

ANGIOPOIETIN-RELATED PROTEIN AND NUCLEIC ACIDS

Throughout this application various publications are referenced. The disclosures of these publications in their entireties are hereby
5 incorporated by reference into this application.

INTRODUCTION

10 The present invention relates generally to the field of genetic engineering and more particularly to genes for receptor tyrosine kinases and their cognate ligands, their insertion into recombinant DNA vectors, and the production of the encoded proteins in recipient strains of microorganisms and recipient eukaryotic cells. More specifically, the present invention is directed to a novel factor which is related to
15 the angiopoietins and may be a regulator of angiogenesis. The novel factor is designated AR-2, (Angiopoietin Related-2) and the present invention is directed as well as to methods of making and using the novel factor. The invention further provides nucleic acid sequences encoding AR-2, and methods for the production of the nucleic acids and
20 the gene products. The novel AR-2 is believed to be a regulator of angiogenesis and thus the factor, as well as nucleic acids encoding it, may be useful in the diagnosis and treatment of certain diseases such as neoplastic diseases involving tumor angiogenesis, wound healing, thromboembolic diseases, atherosclerosis and inflammatory diseases.

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BACKGROUND OF THE INVENTION

The cellular behavior responsible for the development, maintenance,

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and repair of differentiated cells and tissues is regulated, in large part, by intercellular signals conveyed via growth factors and similar ligands and their receptors. The receptors are located on the cell surface of responding cells and they bind peptides or polypeptides known as growth factors as well as other hormone-like ligands. The results of this interaction are rapid biochemical changes in the responding cells, as well as a rapid and a long-term readjustment of cellular gene expression. Several receptors associated with various cell surfaces may bind specific growth factors.

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The phosphorylation of tyrosine residues in proteins by tyrosine kinases is one of the key modes by which signals are transduced across the plasma membrane. Several currently known protein tyrosine kinase genes encode transmembrane receptors for polypeptide growth factors and hormones such as epidermal growth factor (EGF), insulin, insulin-like growth factor-I (IGF-I), platelet derived growth factors (PDGF-A and -B), and fibroblast growth factors (FGFs). (Heldin et al., Cell Regulation, 1: 555-566 (1990); Ullrich, et al., Cell, 61: 243-54 (1990)). In each instance, these growth factors exert their action by binding to the extracellular portion of their cognate receptors, which leads to activation of the intrinsic tyrosine kinase present on the cytoplasmic portion of the receptor. Growth factor receptors of endothelial cells are of particular interest due to the possible involvement of growth factors in several important physiological and pathological processes, such as vasculogenesis, angiogenesis, atherosclerosis, and inflammatory diseases. (Folkman, et al. Science, 235: 442-447 (1987)). Also, the receptors of several hematopoietic growth factors are tyrosine kinases; these include c-fms, which is the colony stimulating

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factor 1 receptor, Sherr, et al., *Cell*, 41: 665-676 (1985), and c-kit, a primitive hematopoietic growth factor receptor reported in Huang, et al., *Cell*, 63: 225-33 (1990).

- 5 The receptor tyrosine kinases have been divided into evolutionary subfamilies based on the characteristic structure of their ectodomains. (Ullrich, et al. *Cell*, 61: 243-54 (1990)). Such subfamilies include, EGF receptor-like kinase (subclass I) and insulin receptor-like kinase (subclass II), each of which contains repeated homologous cysteine-rich sequences in their extracellular domains. A single cysteine-rich region is also found in the extracellular domains of the eph-like kinases. Hirai, et al., *Science*, 238: 1717-1720 (1987); Lindberg, et al. *Mol. Cell. Biol.*, 10: 6316-24 (1990); Lhotak, et al., *Mol. Cell. Biol.* 11: 2496-2502 (1991). PDGF receptors as well as c-fms and c-kit receptor
- 10 tyrosine kinases may be grouped into subclass III; while the FGF receptors form subclass IV. Typical for the members of both of these subclasses are extracellular folding units stabilized by intrachain disulfide bonds. These so-called immunoglobulin (Ig)-like folds are found in the proteins of the immunoglobulin superfamily which contains
- 15 a wide variety of other cell surface receptors having either cell-bound or soluble ligands. Williams, et al., *Ann. Rev. Immunol.*, 6: 381-405 (1988).
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- Receptor tyrosine kinases differ in their specificity and affinity. In
- 25 general, receptor tyrosine kinases are glycoproteins which consist of (1) an extracellular domain capable of binding the specific growth factor(s); (2) a transmembrane domain which usually is an alpha-helical portion of the protein; (3) a juxtamembrane domain where the

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receptor may be regulated by, e.g., protein phosphorylation; (4) a tyrosine kinase domain which is the enzymatic component of the receptor; and (5) a carboxyterminal tail which in many receptors is involved in recognition and binding of the substrates for the tyrosine kinase.

Processes such as alternative exon splicing and alternative choice of gene promoter or polyadenylation sites have been reported to be capable of producing several distinct polypeptides from the same gene. These polypeptides may or may not contain the various domains listed above. As a consequence, some extracellular domains may be expressed as separate, secreted proteins and some forms of the receptors may lack the tyrosine kinase domain and contain only the extracellular domain inserted in the plasma membrane via the transmembrane domain plus a short carboxyl terminal tail.

A gene encoding an endothelial cell transmembrane tyrosine kinase, originally identified by RT-PCR as an unknown tyrosine kinase-homologous cDNA fragment from human leukemia cells, was described by Partanen, et al., Proc. Natl. Acad. Sci. USA, 87: 8913-8917 (1990). This gene and its encoded protein are called "TIE" which is an abbreviation for "tyrosine kinase with Ig and EGF homology domains." Partanen, et al. Mol. Cell. Biol. 12: 1698-1707 (1992).

It has been reported that tie mRNA is present in all human fetal and mouse embryonic tissues. Upon inspection, tie message has been localized to the cardiac and vascular endothelial cells. Specifically, tie mRNA has been localized to the endothelia of blood vessels and

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endocardium of 9.5 to 18.5 day old mouse embryos. Enhanced tie expression was shown during neovascularization associated with developing ovarian follicles and granulation tissue in skin wounds.

Korhonen, et al. Blood 80: 2548-2555 (1992). Thus the TIEs have been suggested to play a role in angiogenesis, which is important for developing treatments for solid tumors and several other angiogenesis-dependent diseases such as diabetic retinopathy, psoriasis, atherosclerosis and arthritis.

Two structurally related rat TIE receptor proteins have been reported to be encoded by distinct genes with related profiles of expression. One gene, termed tie-1, is the rat homolog of human tie. Maisonpierre, et al., Oncogene 8: 1631-1637 (1993). The other gene, tie-2, may be the rat homolog of the murine tek gene which, like tie, has been reported to be expressed in the mouse exclusively in endothelial cells and their presumptive progenitors. Dumont, et al. Oncogene 8: 1293-1301 (1993). The human homolog of tie-2 is described in Ziegler, U.S. Patent No. 5,447,860 which issued on September 5, 1995 (wherein it is referred to as "ork"), which is incorporated in its entirety herein.

Both genes were found to be widely expressed in endothelial cells of embryonic and postnatal tissues. Significant levels of tie-2 transcripts were also present in other embryonic cell populations, including lens epithelium, heart epicardium and regions of mesenchyme. Maisonpierre, et al., Oncogene 8: 1631-1637 (1993).

The predominant expression of the TIE receptor in vascular endothelia suggests that TIE plays a role in the development and maintenance of

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- the vascular system. This could include roles in endothelial cell determination, proliferation, differentiation and cell migration and patterning into vascular elements. Analyses of mouse embryos deficient in TIE-2 illustrate its importance in angiogenesis, particularly for vascular network formation in endothelial cells. Sato, T.N., et al., Nature 376:70-74 (1995). In the mature vascular system, the TIEs could function in endothelial cell survival, maintenance and response to pathogenic influences.
- 10 The TIE receptors are also expressed in primitive hematopoietic stem cells, B cells and a subset of megakaryocytic cells, thus suggesting the role of ligands which bind these receptors in early hematopoiesis, in the differentiation and/or proliferation of B cells, and in the megakaryocytic differentiation pathway. Iwama, et al. Biochem. Biophys. Research Communications 195:301-309 (1993); Hashiyama, et al. Blood 87:93-101 (1996), Batard, et al. Blood 87:2212-2220 (1996).
- 20 Applicants previously identified an angiogenic factor, which was originally called TIE-2 ligand-1 (TL1) but is also referred to as angiopoietin-1 (Ang1), that signals through the TIE-2 receptor and is essential for normal vascular development in the mouse. By homology screening applicants have also identified an Ang1 relative, termed TIE-2 ligand-2 (TL2) or angiopoietin-2 (Ang2), that is a naturally occurring antagonist for Ang1 and the TIE2 receptor. For a description of the cloning and sequencing of TL1 (Ang1) and TL2 (Ang2) as well as for methods of making and uses thereof, reference is hereby made to PCT International Publication No. WO 96/11269 published 18 April 1996 and PCT International Publication No. WO 96/31598 published 10 October

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1996 both in the name of Regeneron Pharmaceuticals, Inc.; and S. Davis, et al., Cell 87: 1161-1169 (1996) each of which is hereby incorporated by reference. The absence of Ang1 causes severe vascular abnormalities in the developing mouse embryo. C. Suri, et al., Cell 87: 1171-1180 (1996). Ang1 and Ang2 provide for naturally occurring positive and negative regulators of angiogenesis. Positive or negative regulation of TIE2 is likely to result in different outcomes depending on the combination of simultaneously acting angiogenic signals.

Applicants have previously identified a family of several related angiogenic factors. These have been designated TIE-2 ligand-1 (TL1) also referred to as angiopoietin-1 (Ang1); TIE-2 ligand-2 (TL2) or angiopoietin-2 (Ang2); Tie ligand-3 (TL3) and Tie ligand-4 (TL4). For descriptions of the structure and functional properties of these four related factors, reference is hereby made to the following publications, each of which is hereby incorporated by reference: U.S. Patent No. 5,643,755, issued 7/1/97 to Davis, et al.; U.S. Patent No. 5,521,073, issued 5/28/96 to Davis, et al.; U.S. Patent No. 5,650,490, issued 7/22/97 to Davis, et al.; U.S. Serial No. 08/348,492, filed 12/2/94, now allowed, date of allowance 8/29/97; U.S. Serial No. 08/418,595, filed 4/6/95, now allowed, date of allowance 11/26/96; U.S. Serial No. 08/665,926, filed 6/19/96, now allowed, date of allowance 12/9/97; PCT International Application No. PCT/US95/12935, filed Oct. 6, 1995, published on April 18, 1996, with Publication No. WO 96/11269; and PCT International Application No. PCT/US96/04806, filed April 5, 1996, published on October 10, 1996, with Publication No. WO96/31598, both PCT applications in the name of Regeneron Pharmaceuticals, Inc.

SUMMARY OF THE INVENTION

The present invention provides for a composition comprising AR-2 substantially free of other proteins. The invention also provides for an isolated nucleic acid molecule encoding AR-2. The isolated nucleic acid may be DNA, cDNA or RNA. The invention also provides for a vector comprising an isolated nucleic acid molecule encoding AR-2. The invention further provides for a host-vector system for the production in a suitable host cell of a polypeptide having the biological activity of AR-2. The suitable host cell may be bacterial, yeast, insect or mammalian. The invention also provides for a method of producing a polypeptide having the biological activity of AR-2 which comprises growing cells of the host-vector system under conditions permitting production of the polypeptide and recovering the polypeptide so produced.

The invention herein described of an isolated nucleic acid molecule encoding AR-2 further provides for the development of the ligand, a fragment or derivative thereof, or another molecule which is a receptor agonist or antagonist, as a therapeutic for the treatment of patients suffering from disorders involving cells, tissues or organs which express the AR-2 receptor. The present invention also provides for an antibody which specifically binds such a therapeutic molecule. The antibody may be monoclonal or polyclonal. The invention also provides for a method of using such a monoclonal or polyclonal antibody to measure the amount of the therapeutic molecule in a sample taken from

a patient for purposes of monitoring the course of therapy.

The present invention also provides for an antibody which specifically binds AR-2. The antibody may be monoclonal or polyclonal. Thus the invention further provides for compositions comprising an antibody which specifically binds AR-2 and a vehicle.

The invention further provides for compositions comprising AR-2 in a vehicle. The invention also provides for a method of regulating angiogenesis in a patient by administering an effective amount of a composition comprising AR-2 in a vehicle.

Alternatively, the invention provides that AR-2 may be conjugated to a cytotoxic agent and a composition prepared therefrom.

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Biologically active AR-2 may be used to promote the growth, survival, migration, and/or differentiation and/or stabilization or destabilization of cells expressing its receptor. Biologically active AR-2 may be used for the in vitro maintenance of AR-2 receptor expressing cells in culture. Alternatively, AR-2 may be used to support cells which are engineered to express its receptor. Further, the AR-2 and its receptor may be used in assay systems to identify agonists or antagonists of the receptor.

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BRIEF DESCRIPTION OF THE FIGURES

FIGURE 1A-1B - Nucleotide and deduced amino acid (single letter code) sequences of TIE ligand-3. The coding sequence starts at position 47.

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The fibrinogen-like domain starts at position 929.

FIGURE 2 - Comparison of Amino Acid Sequences of TIE Ligand Family Members. mAng3 = mTL3 = mouse TIE ligand-3; hAng4 = hTL4 = human

- 5 TIE ligand-4; hAng1 = hTL1 = human TIE-2 ligand1; mAng1 = mTL1 = mouse TIE-2 ligand 1; mAng2 = mTL2 = mouse TIE-2 ligand 2; hAng2 = hTL2 = human TIE-2 ligand 2. The underlined regions indicate conserved regions of homology among the family members.

- 10 FIGURE 3A-3C - Nucleotide and deduced amino acid (single letter code) sequences of TIE ligand-4. Arrow indicates nucleotide position 569.

FIGURE 4A-4B - Nucleotide and deduced amino acid (triple letter code) sequences of Human AR-1.

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FIGURE 5A-5B - Nucleotide and deduced amino acid (single letter code) sequences of mouse uterus cDNA library derived AR-2.

- 20 FIGURE 6 - Nucleotide and deduced amino acid (single letter code) sequences of AR-2 fibrinogen-like domain.

DETAILED DESCRIPTION OF THE INVENTION

- As described in greater detail below, applicants have isolated and
25 identified a novel factor related to the TIE-2 ligands that bind the TIE-2 receptor. The novel factor is referred to herein as AR-2. The TIE ligand family members are referred to herein as TIE-2 ligand 1 (TL1) also known as angiopoietin-1 (Ang1); TIE-2 ligand 2 (TL2) also known

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as angiopoietin-2 (Ang2); TIE ligand-3; and TIE ligand-4. The novel factor is expected to have a function and utility similar to that of the known TIE-2 ligands.

- 5 The present invention comprises novel nucleic acids and their deduced amino acid sequences, as well as functionally equivalent variants thereof comprising naturally occurring allelic variations, as well as proteins or peptides comprising substitutions, deletions or insertional mutants of the described sequences, which retain biological activity.
- 10 Such variants include those in which amino acid residues are substituted for residues within the sequence resulting in a silent change. For example, one or more amino acid residues within the sequence can be substituted by another amino acid(s) of a similar polarity which acts as a functional equivalent, resulting in a silent
- 15 alteration. Substitutes for an amino acid within the sequence may be selected from other members of the class to which the amino acid belongs. For example, the class of nonpolar (hydrophobic) amino acids include alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan and methionine. The polar neutral amino acids include
- 20 glycine, serine, threonine, cysteine, tyrosine, asparagine, and glutamine. The positively charged (basic) amino acids include arginine, lysine and histidine. The negatively charged (acidic) amino acids include aspartic acid and glutamic acid.
- 25 Also included within the scope of the invention are proteins or fragments or derivatives thereof which exhibit the same or similar biological activity as the AR-2 described herein, and derivatives which are differentially modified during or after translation, e.g., by

glycosylation, proteolytic cleavage, linkage to an antibody molecule or other cellular ligand. Functionally equivalent molecules also include molecules that contain modifications, including N-terminal modifications, which result from expression in a particular

5 recombinant host, such as, for example, N-terminal methylation which occurs in certain bacterial (e.g. *E. coli*) expression systems. Functional equivalents also include mutants in which amino acid substitutions are made for cysteine molecules to improve stability of the molecules and to prevent unwanted crosslinking.

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For example, the angiopoietins can be structurally divided into three domains: an N-terminal region lacking in homology to any known structures; an alpha-helical rich coil-coil segment similar to motifs found in many proteins that seem to promote multimerization; and a

15 "fibrinogen-like domain" thus dubbed because it is distantly related to a domain first found in fibrinogen but now noted to be in many other proteins (Davis, S. et al., (1996) Cell 87: 1161-1169). The fibrinogen-like domain represents the most conserved region of the angiopoietins, and recent studies indicate that it comprises the receptor-binding

20 portion of an angiopoietin. In addition, all the information that determines whether an angiopoietin is an agonist or an antagonist appears to reside within the fibrinogen-like domain. For example, when chimeric molecules are made in which the fibrinogen-like domains of angiopoietin-1 and angiopoietin-2 are swapped, agonistic or

25 antagonistic abilities track with the fibrinogen-like domains. The N-terminal and coil-coil regions appear to serve mainly to multimerize the fibrinogen-like domains, which apparently must be clustered to be active. In fact, the N-terminal and coil-coil regions can be substituted

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for by alternative motifs that allow clustering. Thus, the activities of angiopoietin-1 and angiopoietin-2 can be precisely mimicked by surrogates in which the fibrinogen-like domains (FD) of these factors are fused to the constant region of an antibody, resulting in FD-Fc fusions, which can then be clustered using secondary antibodies directed against the Fc. For a general description of the production and use of FD-Fc fusions, see International Publication Number WO 97/48804 published 24 December 1997. Using these techniques, one of skill in the art would be able to similarly make FD-Fc fusions using the fibrinogen-like domain of AR-2. One practical advantage of such surrogates is that native angiopoietins can be difficult to produce recombinantly, while the surrogates can be more easily produced.

Thus the present invention encompasses the nucleotide and deduced amino acid sequence of the fibrinogen-like domain of AR-2 as set forth in Figure 6. As described above, the fibrinogen-like domain can be used to produce surrogates, such as FD-Fc fusions, for the full length sequence. The surrogates have the advantage of easier production while retaining the biological activity of the full length molecule. In addition, to the extent that the amino acid sequence set forth in Figure 6 is identical to the human sequence, it should have the same biological activity as the human sequence and the same immunogenic profile when a composition comprising the AR-2 fibrinogen-like domain, or a derivative thereof, is administered to a human subject.

The present invention also encompasses the nucleotide sequence that encodes the protein described herein as AR-2, as well as host cells, including yeast, bacteria, viruses, and mammalian cells, which are

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genetically engineered to produce the protein, by e.g. transfection, transduction, infection, electroporation, or microinjection of nucleic acid encoding the AR-2 described herein in a suitable expression vector. The present invention also encompasses introduction of the nucleic acid
5 encoding AR-2 through gene therapy techniques such as is described, for example, in Finkel and Epstein FASEB J. 9:843-851 (1995); Guzman, et al. PNAS (USA) 91:10732-10736 (1994).

One skilled in the art will also recognize that the present invention
10 encompasses DNA and RNA sequences that hybridize to a AR-2 encoding sequence, under stringent conditions. The invention also provides for nucleic acid hybridization probes and replication/amplification primers having a AR-2 DNA specific sequence and sufficient to effect specific hybridization with AR-2. Demonstrating specific hybridization
15 generally requires stringent conditions, for example, hybridizing in a buffer comprising 30% formamide in 5 x SSPE (0.18 M NaCl, 0.01 M NaPO₄, pH7.7, 0.001 M EDTA) buffer at a temperature of 42°C and remaining bound when subject to washing at 42°C with 0.2 x SSPE; preferably hybridizing in a buffer comprising 50% formamide in 5 x
20 SSPE buffer at a temperature of 42°C and remaining bound when subject to washing at 42°C with 0.2x SSPE buffer at 42°C. AR-2 homologs can also be distinguished from other polypeptides using alignment algorithms, such as BLASTX (Altschul, et al. (1990) Basic Local Alignment Search Tool, J. Mol. Biol. 215: 403-410).

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Thus, a nucleic acid molecule contemplated by the invention includes one having a sequence deduced from an amino acid sequence of a AR-2 prepared as described herein, as well as a molecule having a sequence

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of nucleic acids that hybridizes to such a nucleic acid sequence, and also a nucleic acid sequence which is degenerate of the above sequences as a result of the genetic code, but which encodes a AR-2 and which has an amino acid sequence and other primary, secondary and tertiary characteristics that are sufficiently duplicative of the AR-2 described herein so as to confer on the molecule the same biological activity as the AR-2 described herein.

Accordingly, the present invention encompasses an isolated and purified nucleic acid molecule comprising a nucleotide sequence encoding a AR-2, wherein the nucleotide sequence is selected from the group consisting of:

- (a) the nucleotide sequence comprising the coding region of AR-2 as set forth in Figure 5A-5B;
- (b) a nucleotide sequence that hybridizes under stringent conditions to the nucleotide sequence of (a); and
- (c) a nucleotide sequence which differs from the sequence of (a) or (b) due to the degeneracy of the genetic code.

The present invention further encompasses an isolated nucleic acid molecule comprising a nucleotide sequence encoding the fibrinogen-like domain of AR-2, wherein the nucleotide sequence is selected from the group consisting of:

- (a) the nucleotide sequence comprising the coding region of the fibrinogen-like domain of AR-2 as set forth in Figure 6;
- (b) a nucleotide sequence that hybridizes under stringent conditions to the nucleotide sequence of (a); and
- (c) a nucleotide sequence which differs from the sequence of

(a) or (b) due to the degeneracy of the genetic code.

The present invention further provides for an isolated AR-2 encoded by an isolated nucleic acid molecule of the invention. The invention also provides for a vector which comprises an isolated nucleic acid molecule comprising a nucleic acid sequence encoding AR-2 or the fibrinogen-like domain of AR-2.

Any of the methods known to one skilled in the art for the insertion of DNA fragments into a vector may be used to construct expression vectors encoding AR-2 using appropriate transcriptional/translational control signals and the protein coding sequences. These methods may include in vitro recombinant DNA and synthetic techniques and in vivo recombinations (genetic recombination). Expression of a nucleic acid sequence encoding AR-2 or peptide fragments thereof may be regulated by a second nucleic acid sequence which is operably linked to the AR-2 encoding sequence such that the AR-2 protein or peptide is expressed in a host transformed with the recombinant DNA molecule. For example, expression of AR-2 described herein may be controlled by any promoter/enhancer element known in the art. Promoters which may be used to control expression of the ligand include, but are not limited to the long terminal repeat as described in Squinto et al., (Cell 65:1-20 (1991)); the SV40 early promoter region (Bernoist and Chambon, Nature 290:304-310), the CMV promoter, the M-MuLV 5' terminal repeat, the promoter contained in the 3' long terminal repeat of Rous sarcoma virus (Yamamoto, et al., Cell 22:787-797 (1980)), the herpes thymidine kinase promoter (Wagner et al., Proc. Natl. Acad. Sci. U.S.A. 78:144-1445 (1981)), the adenovirus promoter, the regulatory sequences of the

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metallothionein gene (Brinster et al., Nature 296:39-42 (1982)); prokaryotic expression vectors such as the β -lactamase promoter (Villa-Komaroff, et al., Proc. Natl. Acad. Sci. U.S.A. 75:3727-3731 (1978)), or the tac promoter (DeBoer, et al., Proc. Natl. Acad. Sci. U.S.A. 80:21-25 (1983)), see also "Useful proteins from recombinant bacteria" in Scientific American, 242:74-94 (1980); promoter elements from yeast or other fungi such as the Gal 4 promoter, the ADH (alcohol dehydrogenase) promoter, PGK (phosphoglycerol kinase) promoter, alkaline phosphatase promoter, and the following animal

transcriptional control regions, which exhibit tissue specificity and have been utilized in transgenic animals; elastase I gene control region which is active in pancreatic acinar cells (Swift et al., Cell 38:639-646 (1984); Ornitz et al., Cold Spring Harbor Symp. Quant. Biol. 50:399-409 (1986); MacDonald, Hepatology 7:425-515 (1987); insulin gene control region which is active in pancreatic beta cells [Hanahan, Nature 315:115-122 (1985)]; immunoglobulin gene control region which is active in lymphoid cells (Grosschedl et al., 1984, Cell 38:647-658; Adames et al., 1985, Nature 318:533-538; Alexander et al., 1987, Mol. Cell. Biol. 7:1436-1444), mouse mammary tumor virus control region which is active in testicular, breast, lymphoid and mast cells (Leder et al., 1986, Cell 45:485-495), albumin gene control region which is active in liver (Pinkert et al., 1987, Genes and Devel. 1:268-276), alpha-fetoprotein gene control region which is active in liver (Krumlauf et al., 1985, Mol. Cell. Biol. 5:1639-1648; Hammer et al., 1987, Science 235:53-58); alpha 1-antitrypsin gene control region which is active in the liver (Kelsey et al., 1987, Genes and Devel. 1:161-171), beta-globin gene control region which is active in myeloid cells (Mogam et al., 1985, Nature 315:338-340; Kollias et al., 1986, Cell 46:89-94); myelin

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basic protein gene control region which is active in oligodendrocytes in the brain (Readhead et al., 1987, Cell 48:703-712); myosin light chain-2 gene control region which is active in skeletal muscle (Shani, 1985, Nature 314:283-286), and gonadotropic releasing hormone gene control
5 region which is active in the hypothalamus (Mason et al., 1986, Science 234:1372-1378). The invention further encompasses the production of antisense compounds which are capable of specifically hybridizing with a sequence of RNA encoding TIE ligand-3 or TIE ligand-4 to modulate its expression. Ecker, U.S. Patent No. 5,166,195, issued November 24, 1992.

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Thus, according to the invention, expression vectors capable of being replicated in a bacterial or eukaryotic host comprising a nucleic acid encoding AR-2 as described herein, are used to transfect a host and thereby direct expression of such nucleic acid to produce AR-2, which
15 may then be recovered in a biologically active form. As used herein, a biologically active form includes a form capable of causing a biological response such as a differentiated function or influencing the phenotype of a cell expressing the receptor for AR-2.

20 Expression vectors containing the gene inserts can be identified by four general approaches: (a) DNA-DNA hybridization, (b) presence or absence of "marker" gene functions, (c) expression of inserted sequences and (d) PCR detection. In the first approach, the presence of a foreign gene inserted in an expression vector can be detected by DNA-DNA
25 hybridization using probes comprising sequences that are homologous to an inserted AR-2 encoding gene. In the second approach, the recombinant vector/host system can be identified and selected based upon the presence or absence of certain "marker" gene functions (e.g.,

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thymidine kinase activity, resistance to antibiotics, transformation phenotype, occlusion body formation in baculovirus, etc.) caused by the insertion of foreign genes in the vector. For example, if a nucleic acid encoding a AR-2 is inserted within the marker gene sequence of the vector, recombinants containing the insert can be identified by the absence of the marker gene function. In the third approach, recombinant expression vectors can be identified by assaying the foreign gene product expressed by the recombinant. Such assays can be based, for example, on the physical or functional properties of a AR-2 gene product, for example, by binding of the AR-2 to its receptor or a portion thereof which may be tagged with, for example, a detectable antibody or portion thereof or by binding to antibodies produced against the AR-2 protein or a portion thereof. Cells of the present invention may transiently or, preferably, constitutively and permanently express AR-2 as described herein. In the fourth approach, DNA nucleotide primers can be prepared corresponding to a AR-2 specific DNA sequence. These primers could then be used to PCR a AR-2 gene fragment. (PCR Protocols: A Guide To Methods and Applications, Edited by Michael A. Innis et al., Academic Press (1990)).

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The recombinant AR-2 may be purified by any technique which allows for the subsequent formation of a stable, biologically active protein. Preferably, the ligand is secreted into the culture medium from which it is recovered. Alternatively, the AR-2 may be recovered from cells either as soluble proteins or as inclusion bodies, from which it may be extracted quantitatively by 8M guanidinium hydrochloride and dialysis in accordance with well known methodology. In order to further purify the AR-2, affinity chromatography, conventional ion exchange

chromatography, hydrophobic interaction chromatography, reverse phase chromatography or gel filtration may be used.

In additional embodiments of the invention, a recombinant AR-2 encoding gene may be used to inactivate or "knock out" the endogenous gene by homologous recombination, and thereby create an AR-2 deficient cell, tissue, or animal. For example, and not by way of limitation, the recombinant AR-2 encoding gene may be engineered to contain an insertional mutation, for example the neo gene, which would inactivate the native AR-2 encoding gene. Such a construct, under the control of a suitable promoter, may be introduced into a cell, such as an embryonic stem cell, by a technique such as transfection, transduction, or injection. Cells containing the construct may then be selected by G418 resistance. Cells which lack an intact AR-2 gene may then be identified, e.g. by Southern blotting, PCR detection, Northern blotting or assay of expression. Cells lacking an intact AR-2 encoding gene may then be fused to early embryo cells to generate transgenic animals deficient in such ligand. Such an animal may be used to define specific in vivo processes, normally dependent upon the factor.

The present invention also provides for antibodies to AR-2 described herein which are useful for detection of the factor in, for example, diagnostic applications. For preparation of monoclonal antibodies directed toward AR-2, any technique which provides for the production of antibody molecules by continuous cell lines in culture may be used. For example, the hybridoma technique originally developed by Kohler and Milstein (1975, *Nature* 256:495-497), as well as the trioma technique, the human B-cell hybridoma technique (Kozbor et al., 1983,

Immunology Today 4:72), and the EBV-hybridoma technique to produce human monoclonal antibodies (Cole et al., 1985, in "Monoclonal Antibodies and Cancer Therapy," Alan R. Liss, Inc. pp. 77-96) and the like are within the scope of the present invention.

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The monoclonal antibodies may be human monoclonal antibodies or chimeric human-mouse (or other species) monoclonal antibodies. Human monoclonal antibodies may be made by any of numerous techniques known in the art (e.g., Teng et al., 1983, Proc. Natl. Acad. Sci. U.S.A. 80:7308-7312; Kozbor et al., 1983, Immunology Today 4:72-79; Olsson et al., 1982, Meth. Enzymol. 92:3-16). Chimeric antibody molecules may be prepared containing a mouse antigen-binding domain with human constant regions (Morrison et al., 1984, Proc. Natl. Acad. Sci. U.S.A. 81:6851, Takeda et al., 1985, Nature 314:452).

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Various procedures known in the art may be used for the production of polyclonal antibodies to epitopes of AR-2. For the production of antibody, various host animals, including but not limited to rabbits, mice and rats can be immunized by injection with AR-2, or a fragment or derivative thereof. Various adjuvants may be used to increase the immunological response, depending on the host species, and including but not limited to Freund's (complete and incomplete), mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanins, dinitrophenol, and potentially useful human adjuvants such as BCG (Bacille Calmette-Guerin) and Corynebacterium parvum.

25

A molecular clone of an antibody to a selected AR-2 epitope can be prepared by known techniques. Recombinant DNA methodology (see e.g., Maniatis et al., 1982, Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York) may be used to
5 construct nucleic acid sequences which encode a monoclonal antibody molecule, or antigen binding region thereof.

The present invention provides for antibody molecules as well as fragments of such antibody molecules. Antibody fragments which
10 contain the idiotype of the molecule can be generated by known techniques. For example, such fragments include but are not limited to: the F(ab')₂ fragment which can be produced by pepsin digestion of the antibody molecule; the Fab' fragments which can be generated by reducing the disulfide bridges of the F(ab')₂ fragment, and the Fab
15 fragments which can be generated by treating the antibody molecule with papain and a reducing agent. Antibody molecules may be purified by known techniques, e.g., immunoabsorption or immunoaffinity chromatography, chromatographic methods such as HPLC (high performance liquid chromatography), or a combination thereof.

20

The present invention further encompasses an immunoassay for measuring the amount of AR-2 in a biological sample by

- a) contacting the biological sample with at least one antibody which specifically binds AR-2 so that the antibody forms a complex
25 with any AR-2 present in the sample; and
- b) measuring the amount of the complex and thereby measuring the amount of the AR-2 in the biological sample.

The present invention also provides for the utilization of AR-2 to support the survival and/or growth and/or migration and/or differentiation of AR-2 receptor expressing cells.

5

Further, the discovery by applicants of AR-2 enables the utilization of assay systems useful for the identification of the AR-2 receptor. Such assay systems would be useful in identifying molecules capable of promoting or inhibiting angiogenesis.

10

The invention further provides for both a method of identifying antibodies or other molecules capable of neutralizing the factor or blocking the binding ability of the factor, as well as the molecules identified by the method. By way of nonlimiting example, the method may be performed via an assay which is conceptually similar to an ELISA assay. For example, AR-2 antibody may be bound to a solid support, such as a plastic multiwell plate. As a control, a known amount of AR-2 which has been Myc-tagged may then be introduced to the well and any tagged AR-2 which binds the antibody may then be identified by means of a reporter antibody directed against the Myc-tag. This assay system may then be used to screen test samples for molecules which are capable of i) binding to the tagged factor or ii) binding to the antibody and thereby blocking binding to the antibody by the tagged factor. For example, a test sample containing a putative molecule of interest together with a known amount of tagged factor may be introduced to the well and the amount of tagged factor which binds to the antibody may be measured. By comparing the amount of bound tagged factor in the test sample to the amount in the control,

20

25

samples containing molecules which are capable of blocking factor binding to the antibody may be identified. The molecules of interest thus identified may be isolated using methods well known to one of skill in the art.

5

Once a blocker of factor binding is found, one of skill in the art would know to perform secondary assays to determine whether the blocker is binding to the antibody or to the factor, as well as assays to determine if the blocker molecule can neutralize the biological activity of the factor. For example, by using a binding assay which employs BIAcore biosensor technology (or the equivalent), in which either AR-2 antibody or AR-2 is covalently attached to a solid support (e.g. carboxymethyl dextran on a gold surface), one of skill in the art would be able to determine if the blocker molecule is binding specifically to the factor or to the antibody.

15

One of skill in the art would be able to produce "factorbodies" which comprise the AR-2 coupled to the Fc domain of IgG ("fFc's"). These factorbodies may be used as targeting agents, in diagnostics or in therapeutic applications, such as targeting agents for tumors and/or associated vasculature as indicated.

20

The invention herein further provides for the development of the AR-2, a fragment or derivative thereof as a therapeutic for the treatment of patients suffering from disorders involving cells, tissues or organs which express the AR-2 receptor. Such molecules may be used in a method of treatment of the human or animal body, or in a method of diagnosis.

25

Because AR-2 has been identified as related to the TIE family of ligands, applicants expect that the AR-2 may be useful for the induction or prevention of vascularization in diseases or disorders where such function is indicated. Such diseases or disorders would include wound healing, ischaemia and diabetes or for preventing or attenuating, for example, tumor growth. The AR-2 may be tested in animal models and used therapeutically as described for other agents, such as vascular endothelial growth factor (VEGF). Ferrara, et al. U.S. Patent No. 5,332,671 issued July 26, 1994. The Ferrara reference, as well as other studies, describe in vitro and in vivo studies that may be used to demonstrate the effect of an angiogenic factor in enhancing blood flow to ischemic myocardium, enhancing wound healing, and in other therapeutic settings wherein neoangiogenesis is desired. [See Sudo, et al. European Patent Application 0 550 296 A2 published July 7, 1993; Banai, et al. Circulation 89:2183-2189 (1994); Unger, et al. Am. J. Physiol. 266:H1588-H1595 (1994); Lazarous, et al. Circulation 91:145-153 (1995)]. According to the invention, AR-2 may be used alone or in combination with one or more additional pharmaceutically active compounds such as, for example, VEGF or basic fibroblast growth factor (bFGF), as well as cytokines, neurotrophins, etc.

Antagonists of the AR-2, such as antibodies or receptorbodies, would be useful to prevent or attenuate its biological activity. These agents may be used alone or in combination with other compositions.

In addition, AR-2 may be useful for the delivery of toxins to a receptor bearing cell. Where the AR-2 receptor is associated with a disease

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state, AR-2 may be useful as diagnostic reagents for detecting the disease by, for example, tissue staining or whole body imaging. Such reagents include radioisotopes, flurochromes, dyes, enzymes and biotin. Such diagnostics or targeting agents may be prepared as described in

5 Allitalo, et al. WO 95/26364 published October 5, 1995 and Burrows, F. and P. Thorpe, PNAS (USA) 90:8996-9000 (1993) which is hereby incorporated by reference in its entirety.

10 The AR-2 of the present invention may be used alone, or in combination with another pharmaceutically active agent such as, for example, cytokines, neurotrophins, interleukins, etc. In a preferred embodiment, the AR-2 may be used in conjunction with any of a number of the above referenced factors which are known to induce stem cell or other hematopoietic precursor proliferation, or factors acting on later cells

15 in the hematopoietic pathway, including, but not limited to, hemopoietic maturation factor, thrombopoietin, stem cell factor, erythropoietin, G-CSF, GM-CSF, etc.

20 The present invention also provides for compositions comprising the AR-2 described herein, peptide fragments thereof, or derivatives in a vehicle. The AR-2, peptide fragments, or derivatives may be administered systemically or locally. Any appropriate mode of administration known in the art may be used, including, but not limited

25 to, intravenous, intrathecal, intraarterial, intranasal, oral, subcutaneous, intraperitoneal, or by local injection or surgical implant. Sustained release formulations are also provided for.

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The present invention also provides for an antibody which specifically binds such a therapeutic molecule. The antibody may be monoclonal or polyclonal. The invention also provides for a method of using such a monoclonal or polyclonal antibody to measure the amount of the
5 therapeutic molecule in a sample taken from a patient for purposes of monitoring the course of therapy.

The invention further provides for a composition comprising a AR-2 and a cytotoxic agent conjugated thereto. In one embodiment, the cytotoxic
10 agent may be a radioisotope or toxin.

The invention also provides for an antibody which specifically binds a AR-2. The antibody may be monoclonal or polyclonal.

- 15 The invention further provides for a method of purifying AR-2 comprising:
- a) coupling at least one AR-2 binding substrate to a solid matrix;
 - b) incubating the substrate of a) with a cell lysate so that the
20 substrate forms a complex with any AR-2 in the cell lysate;
 - c) washing the solid matrix; and
 - d) eluting the AR-2 from the coupled substrate.

The substrate may be any substance that specifically binds the AR-2.
25 In one embodiment, the substrate is selected from the group consisting of anti-AR-2 antibody, AR-2 receptor and AR-2 receptorbody.

The invention also provides for a composition comprising AR-2 in a

vehicle, as well as a method of regulating angiogenesis in a patient comprising administering to the patient an effective amount of the therapeutic composition.

- 5 In addition, the present invention provides for a method for identifying a cell which expresses AR-2 receptor which comprises contacting a cell with a detectably labeled AR-2 or factorbody, under conditions permitting binding of the detectably labeled factor to the AR-2 receptor and determining whether the detectably labeled factor is
- 10 bound to the AR-2 receptor, thereby identifying the cell as one which expresses AR-2 receptor. The present invention also provides for a composition comprising a AR-2 and a cytotoxic agent conjugated thereto. The cytotoxic agent may be a radioisotope or toxin.
- 15 The invention also provides a method of detecting expression of AR-2 by a cell which comprises obtaining mRNA from the cell, contacting the mRNA so obtained with a labeled nucleic acid molecule encoding AR-2, under hybridizing conditions, determining the presence of mRNA hybridized to the labeled molecule, and thereby detecting the
- 20 expression of the AR-2 in the cell.

The invention further provides a method of detecting expression of AR-2 in tissue sections which comprises contacting the tissue sections with a labeled nucleic acid molecule encoding a AR-2, under hybridizing

25 conditions, determining the presence of mRNA hybridized to the labelled molecule, and thereby detecting the expression of AR-2 in tissue sections.

EXAMPLE 1 - ISOLATION AND SEQUENCING OF FULL LENGTH cDNA
CLONE ENCODING MAMMALIAN TIE LIGAND-3

5 TIE ligand-3 (TL3) was cloned from a mouse BAC genomic library (Research Genetics) by hybridizing library duplicates, with either mouse TL1 or mouse TL2 probes corresponding to the entire coding sequence of those genes. Each copy of the library was hybridized using
10 phosphate buffer at 55°C overnight. After hybridization, the filters were washed using 2xSSC, 0.1% SDS at 60°C, followed by exposure of X ray film to the filters. Strong hybridization signals were identified corresponding to mouse TL1 and mouse TL2. In addition, signals were identified which weakly hybridized to both mouse TL1 and mouse TL2.
15 DNA corresponding to these clones was purified, then digested with restriction enzymes, and two fragments which hybridized to the original probes were subcloned into a bacterial plasmid and sequenced. The sequence of the fragments contained two exons with homology to both mouse TL1 and mouse TL2. Primers specific for these sequences
20 were used as PCR primers to identify tissues containing transcripts corresponding to TL3. A PCR band corresponding to TL3 was identified in a mouse uterus cDNA library in lambda gt-11. (Clontech Laboratories, Inc., Palo Alto, CA).
25 Plaques were plated at a density of 1.25×10^6 /20x20 cm plate and replica filters taken following standard procedures (Sambrook, et al., Molecular Cloning: A Laboratory Manual, 2nd Ed., page 8.46, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York). Duplicate filters

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were screened at "normal" stringency (2 x SSC, 65°C) with a 200 bp PCR radioactive probe made to the mouse TL3 sequence. Hybridization was at 65°C in a solution containing 0.5 mg/ml salmon sperm DNA.

Filters were washed in 2 x SSC at 65°C and exposed for 6 hours to X-

5 ray film. Two positive clones that hybridized in duplicate were picked. EcoRI digestion of phage DNA obtained from these clones indicated two independent clones with insert sizes of approximately 1.2 kb and approximately 2.2 kb. The 2.2kb EcoRI insert was subcloned into the EcoRI site of pBluescript KS (Stratagene). Sequence analysis
10 showed that the longer clone was lacking an initiator methionine and signal peptide but otherwise encoded a probe homologous to both mouse TL1 and mouse TL2.

Two TL3-specific PCR primers were then synthesised as follows:

15 US2: cctctgggctcgccagtttgtagg

US1: ccagctggcagatcagg

The following PCR reactions were performed using expression libraries derived from the mouse cell lines C2C12ras and MG87. In the primary
20 PCR reaction, the specific primer US2 was used in conjunction with vector-specific oligos to allow amplification in either orientation.

PCR was in a total volume of 100µl using 35 cycles of 94°C, 1 min;

42°C or 48°C for 1 min; 72°C, 1 min. The secondary PCR reaction

included the second specific primer, US1, which is contained within the

25 primary PCR product, in conjunction with the same vector oligos. The secondary reactions were for 30 cycles, using the same temperatures and times as previous. PCR products were gel isolated and submitted for sequence analysis. On the basis of sequences obtained from a total

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of four independent PCR reactions using two different cDNA libraries, the 5' end of the TL3 sequence was deduced. Northern analysis revealed moderate to low levels of mouse TL3 transcript in mouse placenta. The expression of mouse TL3 consisted of a transcript of approximately 3
5 kb. The full length TL3 coding sequence is set forth in Figure 1A-1B.

The mouse TL3 sequence may then be used to obtain a human clone containing the coding sequence of its human counterpart by hybridizing either a human genomic or cDNA library with a probe corresponding to
10 mouse TL3 as has been described previously, for example, in Example 8 in International Publication No. WO 96/31598 published 10 October 1996.

15 **EXAMPLE 2 - ISOLATION OF FULL LENGTH GENOMIC CLONE ENCODING HUMAN TIE LIGAND-4**

TIE ligand-4 (TL4) was cloned from a human BAC genomic library (BAC
20 HUMAN (II), Genome Systems Inc.) by hybridizing library duplicates, with either a human TL1 radioactive probe corresponding to the entire fibrinogen coding sequence of TL1 (nucleotides 1153 to 1806) or a mouse TL3 radioactive probe corresponding to a segment of 186
nucleotides from the fibrinogen region of mouse TL3 (nucleotides 1307
25 to 1492 of Figure 1A-1B). Each probe was labeled by PCR using exact oligonucleotides and standard PCR conditions, except that dCTP was replaced by P³²dCTP. The PCR mixture was then passed through a gel filtration column to separate the probe from free P³² dCTP. Each copy of the library was hybridized using phosphate buffer, and radioactive

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probe at 55°C overnight using standard hybridization conditions. After hybridization, the filters were washed using 2xSSC, 0.1% SDS at 55°C, followed by exposure of X ray film. Strong hybridization signals were observed corresponding to human TL1. In addition, signals were

5 identified which weakly hybridized to both human TL1 and mouse TL3. DNA corresponding to these clones was purified using standard procedures, then digested with restriction enzymes, and one fragment which hybridized to the original probes was subcloned into a bacterial plasmid and sequenced. The sequence of the fragments contained one
10 exon with homology to both human TL1 and mouse TL3 and other members of the TIE ligand family. Primers specific for these sequences may be used as PCR primers to identify tissues containing transcripts corresponding to TL4.

15 The complete sequence of human TL4 may be obtained by sequencing the full BAC clone contained in the deposited bacterial cells. Exons may be identified by homology to known members of the TIE-ligand family such as TL1, TL2 and TL3. The full coding sequence of TL4 may then be determined by splicing together the exons from the TL4 genomic clone
20 which, in turn, may be used to produce the TL4 protein. Alternatively, the exons may be used as probes to obtain a full length cDNA clone, which may then be used to produce the TL4 protein. Exons may also be identified from the BAC clone sequence by homology to protein domains such as fibrinogen domains, coiled coil domains, or protein signals such
25 as signal peptide sequences. Missing exons from the BAC clone may be obtained by identification of contiguous BAC clones, for example, by using the ends of the deposited BAC clone as probes to screen a human genomic library such as the one used herein, by using the exon sequence

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contained in the BAC clone to screen a cDNA library, or by performing either 5' or 3' RACE procedure using oligonucleotide primers based on the TL4 exon sequences.

5 Identification of Additional TIE Ligand Family Members

The novel TIE ligand-4 sequence may be used in a rational search for additional members of the TIE ligand family using an approach that takes advantage of the existence of conserved segments of strong
10 homology between the known family members. For example, an alignment of the amino acid sequences of the TIE ligands shows several regions of conserved sequence (see underlined regions of Figure 2). Degenerate oligonucleotides essentially based on these boxes in combination with either previously known or novel TIE ligand homology
15 segments may be used to identify new TIE ligands.

The highly conserved regions among TL1, TL2 and TL3 may be used in designing degenerate oligonucleotide primers with which to prime PCR
20 reactions using cDNAs. cDNA templates may be generated by reverse transcription of tissue RNAs using oligo d(T) or other appropriate primers. Aliquots of the PCR reactions may then be subjected to electrophoresis on an agarose gel. Resulting amplified DNA fragments may be cloned by insertion into plasmids, sequenced and the DNA
sequences compared with those of all known TIE ligands.

25

Size-selected amplified DNA fragments from these PCR reactions may be cloned into plasmids, introduced into E. coli by electroporation, and transformants plated on selective agar. Bacterial colonies from PCR

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transformation may be analyzed by sequencing of plasmid DNAs that are purified by standard plasmid procedures.

Cloned fragments containing a segment of a novel TIE ligand may be used as hybridization probes to obtain full length cDNA clones from a cDNA library. For example, the human TL4 genomic sequence may be used to obtain a human cDNA clone containing the complete coding sequence of human TL4 by hybridizing a human cDNA library with a probe corresponding to human TL4 as has been described previously.

EXAMPLE 3 - Cloning of the full coding sequence of hTL4

Both 5' and 3' coding sequence from the genomic human TL-4 clone encoding human TIE ligand-4 (hTL-4 ATCC Accession No. 98095) was obtained by restriction enzyme digestion, Southern blotting and hybridization of the hTL-4 clone to coding sequences from mouse TL3, followed by subcloning and sequencing the hybridizing fragments. Coding sequences corresponding to the N-terminal and C-terminal amino acids of hTL4 were used to design PCR primers (shown below), which in turn were used for PCR amplification of TL4 from human ovary cDNA. A PCR band was identified as corresponding to human TL4 by DNA sequencing using the ABI 373A DNA sequencer and Taq Dideoxy Terminator Cycle Sequencing Kit (Applied Biosystems, Inc., Foster City, CA). The PCR band was then subcloned into vector pCR-script and several plasmid clones were analyzed by sequencing. The complete human TL4 coding sequence was then compiled and is shown in Figure 3A-3C. In another embodiment of the invention, the nucleotide at position 569 is changed from A to G, resulting in an amino acid change

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from Q to R.

The PCR primers used as described above were designed as follows:

hTL4atg 5'-gcatgctatctcgagccaccATGCTCTCCCAGCTAGCCATGCTGCAG-3'

5

hTL4not 5'-

gtgtcgacgcggccgctctagatcagacTTAGATGTCCAAAGGCCGTATCATCAT-3'

10

Lowercase letters indicate "tail" sequences added to the PCR primers to facilitate cloning of the amplified PCR fragments.

EXAMPLE 4 - CLONING OF A NOVEL FACTOR RELATED TO THE TIE
FAMILY OF LIGANDS

15 Human AR-1 was cloned from a human BAC genomic library (Human Genome Sciences, Inc., Cat. No. FBAC 4435, Release II) by hybridizing library duplicates with a mouse TL3 probe. The probe was labeled by PCR using exact oligonucleotides and standard PCR conditions, except that dCTP was replaced by P³²dCTP. The PCR mixture was then passed
20 through a gel filtration column to separate the probe from free P³²dCTP. The library was hybridized using phosphate buffer at 55°C overnight. After hybridization, the filters were washed using 2xSSC, 0.1% SDS at 60°C, followed by exposure of X-ray film to the filters. Strong hybridization signals were identified corresponding to mouse
25 TL3. Genomic DNA corresponding to these clones was purified, digested with restriction enzymes, and subcloned into a bacterial plasmid and sequenced. The sequence of the fragment contained a portion of the 3' end of the human AR-1 gene. To obtain the 5' end of the clone, the 3'

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sequence was used to design oligonucleotide primers for use in a standard RACE procedure. The RACE product was sequenced and found to contain the upstream sequence of the gene including the 5' untranslated sequence. To obtain the full length human AR-1 clone, the 5' sequence
5 was used to design oligonucleotide primers that were used in conjunction with Adaptor primer 1 and Adaptor primer 2 (Clontech Laboratories, Inc., Catalog # 7413-1) to amplify Human Skeletal Muscle Marathon-Ready™ cDNA (Clontech Laboratories, Inc., Catalog # 7413-1). The amplification product was cloned into the pMT21 vector and
10 sequenced. The nucleotide and deduced amino acid sequence of human AR-1 is set forth in Figure 4A-4B.

EXAMPLE 5 - CLONING OF A SECOND NOVEL FACTOR RELATED TO THE
TIE FAMILY OF LIGANDS

15 A second distant angiopoietin relative has been identified via a standard homology-based PCR screen for new angiopoietins. AR-2 was initially identified during a PCR screen using degenerate primers for conserved angiopoietin sequences PSGEYW and WWFDAC. A PCR
fragment corresponding to AR-2 was then used as a probe to identify a
20 partial cDNA clone from a mouse uterus cDNA library (Clontech Cat# ML 1022B). The partial nucleotide and deduced amino acid sequence of AR-2 is set forth in Figures 5A-5B. Figure 6 sets forth the nucleotide and deduced amino acid sequence of the fibrinogen-like domain of AR-2.

The present invention is not to be limited in scope by the specific
25 embodiments described herein. Indeed, various modifications of the

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invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description and accompanying figures. Such modifications are intended to fall within the scope of the appended claims.

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What is claimed is:

1. An isolated nucleic acid molecule comprising a nucleotide sequence encoding the fibrinogen-like domain of AR-2, wherein the nucleotide sequence is selected from the group consisting of:
 - (a) the nucleotide sequence comprising the coding region of the fibrinogen-like domain of AR-2 as set forth in Figure 6;
 - (b) a nucleotide sequence that hybridizes under stringent conditions to the nucleotide sequence of (a); and
 - (c) a nucleotide sequence which differs from the sequence of (a) or (b) due to the degeneracy of the genetic code.
2. A vector which comprises a nucleic acid molecule of claim 1.
3. A vector according to claim 2, wherein the nucleic acid molecule is operatively linked to an expression control sequence capable of directing its expression in a host cell.
4. A vector according to claim 3, which is a plasmid.
5. Isolated AR-2 fibrinogen-like domain having the amino acid sequence set forth in Figure 6.
6. Isolated AR-2 fibrinogen-like domain encoded by a nucleic acid molecule of claim 1.
7. A host-vector system for the production of AR-2 which comprises a vector of claim 3, in a host cell.

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8. A host-vector system according to claim 7, wherein the host cell is a bacterial, yeast, insect or mammalian cell.
9. A method of producing AR-2 which comprises growing cells of the host-vector system of claim 8, under conditions permitting production of the AR-2, and recovering the AR-2 so produced.
10. An antibody which specifically binds the AR-2 of claim 5 or 6.
11. An antibody according to claim 10, which is a monoclonal antibody.
12. A conjugate comprising the isolated AR-2 of claim 5 or 6, conjugated to a cytotoxic agent.
13. A conjugate according to claim 12, wherein the cytotoxic agent is a radioisotope or toxin.
14. A composition comprising the AR-2 of claim 5 or 6, and a carrier.
15. A composition comprising an antibody of claim 11, and a carrier.
16. A composition comprising a conjugate of claim 12, and a carrier.
17. A factorbody comprising isolated AR-2 fibrinogen-like domain of claim 5 or 6, fused to an immunoglobulin constant region.
18. The factorbody of claim 17, wherein the immunoglobulin constant region is the Fc portion of IgG.

19. A nucleic acid according to claim 1 which is substantially as hereinbefore described in any one of the foregoing examples.
20. A vector according to claim 2 which is substantially as hereinbefore described
5 in any one of the foregoing examples.
21. A polypeptide according to claim 5 or 6 which is substantially as hereinbefore described in any one of the foregoing examples.
- 10 22. A host-vector system according to claim 7 which is substantially as hereinbefore described in any one of the foregoing examples.
23. A method according to claim 9 which is substantially as hereinbefore described in any one of the foregoing examples.
- 15 24. An antibody according to claim 10 which is substantially as hereinbefore described in any one of the foregoing examples.
25. A conjugate according to claim 12 which is substantially as hereinbefore
20 described in any one of the foregoing examples.
26. A composition according to claim 14, 15 or 16 which is substantially as hereinbefore described in any one of the foregoing examples.
- 25 27. A factor body according to claim 17 which is substantially as hereinbefore described in any one of the foregoing examples.