bonds between the two subunits (b) are added.

15. A DNA molecule encoding a hybrid protein in accordance with claim 1.

16. An expression vector containing a DNA molecule in accordance with claim 15.

17. A host cell containing an expression vector in accordance with claim 16 and capable of expressing said hybrid protein.

18. A method for producing hybrid protein comprising culturing a host cell in accordance with claim 17 and recovering the hybrid protein expressed thereby.

19. A pharmaceutical composition comprising a hybrid protein in accordance with claim 1 and a pharmaceutically acceptable carrier and/or excipient.

20. A method for inducing follicular maturation, comprising administering a pharmaceutical composition comprising the hybrid protein of claim 8 to a subject in need thereof.

This Sheet Has Been Replaced

