(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
25 May 2001 (25.05.2001)

PCT

(10) International Publication Number
WO 01/36637 A1

(51) International Patent Classification[7]: C12N 15/12, 15/62, C07K 14/705

(21) International Application Number: PCT/US00/31459

(22) International Filing Date:
14 November 2000 (14.11.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
09/442,029 17 November 1999 (17.11.1999) US

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(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:
— *With international search report.*
— *Before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments.*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 01/36637 A1

(54) Title: RECEPTOR ACTIVATOR OF NF-KAPPA B

(57) Abstract: Isolated receptors, DNAs encoding such receptors, and pharmaceutical compositions made therefrom, are disclosed. The isolated receptors can be used to regulate an immune response. The receptors are also useful in screening for inhibitors thereof.

TITLE

Receptor Activator of NF-κB

CROSS-REFERENCE TO RELATED PATENT APPLICATIONS

This application is a continuation-in-part of USSN 09/442,029, filed November 17, 1999, which is a continuation-in-part of USSN 08/995,659, filed December 22, 1997, and a continuation-in-part of 08/996,139 filed December 22, 1997, both of which which are continuations-in-part of USSN 60/064,671, filed October 14, 1997, and USSN 08/813,509, filed March 7, 1997 (converted to provisional application USSN 60/077,181, on August 18, 1997) and USSN 08/772,330, filed December 23, 1996 (converted to provisional application USSN 60/059,978, on July 24, 1997).

TECHNICAL FIELD OF THE INVENTION

The present invention relates generally to the field of cytokine receptors, and more specifically to cytokine receptor/ligand pairs having immunoregulatory activity.

BACKGROUND OF THE INVENTION

Efficient functioning of the immune system requires a fine balance between cell proliferation and differentiation and cell death, to ensure that the immune system is capable of reacting to foreign, but not self-antigens. Integral to the process of regulating the immune and inflammatory response are various members of the Tumor Necrosis Factor (TNF) Receptor/Nerve Growth Factor Receptor superfamily (Smith et al., *Science* 248:1019; 1990). This family of receptors includes two different TNF receptors (Type I and Type II; Smith et al., *supra*; and Schall et al., *Cell* 61:361, 1990), nerve growth factor receptor (Johnson et al., *Cell* 47:545, 1986), B cell antigen CD40 (Stamenkovic et al., *EMBO J.* 8:1403, 1989), CD27 (Camerini et al., *J. Immunol.* 147:3165, 1991), CD30 (Durkop et al., *Cell* 68:421, 1992), T cell antigen OX40 (Mallett et al., *EMBO J.* 9:1063, 1990), human *Fas* antigen (Itoh et al., *Cell* 66:233, 1991), murine 4-1BB receptor (Kwon et al., *Proc. Natl. Acad. Sci. USA* 86:1963, 1989) and a receptor referred to as Apoptosis-Inducing Receptor (AIR; USSN 08/720,864, filed October 4, 1996).

CD40 is a receptor present on B lymphocytes, epithelial cells and some carcinoma cell lines that interacts with a ligand found on activated T cells, CD40L (USSN 08/249,189, filed May 24, 1994). The interaction of this ligand/receptor pair is essential for both the cellular and humoral immune response. Signal transduction via CD40 is mediated through the association of the cytoplasmic domain of this molecule with members of the TNF receptor-associated factors (TRAFs; Baker and Reddy, *Oncogene* 12:1, 1996). It has recently been found that mice that are defective in TRAF3 expression

due to a targeted disruption in the gene encoding TRAF3 appear normal at birth but develop progressive hypoglycemia and depletion of peripheral white cells, and die by about ten days of age (Xu et al., *Immunity* 5:407, 1996). The immune responses of chimeric mice reconstituted with $TRAF3^{-/-}$ fetal liver cells resemble those of CD40-deficient mice, although $TRAF3^{-/-}$ B cells appear to be functionally normal.

The critical role of TRAF3 in signal transduction may be in its interaction with one of the other members of the TNF receptor superfamily, for example, CD30 or CD27, which are present on T cells. Alternatively, there may be other, as yet unidentified members of this family of receptors that interact with TRAF3 and play an important role in postnatal development as well as in the development of a competent immune system. Identifying additional members of the TNF receptor superfamily would provide an additional means of regulating the immune and inflammatory response, as well as potentially providing further insight into post-natal development in mammals.

SUMMARY OF THE INVENTION

The present invention provides a novel receptor, referred to as RANK (for receptor activator of NF-κB), that is a member of the TNF receptor superfamily. RANK is a Type I transmembrane protein having 616 amino acid residues that interacts with TRAF1, TRAF2, TRAF3, TRAF5, and TRAF6. Triggering of RANK by over-expression, co-expression of RANK and membrane bound RANK ligand (RANKL), and with addition of soluble RANKL or agonistic antibodies to RANK results in the upregulation of the transcription factor NF κB, a ubiquitous transcription factor that is most extensively utilized in cells of the immune system.

Soluble forms of the receptor can be prepared and used to interfere with signal transduction through membrane-bound RANK, and hence upregulation of NF-κB; accordingly, pharmaceutical compositions comprising soluble forms of the novel receptor are also provided. Inhibition of NF-κB by RANK antagonists may be useful in ameliorating negative effects of an inflammatory response that result from triggering of RANK, for example in treating toxic shock or sepsis, graft-versus-host reactions, acute inflammatory reactions, and the effects of excess bone resorption. Soluble forms of the receptor will also be useful in *in vitro* and *in vivo* based screening tests for agonists or antagonists of RANK activity.

The cytoplasmic domain of RANK will be useful in developing assays for inhibitors of signal transduction, for example, for screening for molecules that inhibit interaction of RANK with TRAF1, TRAF2, TRAF3, TRAF5 and in particular TRAF6. Deleted forms and fusion proteins comprising the novel receptor are also disclosed.

Further encompassed by the present invention are gene therapy methods to correct gene-activating mutations, associated with e.g. familial expansile osteolysis (FEO) and

early onset Paget's disease of bone (EP). Also included are gene therapy methods to correct gene activating or inactivating mutations associated with other syndromes in which the RANK gene encodes overactive, inactive, insufficiently active, or no RANK. Gene therapy to correct such mutations or defective genes includes isolating hematopoietic stem cells from an individual afflicted with a syndrome associated with RANK gene mutation, transfecting the isolated cells with a transfection vector that expresses a suitably active RANK, and administering the transfected cells expressing RANK to the individual.

The present invention also encompasses DNA and proteins encoded by the DNA that are associated with FEO and EP, methods for identifying individuals who are afflicted with FEO or EP, and therapeutic uses of the DNA and proteins that are associated with these diseases..

The present invention also identifies a counterstructure, or ligand, for RANK, referred to as RANKL. RANKL is a Type 2 transmembrane protein with an intracellular domain of less than about 50 amino acids, a transmembrane domain and an extracellular domain of from about 240 to 250 amino acids. Similar to other members of the TNF family to which it belongs, RANKL has a 'spacer' region between the transmembrane domain and the receptor binding domain that is not necessary for receptor binding. Accordingly, soluble forms of RANKL can comprise the entire extracellular domain or fragments thereof that include the receptor binding region.

These and other aspects of the present invention will become evident upon reference to the following detailed description of the invention.

DETAILED DESCRIPTION OF THE INVENTION

A novel partial cDNA insert with a predicted open reading frame having some similarity to CD40 was identified in a database containing sequence information from cDNAs generated from human bone marrow-derived dendritic cells (DC). The insert was used to hybridize to colony blots generated from a DC cDNA library containing full-length cDNAs. Several colony hybridizations were performed, and two clones (SEQ ID NOs:1 and 3) were isolated. SEQ ID NO:5 shows the nucleotide and amino acid sequence of a predicted full-length protein based on alignment of the overlapping sequences of SEQ ID NOs:1 and 3.

RANK is a member of the TNF receptor superfamily; it most closely resembles CD40 in the extracellular region. Similar to CD40, RANK associates with TRAF2 and TRAF3 (as determined by co-immunoprecipitation assays substantially as described by Rothe et al., *Cell* 83:1243, 1995). TRAFs are critically important in the regulation of the immune and inflammatory response. Through their association with various members of the TNF receptor superfamily, a signal is transduced to a cell. That signal results in the

proliferation, differentiation or apoptosis of the cell, depending on which receptor(s) is/are triggered and which TRAF(s) associate with the receptor(s); different signals can be transduced to a cell via coordination of various signaling events. Thus, a signal transduced through one member of this family may be proliferative, differentiative or apoptotic, depending on other signals being transduced to the cell, and/or the state of differentiation of the cell. Such exquisite regulation of this proliferative/apoptotic pathway is necessary to develop and maintain protection against pathogens; imbalances can result in autoimmune disease.

RANK is expressed on epithelial cells, osteoclast precursors, some B cell lines, and on activated T cells. However, its expression on activated T cells is late, about four days after activation. This time course of expression coincides with the expression of Fas, a known agent of apoptosis. RANK may act as an anti-apoptotic signal, rescuing cells that express RANK from apoptosis as CD40 is known to do. Alternatively, RANK may confirm an apoptotic signal under the appropriate circumstances, again similar to CD40. RANK and its ligand are likely to play an integral role in regulation of the immune and inflammatory response.

Moreover, the post-natal lethality of mice having a targeted disruption of the TRAF3 gene demonstrates the importance of this molecule not only in the immune response, but also in development. The isolation of RANK, as a protein that associates with five of the 6 known TRAFs, and its ligand will allow further definition of this signaling pathway, and development of diagnostic and therapeutic modalities for use in the area of autoimmune and/or inflammatory disease. The RANK/TRAF association can be utilized in screening methodologies to discover therapeutics, including small molecules and peptides, that modulate RANK/TRAF interaction and thus effect the activities that result from the interaction. The Examples below demonstrate that TRAF6 is particularly important in mediating RANK signaling. Therapeutics that inhibit RANK/TRAF6 interaction are immunosuppressants and anti-inflammatory agents. Compounds that interfere with RANK/TRAF6 interactions are also useful for modulating the formation of osteoclasts from osteoclast precursors, modulating osteoclast function and activities, and as inhibitors of diseases associated with excess bone resorption.

Familial expansile osteolysis (FEO) is a rare autosomal dominant bone dysplasia with similarities to Paget's disease of bone. These diseases are characterized by focal area of increased bone remodeling that leads to deformity and disability. The FEO gene and the gene associated with familial Paget's disease of the bone map to chromosome 18q21 which is the same location that includes the RANK gene. In connection with the discovery of the FEO gene and the gene responsible for early onset Paget's disease of bone, the present invention further includes FEO RANK and EP RANK DNA and encoded polypeptides that include RANK insertion mutations which cause FEO and EP.

This discovery further demonstrates the importance of the osteoblast/osteoclastogenesis pathway in disease and is the basis of gene therapy methods of the present invention and methods for diagnosing early onset Paget's disease of the bone and FEO.

DNAs, Proteins and Analogs

The present invention provides isolated RANK polypeptides and analogs (or muteins) thereof having an activity exhibited by the native molecule (i.e, RANK muteins that bind specifically to a RANK ligand expressed on cells or immobilized on a surface or to RANK-specific antibodies; soluble forms thereof that inhibit RANK ligand-induced signaling through RANK, and muteins that cause disease and because they are inactive or have increased RANK activity or decreased RANK activity). Such proteins are substantially free of contaminating endogenous materials and, optionally, without associated native-pattern glycosylation. Derivatives of RANK within the scope of the invention also include various structural forms of the primary proteins that retain biological activity. Due to the presence of ionizable amino and carboxyl groups, for example, a RANK protein may be in the form of acidic or basic salts, or may be in neutral form. Individual amino acid residues may also be modified by oxidation or reduction. The primary amino acid structure may be modified by forming covalent or aggregative conjugates with other chemical moieties, such as glycosyl groups, lipids, phosphate, acetyl groups and the like, or by creating amino acid sequence mutants. Covalent derivatives are prepared by linking particular functional groups to amino acid side chains or at the N- or C-termini.

Derivatives of RANK may also be obtained by the action of cross-linking agents, such as M-maleimidobenzoyl succinimide ester and N-hydroxysuccinimide, at cysteine and lysine residues. The inventive proteins may also be covalently bound through reactive side groups to various insoluble substrates, such as cyanogen bromide-activated, bisoxirane-activated, carbonyldiimidazole-activated or tosyl-activated agarose structures, or by adsorbing to polyolefin surfaces (with or without glutaraldehyde cross-linking). Once bound to a substrate, the proteins may be used to selectively bind (for purposes of assay or purification) antibodies raised against the proteins or against other proteins which are similar to RANK or RANKL, as well as other proteins that bind RANK or RANKL or homologs thereof.

Soluble forms of RANK are also within the scope of the invention. The nucleotide and predicted amino acid sequence of RANK is shown in SEQ ID NOs:1 through 6. Computer analysis indicated that the protein has an N-terminal signal peptide; the predicted cleavage site follows residue 24, thus generating a mature protein whose first amino acid is amino acid residue 25. Those skilled in the art will recognize that the actual cleavage site may be different than that predicted by computer analysis. Thus, the N-terminal amino acid of the cleaved peptide is expected to be within about five amino

acids on either side of the predicted, preferred cleavage site following residue 24. Moreover a soluble form beginning with amino acid 33 was prepared; this soluble form bound RANKL. The signal peptide is predicted to be followed by a 188 amino acid extracellular domain, a 21 amino acid transmembrane domain, and a 383 amino acid cytoplasmic tail.

Soluble RANK comprises the signal peptide and the extracellular domain (residues 1 to 213 of SEQ ID NO:6) or a fragment thereof. Alternatively, a different signal peptide can be substituted for the native leader, beginning with residue 1 and continuing through a residue selected from the group consisting of amino acids 24 through 33 (inclusive) of SEQ ID NO:6. Fragments of the extracellular or cytoplasmic domain can be prepared using known techniques to isolate a desired portion of the domain region of interest. For example, one such technique includes comparing the extracellular or cytoplasmic domain with those of other members of the TNFR family and selecting forms similar to those prepared for other family members. Alternatively, unique restriction sites or PCR techniques that are known in the art can be used to prepare numerous truncated forms which can be expressed and analyzed for activity.

Also included within the scope of the invention are fragments or derivatives of the intracellular domain of RANK. Such fragments are prepared by any of the herein-mentioned techniques, and include, but are not limited to, peptides that are identical to the full cytoplasmic domain of RANK as shown in SEQ ID NO:6, (amino acid 234-616) or of murine RANK as shown in SEQ ID NO:15, and those that comprise a portion of the cytoplasmic region. As described in Examples 19 and 20, truncated forms of the RANK cytoplasmic domain have been identified as TRAF binding sites. Such fragments include the COOH-terminal 72 amino acids (amino acids 545-616) which interacts with TRAFs 1, 2, 3, 5 and 6, amino acids 339-422 which interacts with TRAF6, amino acids 339-362 which interacts with TRAF6, and all of SEQ ID NO:6. Similarly, fragments of the cytoplasmic domain of muRANK (SEQ ID NO:14 and SEQ ID NO:15) that are conserved regions with SEQ ID NO:5 and SEQ ID NO:6, respectively, are important for TRAF binding, and encompassed by the present invention. Accordingly, a PCR-based technique was developed to facilitate preparation of various C-terminal truncations that would retain the conserved regions.

All techniques used in preparing soluble forms may also be used in preparing fragments or analogs of the cytoplasmic domain (i.e., RT-PCR techniques or use of selected restriction enzymes to prepare truncations). DNAs encoding all or a fragment of the intracytoplasmic domain will be useful in identifying other proteins that are associated with RANK signaling, for example using the immunoprecipitation techniques described herein, or another technique such as a yeast two-hybrid system (Rothe et al., supra).

Other derivatives of the RANK proteins within the scope of this invention include covalent or aggregative conjugates of the proteins or their fragments with other proteins or polypeptides, such as by synthesis in recombinant culture as N-terminal or C-terminal fusions. For example, the conjugated peptide may be a signal (or leader) polypeptide sequence at the N-terminal region of the protein which co-translationally or post-translationally directs transfer of the protein from its site of synthesis to its site of function inside or outside of the cell membrane or wall (e.g., the yeast α-factor leader).

Protein fusions can comprise peptides added to facilitate purification, identification, and function of RANK proteins, RANK homologs (e.g., poly-His), or RANK fragments, including fragments of the cytoplasmic domain and extracellular domain. The amino acid sequence of the inventive proteins can also be linked to an identification peptide such as that described by Hopp et al., *Bio/Technology* 6:1204 (1988). Such highly antigenic identification peptides provide an epitope reversibly bound by a specific monoclonal antibody, enabling rapid assay and facile purification of expressed recombinant protein. The sequence of Hopp et al. is also specifically cleaved by bovine mucosal enterokinase, allowing removal of the peptide from the purified protein. Fusion proteins capped with such peptides may also be resistant to intracellular degradation in *E. coli*.

Fusion proteins further comprise the amino acid sequence of a RANK or RANK fragment linked to an immunoglobulin Fc region (RANK.Fc). An exemplary Fc region is a human IgG_1 having an amino acid sequence set forth in SEQ ID NO:8. Fragments of an Fc region may also be used, as can Fc muteins. For example, certain residues within the hinge region of an Fc region are critical for high affinity binding to FcγRI. Canfield and Morrison (*J. Exp. Med.* 173:1483; 1991) reported that $Leu_{(234)}$ and $Leu_{(235)}$were critical to high affinity binding of IgG_3 to FcγRI present on U937 cells. Similar results were obtained by Lund et al. (*J. Immunol.* 147:2657, 1991; *Molecular Immunol.* 29:53, 1991). Such mutations, alone or in combination, can be made in an IgG_1 Fc region to decrease the affinity of IgG_1 for FcR. Depending on the portion of the Fc region used, a fusion protein may be expressed as a dimer, through formation of interchain disulfide bonds. If the fusion proteins are made with both heavy and light chains of an antibody, it is possible to form a protein oligomer with as many as four RANK regions.

In another embodiment, RANK proteins and RANK fragments further comprise an oligomerizing peptide such as a leucine zipper domain. Leucine zippers were originally identified in several DNA-binding proteins (Landschulz et al., *Science* 240:1759, 1988). Leucine zipper domain is a term used to refer to a conserved peptide domain present in these (and other) proteins, which is responsible for dimerization of the proteins. The leucine zipper domain (also referred to herein as an oligomerizing, or oligomer-forming, domain) comprises a repetitive heptad repeat, with four or five leucine

residues interspersed with other amino acids. Examples of leucine zipper domains are those found in the yeast transcription factor GCN4 and a heat-stable DNA-binding protein found in rat liver (C/EBP; Landschulz et al., *Science* 243:1681, 1989). Two nuclear transforming proteins, *fos* and *jun*, also exhibit leucine zipper domains, as does the gene product of the murine proto-oncogene, *c-myc* (Landschulz et al., *Science* 240:1759, 1988). The products of the nuclear oncogenes *fos* and *jun* comprise leucine zipper domains preferentially form a heterodimer (O'Shea et al., *Science* 245:646, 1989; Turner and Tjian, *Science* 243:1689, 1989). The leucine zipper domain is necessary for biological activity (DNA binding) in these proteins.

The fusogenic proteins of several different viruses, including paramyxovirus, coronavirus, measles virus and many retroviruses, also possess leucine zipper domains (Buckland and Wild, *Nature* 338:547,1989; Britton, *Nature* 353:394, 1991; Delwart and Mosialos, *AIDS Research and Human Retroviruses* 6:703, 1990). The leucine zipper domains in these fusogenic viral proteins are near the transmembrane region of the proteins; it has been suggested that the leucine zipper domains could contribute to the oligomeric structure of the fusogenic proteins. Oligomerization of fusogenic viral proteins is involved in fusion pore formation (Spruce et al, *Proc. Natl. Acad. Sci. U.S.A.* 88:3523, 1991). Leucine zipper domains have also been recently reported to play a role in oligomerization of heat-shock transcription factors (Rabindran et al., *Science* 259:230, 1993).

Leucine zipper domains fold as short, parallel coiled coils. (O'Shea et al., *Science* 254:539; 1991) The general architecture of the parallel coiled coil has been well characterized, with a "knobs-into-holes" packing as proposed by Crick in 1953 (*Acta Crystallogr.* 6:689). The dimer formed by a leucine zipper domain is stabilized by the heptad repeat, designated $(abcdefg)_n$ according to the notation of McLachlan and Stewart (*J. Mol. Biol.* 98:293; 1975), in which residues *a* and *d* are generally hydrophobic residues, with *d* being a leucine, which line up on the same face of a helix. Oppositely-charged residues commonly occur at positions *g* and *e*. Thus, in a parallel coiled coil formed from two helical leucine zipper domains, the "knobs" formed by the hydrophobic side chains of the first helix are packed into the "holes" formed between the side chains of the second helix.

The leucine residues at position *d* contribute large hydrophobic stabilization energies, and are important for dimer formation (Krystek et al., *Int. J. Peptide Res.* 38:229, 1991). Lovejoy et al. recently reported the synthesis of a triple-stranded α-helical bundle in which the helices run up-up-down (*Science* 259:1288, 1993). Their studies confirmed that hydrophobic stabilization energy provides the main driving force for the formation of coiled coils from helical monomers. These studies also indicate that electrostatic interactions contribute to the stoichiometry and geometry of coiled coils.

Several studies have indicated that conservative amino acids may be substituted for individual leucine residues with minimal decrease in the ability to dimerize; multiple changes, however, usually result in loss of this ability (Landschulz et al., *Science* 243:1681, 1989; Turner and Tjian, *Science* 243:1689, 1989; Hu et al., *Science* 250:1400, 1990). van Heekeren et al. reported that a number of different amino residues can be substituted for the leucine residues in the leucine zipper domain of GCN4, and further found that some GCN4 proteins containing two leucine substitutions were weakly active (*Nucl. Acids Res.* 20:3721, 1992). Mutation of the first and second heptadic leucines of the leucine zipper domain of the measles virus fusion protein (MVF) did not affect syncytium formation (a measure of virally-induced cell fusion); however, mutation of all four leucine residues prevented fusion completely (Buckland et al., *J. Gen. Virol.* 73:1703, 1992). None of the mutations affected the ability of MVF to form a tetramer.

Amino acid substitutions in the *a* and *d* residues of a synthetic peptide representing the GCN4 leucine zipper domain have been found to change the oligomerization properties of the leucine zipper domain (Alber, Sixth Symposium of the Protein Society, San Diego, CA). When all residues at position *a* are changed to isoleucine, the leucine zipper still forms a parallel dimer. When, in addition to this change, all leucine residues at position *d* are also changed to isoleucine, the resultant peptide spontaneously forms a trimeric parallel coiled coil in solution. Substituting all amino acids at position *d* with isoleucine and at position *a* with leucine results in a peptide that tetramerizes. Peptides containing these substitutions are still referred to as leucine zipper domains.

The present invention also includes RANK with or without associated native-pattern glycosylation. Proteins expressed in yeast or mammalian expression systems, e.g., COS-7 cells, may be similar or slightly different in molecular weight and glycosylation pattern than the native molecules, depending upon the expression system. Expression of DNAs encoding the inventive proteins in bacteria such as *E. coli* provides non-glycosylated molecules. Functional mutant analogs of RANK protein having inactivated N-glycosylation sites can be produced by oligonucleotide synthesis and ligation or by site-specific mutagenesis techniques. These analog proteins can be produced in a homogeneous, reduced-carbohydrate form in good yield using yeast expression systems. N-glycosylation sites in eukaryotic proteins are characterized by the amino acid triplet Asn-A_1-Z, where A_1 is any amino acid except Pro, and Z is Ser or Thr. In this sequence, asparagine provides a side chain amino group for covalent attachment of carbohydrate. Such a site can be eliminated by substituting another amino acid for Asn or for residue Z, deleting Asn or Z, or inserting a non-Z amino acid between A_1 and Z, or an amino acid other than Asn between Asn and A_1.

RANK protein derivatives may also be obtained by mutations of the native RANK or subunits thereof. A RANK mutated protein, as referred to herein, is a polypeptide homologous to a native RANK protein, but which has an amino acid sequence different from the native protein because of one or a plurality of deletions, insertions or substitutions. The effect of any mutation made in a DNA encoding a mutated protein may be easily determined by analyzing the ability of the mutated protein to bind its counterstructure in a specific manner. Moreover, activity of RANK analogs, muteins or derivatives can be determined by any of the assays described herein (for example, inhibition of the ability of RANK to activate transcription, as shown, e.g., in Example 5).

The present invention encompasses RANK DNAs containing mutations consisting of duplications in the signal peptide of RANK, e.g., the mutations associated with FEO and EP. The invention also encompasses the polypeptides encoded by isolated RANK DNAs having mutations in their signal peptide coding region. Such mutations in the RANK gene will result in duplications encompassing between one and ten consecutive amino acids in the signal peptide of the wild-type RANK protein. Thus, the duplicated amino acids are located within residues 1-34 of SEQ ID NO:6.

As described in Example 25, FEO and EP mutations were identified by screening families of individuals diagnosed with FEO and EP for mutations in all the exons, intron-exon junction, 5' and 3' UTRs and promoter region of the RANK gene, using direct sequencing of PCR products. The screening tests led to the discovery of RANK gene mutations that include tandem duplication of bases 84 to 101 (84dup18) of SEQ ID NO:5 in FEO and duplication of bases 75 to 101 (75dup27) of SEQ ID NO:5 in EP. The FEO and EP duplications produce share the same 3' endpoint at base 101. These duplications in the gene result in a 6 or 9 amino acid duplication in the signal peptide of the FEO and EP RANK proteins, respectively. The full FEO RANK DNA sequence of the present invention is shown in SEQ ID NO:20. The full EP RANK DNA sequence of the present invention is shown in SEQ ID NO:22. The coding regions of SEQ ID NOs:20 and 22 extend from nucleotides 39-1904 and 39-1913, respectively. The encoded polypeptides of SEQ ID NO:20 and SEQ ID NO:22 are shown in SEQ ID NO:21 and SEQ ID NO:23, respectively.

The invention also provides recombinant expression vectors containing the coding regions of the mutant RANK DNAs of SEQ ID NOS:20 and 22, as well as vectors containing DNAs encoding only the extracellular portions of the mutated FEO and EP RANK proteins. If desired, such vectors can be designed to direct the secretion of the expressed RANK proteins. Provided also are host cells transfected or transformed with such vectors, and processes for preparing the mutant polypeptides that are expressed by these transformed host cells.

In addition, the invention provides RANK polypeptides having the amino acid sequences shown in SEQ ID NOS:21 and 23, as well as RANKL-binding or TRAF-binding fragments thereof. Provided also are antibodies capable of specifically binding with epitopes located at the NH_2-terminus of RANK EP and RANK FEO. These epitopes encompass the uncleaved signal peptides containing duplicated amino acids that are characteristic of these mutant proteins, i.e., amino acids 1-40 of SEQ ID NO:21 (FEO) and amino acids 1-43 of SEQ ID NO:23 (EP). The antibodies are prepared according to standard procedures using RANK EP or RANK FEO or fragments thereof as the source of antigen.

The FEO mutant protein of SEQ ID NO:21 has a predicted signal peptide cleavage site that follows any one of amino acid residues 30-39 (of SEQ ID NO:21), and the EP mutant protein of SEQ ID NO:23 has a predicted signal peptide cleavage site that follows any one of amino acid residues 33-42 (of SEQ ID NO:23). When FEO RANK and EP RANK were expressed as recombinant proteins, however, no signal peptide cleavage was detected. Example 26 describes expression analysis of wild type, FEO and EP RANK using metabolically labeled transfected mammalian cells. The results showed that relative to wild type RANK, both FEO RANK and EP RANK were approximately 3 kilodaltons larger than wild type RANK when the labeled and immunoprecipitated wild type and mutant RANK proteins were resolved on polyacrylamide Tris-glycine-SDS gels. The extra 3 kilodaltons in molecular weight of the mutant RANK proteins compared to wild type RANK is consistent with the lack of cleavage of the approximately 30 amino acid predicted signal peptides of the mutant RANK proteins. This lack of appropriate signal peptide cleavage may result in the inappropriate intracellular trafficking of the mutant RANK proteins, possibly leading to abnormal accumulation of mutant RANK proteins within intracellular compartments. This abnormal accumulation may in turn lead to self-association of mutant RANK molecules causing elevated levels of constitutive RANK signal transduction.

One consequence of RANK signal transduction is the activation of the transcription factor NF-κB. This activation can be assessed in a co-transfection assay to study the effects of mutations in RANK on RANK signal transduction. Example 27 describes expression analysis of mutant RANK using mammalian cells co-transfected with an NF-κB responsive luciferase reporter plasmid and wild type RANK, FEO RANK or EP RANK. The results show that the six and nine amino acid duplications in the RANK signal peptide found in FEO and EP RANK, respectively, result in a higher level of luciferase activity compared with wild type RANK, indicating that these mutations result in increased RANK activity.

The discovery that RANK mutations are related to bone disease suggests that restoring normal RANK function using gene therapy techniques is a therapeutic approach

to treating such bone diseases. Such treatment can include using gene transfer tecniques to introduce a gene encoding RANK having normal biological function into cells obtained from an individual with FEO, EP or other disorders associated with a mutated RANK gene. Suitable recipient cells include hematopoietic stem cells, or partially differentiated cells that are precursors of mature B cells, T cells, epithelial cells or osteoclasts. After isolating and transfecting the cells with a normal RANK gene under circumstances in which the mutant gene is inactivated, the cells can be re-administered to the individual to obtain normal RANK expression to ameliorate the effects of the mutant RANK gene.

Analogs of the inventive proteins may be constructed by, for example, making various substitutions of residues or sequences or deleting terminal or internal residues or sequences not needed for biological activity. For example, cysteine residues can be deleted or replaced with other amino acids to prevent formation of incorrect intramolecular disulfide bridges upon renaturation. Other approaches to mutagenesis involve modification of adjacent dibasic amino acid residues to enhance expression in yeast systems in which KEX2 protease activity is present.

When a deletion or insertion strategy is adopted, the potential effect of the deletion or insertion on biological activity should be considered. Subunits of the inventive proteins may be constructed by deleting terminal or internal residues or sequences. Soluble forms of RANK can be readily prepared and tested for their ability to inhibit RANK-induced NF-κB activation. Polypeptides corresponding to the cytoplasmic regions, and fragments thereof (for example, a death domain) can be prepared by similar techniques. Additional guidance as to the types of mutations that can be made is provided by a comparison of the sequence of RANK to proteins that have similar structures, as well as by performing structural analysis of the inventive RANK proteins.

Generally, substitutions should be made conservatively; i.e., the most preferred substitute amino acids are those which do not affect the biological activity of RANK (for example, the ability of the inventive proteins to bind antibodies to the corresponding native protein in a substantially equivalent manner, the ability to bind the counterstructure in substantially the same manner as the native protein, the ability to transduce a RANK signal, or ability to induce NF-κB activation upon overexpression in transient transfection systems). Examples of conservative substitutions include substitution of amino acids outside of the binding domain(s) (either ligand/receptor or antibody binding areas for the extracellular domain, or regions that interact with other, intracellular proteins for the cytoplasmic domain), and substitution of amino acids that do not alter the secondary and/or tertiary structure of the native protein. Additional examples include substituting one aliphatic residue for another, such as Ile, Val, Leu, or Ala for one another, or substitutions of one polar residue for another, such as between Lys and Arg; Glu and Asp; or Gln and Asn. Other such conservative substitutions, for example, substitutions of

entire regions having similar hydrophobicity characteristics, are well known and within the scope of the invention.

Mutations in nucleotide sequences constructed for expression of analog proteins or fragments thereof must, of course, preserve the reading frame phase of the coding sequences and preferably will not create complementary regions that could hybridize to produce secondary mRNA structures such as loops or hairpins which would adversely affect translation of the mRNA.

Not all mutations in the nucleotide sequence which encodes a RANK protein or fragments thereof will be expressed in the final product, for example, nucleotide substitutions may be made to enhance expression, primarily to avoid secondary structure loops in the transcribed mRNA (see EPA 75,444A, incorporated herein by reference), or to provide codons that are more readily translated by the selected host, e.g., the well-known *E. coli* preference codons for *E. coli* expression.

Although a mutation site may be predetermined, it is not necessary that the nature of the mutation *per se* be predetermined. For example, in order to select for optimum characteristics of mutants, random mutagenesis may be conducted and the expressed mutated proteins screened for the desired activity. Mutations can be introduced at particular loci by synthesizing oligonucleotides containing a mutant sequence, flanked by restriction sites enabling ligation to fragments of the native sequence. Following ligation, the resulting reconstructed sequence encodes an analog having the desired amino acid insertion, substitution, or deletion.

Alternatively, oligonucleotide-directed site-specific mutagenesis procedures can be employed to provide an altered gene having particular codons altered according to the substitution, deletion, or insertion required. Exemplary methods of making the alterations set forth above are disclosed by Walder et al. (*Gene 42*:133, 1986); Bauer et al. (*Gene 37*:73, 1985); Craik (*BioTechniques*, January 1985, 12-19); Smith et al. (*Genetic Engineering: Principles and Methods*, Plenum Press, 1981); and U.S. Patent NOs. 4,518,584 and 4,737,462 disclose suitable techniques, and are incorporated by reference herein.

Other embodiments of the inventive proteins include RANK polypeptides encoded by DNAs capable of hybridizing to the DNA of SEQ ID NO:6 under moderately stringent conditions (prewashing solution of 5 X SSC, 0.5% SDS, 1.0 mM EDTA (pH 8.0) and hybridization conditions of 50°C, 5 X SSC, overnight) to the DNA sequences encoding RANK, or more preferably under stringent conditions (for example, hybridization in 6 X SSC at 63°C overnight; washing in 3 X SSC at 55°C), and other sequences which are degenerate to those which encode the RANK.

In one embodiment, RANK polypeptides are at least about 70% identical in amino acid sequence to the amino acid sequence of native RANK protein as set forth in SEQ ID

NO:5. In a preferred embodiment, RANK polypeptides are at least about 80% identical in amino acid sequence to the native form of RANK; most preferred polypeptides are those that are at least about 90% identical to native RANK.

Amino acid sequence identity as used herein is the number of aligned amino acids which are identical, divided by the total number of amino acids in the shorter of the two sequences being compared. After aligning the two amino acid sequences, percent identity is calculated according to the following algorithm: (1) a unary comparison matrix (containing a value of 1 for identities and 0 for non-identities) (see, e.g., Gribskov and Burgess, *Nucl. Acids Res. 14:*6745, 1986, as described by Schwartz and Dayhoff, eds., *Atlas of Protein Sequence and Structure,* National Biomedical Research Foundation, pp. 353-358, 1979); (2) a penalty of 3.0 for each gap and an additional 0.10 penalty for each symbol in each gap; and (3) no penalty for end gaps. A number of computer programs are available commercially for aligning sequences and determining sequence identities. When the aforedescribed algorithm is used, the same percent identity is expected to be obtained regardless of which computer program is employed to align the sequences and perform the calculations. Computer programs suitable for running this algorithm include the GAP program, version 6.0, described by Devereux et al. (*Nucl. Acids Res.* 12:387, 1984), or the BESTFIT program, both of which are available from the University of Wisconsin Genetics Computer Group (UWGCG) as part of the GCG computer package for sequence manipulation. For fragments derived from the RANK protein, the identity is calculated based on that portion of the RANK protein that is present in the fragment

The biological activity of RANK analogs or muteins can be determined by testing the ability of the analogs or muteins to inhibit activation of transcription, for example as described in the Examples herein. Alternatively, suitable assays, for example, an enzyme immunoassay or a dot blot, employing an antibody that binds native RANK, or a soluble form of RANKL, can be used to assess the activity of RANK analogs or muteins, as can assays that employ cells expressing RANKL. Suitable assays also include, for example, signal transduction assays and methods that evaluate the ability of the cytoplasmic region of RANK to associate with other intracellular proteins (i.e., TRAFs 2 and 3) involved in signal transduction will also be useful to assess the activity of RANK analogs or muteins. Such methods are well known in the art.

Fragments of the RANK nucleotide sequences are also useful. In one embodiment, such fragments comprise at least about 17 consecutive nucleotides, preferably at least about 25 nucleotides, more preferably at least 30 consecutive nucleotides, of the RANK DNA disclosed herein. DNA and RNA complements of such fragments are provided herein, along with both single-stranded and double-stranded forms of the RANK DNA of SEQ ID NO:5, and those encoding the aforementioned polypeptides. A fragment of RANK DNA generally comprises at least about 17

nucleotides, preferably from about 17 to about 30 nucleotides. Such nucleic acid fragments (for example, a probe corresponding to the extracellular domain of RANK) are used as a probe or as primers in a polymerase chain reaction (PCR).

The probes also find use in detecting the presence of RANK nucleic acids in *in vitro* assays and in such procedures as Northern and Southern blots. Cell types expressing RANK can be identified as well. Such procedures are well known, and the skilled artisan can choose a probe of suitable length, depending on the particular intended application. For PCR, 5' and 3' primers corresponding to the termini of a desired RANK DNA sequence are employed to amplify that sequence, using conventional techniques. PCR primers generally are between 15 and 50 nucleotides in length, though if desired, shorter or longer primers may be used.

Other useful fragments of the RANK nucleic acids are antisense or sense oligonucleotides comprising a single-stranded nucleic acid sequence (either RNA or DNA) capable of binding to target RANK mRNA (sense) or RANK DNA (antisense) sequences. The ability to create an antisense or a sense oligonucleotide, based upon a cDNA sequence for a given protein is described in, for example, Stein and Cohen, *Cancer Res.* 48:2659, 1988 and van der Krol et al., *BioTechniques* 6:958, 1988. Nucleic acid fragments such as these be used, for example, to partly or completely abrogate RANK expression, e.g., by interfering with transcription or translation.

Uses of DNAs, Proteins and Analogs

The RANK DNAs, proteins, fragments, muteins and analogs described herein have numerous uses, including the preparation of pharmaceutical compositions, as therapeutics in such compositions, for infecting target cells for use in gene therapy applications, in diagnosing diseases associated with RANK and as targets for use in screening assays. For example, soluble forms of RANK will be useful as antagonists of RANK-mediated NF-κB activation, as well as to inhibit transduction of a signal via RANK. RANK compositions (both protein and DNAs) will also be useful in development of both agonistic and antagonistic antibodies to RANK. RANK DNA and DNA encoding RANK fragments are useful in preparing recombinant RANK and RANK fragments utilizing host cells transformed with vectors that include the DNA.

RANK fragments and RANK oligonucleotides are useful in diagnosing diseases associated with RANK, e.g. FEO and EP. In particular, RANK oligonucleotides that include the above described tandem duplication mutations can be prepared and used in hybridization assays to confirm the presence of the mutations in patients suspected of being afflicted with FEO or EP.

NF-κB reporter assays described in Example 27 demonstrate an increase in reporter activation by cells transfected with cDNA encoding mutant FEO or EP RANK.

Accordingly, RANK molecules that include these mutations (e.g. the RANK of SEQ ID NO:20 and SEQ ID NO:22) are useful as therapeutics for indications in which a more active RANK is advantageous. For example, cells expressing a more active RANK can be used to boost osteoclast activity in patients with certain bone remodeling dysfunctions such as osteopetrosis.

The RANK cytoplasmic domain and soluble fragments of the RANK cytoplasmic domain are inhibitors of TRAF/RANK interactions and find utility in *in vitro* screening assays and *in vivo* therapeutics. As therapeutics that are cell membrane permeable, the RANK cytoplasmic domain and amino acid fragments of the RANK cytoplasmic domain that are necessary for TRAF binding can be administered to bind one or more TRAFs and antagonize RANK mediated triggering of the NF-κB and/or JNK signaling pathways. (See examples 20 and 21). Advantageously, soluble fragments that specifically disrupt, for example, the TRAF6/RANK association or inhibit the TRAF6/RANK association are not likely to effect signaling from other TNF receptors and are useful immunosuppressants or anti-inflammatory agents. Similarly, fragments that enhance or increase TRAF6/RANK association and the resulting TRAF6 signaling can be useful immuno upregulators, e.g. provide increased DC function. Additionally, a soluble RANK cytoplasmic domain fragment that specifically interrupts the TRAF6 mediated RANK signaling that leads to osteoclast differentiation and function can be useful in inhibiting osteoclast formation, and osteoclast activity as well as the effects of excessive bone resorption.

Fragments of the RANK cytoplasmic domain useful in pharmaceutical compositions include those fragments that are capable of binding TRAFS 1, 2, 3, 5, and 6. (See Examples 19 and 20). With respect to TRAF6 activity and association, useful RANK cytoplasmic fragments include the COOH-terminal 72 amino acids (amino acids 545-616 of SEQ ID NO:6), amino acids 339-422 of SEQ ID NO:6, and amino acids 339-362 of SEQ ID NO:6. RANK fragments that are modulators of RANK association with TRAFS1, 2, 3 and 5 include the COOH-terminal 72 amino acids (amino acids 545-616 of SEQ ID NO:6).

The present invention encompasses methods for screening test compounds for their ability to modulate TRAF/RANK interactions and their ability to modulate activities mediated by TRAF/RANK interactions. Additionally, the present invention includes compounds that modulate RANK/TRAF interactions and that are identified by the screening methods of the present invention. In general, screening methods involve allowing a RANK or RANK polypeptide fragment that is known to bind or interact with a TRAF, to interact with the TRAF under conditions in which the RANK is known to bind or interact with the TRAF. The RANK/TRAF interaction is allowed to occur in the presence of a test compound or the test compound is allowed to contact the RANK/TRAF

subsequent to their interaction. By observing the effect that the test compound has on the known binding characteristics of the RANK or the RANK fragment, compounds that enhance RANK/TRAF binding or inhibit RANK/TRAF binding can be identified.

Typical test compounds are small molecules or peptides and may be part of extensive small molecule libraries developed for use in screening methods. RANK proteins that may be used in screening methods include full length RANK, the RANK cytoplasmic domain, RANK fragments, and RANK mutants or RANK analogs that bind a TRAF. Particularly useful RANK fragments include the cytoplasmic domain and the cytoplasmic domain fragments identified above. Particularly, useful TRAFs include TRAF6 and TRAF6/TRAF3, TRAF6/TRAF2 heterocomplexes. TRAFs may be recombinantly prepared and used directly in cells or isolated. Similarly, native TRAFs can be used in cells or isolated and used in *in vitro* assays.

Specific screening methods are known in the art and many are extensively incorporated in high throughput test systems so that large numbers of test compounds can be screened within a short amount of time. Suitable screening methods can be performed in a variety of formats including, but not limited to, binding assay screens, functional assay screens and cell based screens. By observing the affect that test compounds have on TRAF/RANK binding in binding assays, on TRAF/RANK mediated activity in functional tests and in cell based screens, compounds are identified that are potential therapeutics because they can modulate the TRAF/RANK interaction.

Binding assays and their use in screening methodologies are known in the art. For example, U.S. patent no. 5,767,244 (incorporated herein by reference) describes methods useful for screening compounds that are active at the level of a TRAF6 modulatable cellular function. In particular, RANK binding assays can be used to screen for test compounds that are capable of modulating TRAF/RANK binding. Suitable assays include standard protein-protein interaction tests that demonstrate the presence or absence of protein-protein interactions and measure binding affinities (See, for example, Example 22). Typically, such binding assays involve incubating a test mixture under conditions in which the desired TRAF and RANK protein, RANK fragment or RANK analog binds with a known binding affinity. Forms of RANK that are particularly useful in screening for modulators of TRAF6/RANK interaction include the full length RANK cytoplasmic domain (amino acids 234-616 of SEQ ID NO:6) and fragments of the RANK cytoplasmic domain that are capable of binding TRAF6 (see Examples 19 and 20). Such binding fragments include the COOH-terminal 72 amino acids (amino acids 545-616 of SEQ ID NO:6), amino acids 339-362 of SEQ ID NO:6, and amino acids 339-422 of SEQ ID NO:6. Forms of RANK that are useful in screening for modulators of RANK association with TRAFS1, 2, 3 and 5 include the COOH-terminal 72 amino acids (amino acids 545-616 of SEQ ID NO:6). Fragments of the COOH-terminal 72 amino acids that include

amino acids 571-573 are useful in screening for modulators of RANK association with TRAF3. Similarly, fragments of the COOH-terminal 72 amino acids that include amino acids 609-610 are useful in screening for modulators of RANK association with TRAF1, 2 and 5. Modulators that inhibit TRAF/RANK association are useful TRAF/RANK antagonists and those that enhance TRAF/RANK association are useful agonists of activities mediated by the TRAF/RANK interaction.

The present invention includes polypeptides that bind one or more TRAFs and have an amino acid sequence that is at least 80% identical to amino acids 545-616, amino acids 339-442, or amino acids 234-616 of SEQ ID. Identity can be determined using the GAP computer program as described above.

Protein-protein interactions can be observed and measured in binding assays using a variety of detection methodologies that include, but are not limited to, surface plasmon resonance (Biacore), radioimmune based assays, and fluorescence polarization binding assays. When performed in the presence of a test compound, the ability of the test compound to modulate (e.g. inhibit or enhance) the protein-protein binding affinity is measured. Test compounds shown to inhibit TRAF/RANK interaction are therapeutic inhibitors of RANK mediated activities. For example, inhibitors of TRAF6/RANK associations are useful as antagonists of RANK mediated triggering of the NF-κB and/or JNK signaling pathways and can be immunosupressants or anti-inflammatory therapeutics. TRAF6 binding inhibitors shown to interrupt the RANK/RANKL signaling process that leads to osteoclast differentiation are also suitable therapeutics for the treatment of bone loss and the effects of excess bone resorption such as hypercalcemia.

RANK protein, RANK fragments (including RANK cytoplasmic domain fragments identified above), mutants and analogs are also useful in cell based assay methods that screen for test compounds which are inhibitors or modulators of the TRAF/RANK interactions. Advantageously, cell based assays are mechanism based and can be designed to assay test compounds for their cell membrane permeability characteristics; their ability to modulate TRAF/RANK interactions; their ability to selectivity modulate a specific TRAF/RANK mediated activity; and their cell toxicity characteristics. A number of cell based methods are known in the art. Many of the assays are based upon a yeast two-hybrid assay or mammalian two-hybrid assay. (See White, *Proc. Natl. Acad. Sci. USA 93*:10001-10003, 1996). Yeast two hybrid assays as they relate to selecting small molecule inhibitors of protein-protein interactions are described in Huang et al. *Proc. Natl. Acad. Sci 94*:13396-13401, 1997. Typically, these assays involve expressing proteins (e.g. RANK and TRAF6) whose interaction triggers a reporter gene. Test compounds that are cell permeable can be identified for their ability to modulate the RANK/TRAF6 interaction as noted by a difference in the reporter gene

triggering as compared with the reporter gene triggering in the absence of the test compound.

Additional assays that are useful for discovering modulators of RANK/TRAF interactions include *in vivo* functional assays. For example, test compounds can be screened for their ability to inhibit osteoclast maturation and/or osteoclast activity by incubating osteoclast precursors in the presence of CSF-1, RANKL, and a test compound of interest under conditions known to induce osteoclast formation or under conditions known to induce osteoclast function. Functional assays that result in inhibited or no osteoclast formation or activity can be determined by standard TRAP assays as described in Example 23.

DNAs of the present invention are useful for the expression of recombinant proteins, and as probes for analysis (either quantitative or qualitative) of the presence or distribution of RANK transcripts and the presence of RANK mutations, e.g. those associated with FEO and EP. RANK DNA of the present invention is particularly useful in gene therapy applications in which a gene encoding normal functioning RANK is introduced into appropriate cells obtained from an individual with a RANK associated disease. Such diseases include, for example, FEO and EP, which result from autosomal dominant mutations in RANK. To treat FEO and EP, stem cells are first isolated from the patient, then the patient is subjected to radiation to ablate their bone marrow cells. The isolated stem cells are treated to replace the defective gene with a copy of wild-type RANK, then the cells can be re-administered to the individual, thus ameliorating the effects of the disease in the individual. Numerous methods have been developed for introducing exogenous genes into mammalian cells, such as by transfection or by infection. The defective copy of RANK in the recipient cells can be replaced by directed recombination with the transfecting plasmid. Alternatively, cells from a RANK+/RANK+ immunologically compatible donor can be used to reconstitute the patient's bone marrow. These transduction methods may be physical in nature, or they may rely on the use of recombinant retroviral vectors encoding DNA which can be transcribed to RNA, packed into infectious viral particles and used to infect target cells and thereby deliver the desired genetic material.

Many different types of mammalian gene transfer and expression vectors have been developed (see, Miller and Calos, eds., "Gene Transfer Vectors for Mammalian Cells," *Current Comm. Mol. Biol.*, Cold Spring Harbor Laboratory, New York, 1987). Naked DNA can be physically introduced into mammalian cells by transfection using any one of a number of techniques including, but not limited to, calcium phosphate transfection (Berman et al., *Proc. Natl. Acad. Sc. USA 84* 81:7176, 1984); DEAE-Dextran transfect, protoplast fusion (Deans et al., *Proc. Natl. Acad. Sci. USA 84* 81:1292, 1984); electroporation (Potter et al., *Proc. Natl. Acad. Sci USA 84* 81:7176, 1984); lipofection

(Felgner et al., *Proc. Natl. Acad. Sci. USA 84*:7413, 1987), polybrene transfection (Kawai and Nishzawa, *Mol. Cell. Biol. 4*:1172, 1984) and direct gene transfer by lasermicropuncture of cell membranes (Tao et al., Proc. *Natl. Acad. Sci. USA 84*:4180, 1987).

Various infection techniques have been developed which utilize recombinant infectious, virus particles for gene delivery. This represents a preferred approach to the present invention. The viral vectors which have been used in this manner include virus vectors derived from simian virus 40 (SV40; Karlsson et al., *Proc. Natl. Acad. Sci. USA 84* 82:158, 1985); adenoviruses (Karlsson et al., *EMBO J.* 5:2377, 1986); adeno-associated virus (LaFace et al., *Virology 162*:483, 1988) and retroviruses (Coffin, 1985, p17-71 in Weiss et al (eds.), *RNA Tumor Viruses*, 2nd ed., Vol 2. Cold Spring Harbor Laboratory, New York.

Gene transfer and expression methods are numerous and essentially function to introduce and express genetic material in mammalian cells. Several of the above described techniques have been used to transduce hematopoietic or lymphoid cells, including calcium phosphate transfection (Berman et al., *supra*, 1984); protoplast fusion (Deans et al., *supra* 1984); electroporation (Cann et al. *Oncogene 3*:123, 1988) and infection with recombinant adenovirus (Karlsson et al., *supra*; Ruether et al. *Mol. Cell Biol. 6*:123, 1986); adeno-associated virus (LaFace et al., *supra*); and, retrovirus vector (Overell et al., *Oncogene 4*:1425, 1989). Primary T lymphocytes have been successfully transduced by electroporation (Cann et al., *supra,* 1988) and by retroviral infection (Nishihara et al., *Cancer Res 48*:4730, 1988); Kasid et al., *supra*, 1990).

The inventive proteins will also be useful in preparing kits that are used to detect soluble RANK or RANKL, or monitor RANK-related activity, for example, in patient specimens. RANK proteins will also find uses in monitoring RANK-related activity in other samples or compositions, as is necessary when screening for antagonists or mimetics of this activity (for example, peptides or small molecules that inhibit or mimic, respectively, the interaction). A variety of assay formats are useful in such kits, including (but not limited to) ELISA, dot blot, solid phase binding assays (such as those using a biosensor), rapid format assays and bioassays.

The purified RANK according to the invention will facilitate the discovery of inhibitors of RANK, and thus, inhibitors of an inflammatory response (via inhibition of NF-κB activation). The use of a purified RANK polypeptide in the screening for potential inhibitors is important and can virtually eliminate the possibility of interfering reactions with contaminants. Such a screening assay can utilize the extracellular domain of RANK, the intracellular domain, or a fragment of either of these polypeptides. Detecting the inhibiting activity of a molecule would typically involve use of a soluble form of RANK derived from the extracellular domain in a screening assay to detect

molecules capable of binding RANK and inhibiting binding of, for example, an agonistic antibody or RANKL, or using a polypeptide derived from the intracellular domain in an assay to detect inhibition of the interaction of RANK and other, intracellular proteins involved in signal transduction.

Moreover, in vitro systems can be used to ascertain the ability of molecules to antagonize or agonize RANK activity. Included in such methods are uses of RANK chimeras, for example, a chimera of the RANK intracellular domain and an extracellular domain derived from a protein having a known ligand. Utilizing the known ligand to transduce a signal can then monitor the effects on signal transduction of various molecules.

In addition, RANK polypeptides can also be used for structure-based design of RANK-inhibitors. Such structure-based design is also known as "rational drug design." The RANK polypeptides can be three-dimensionally analyzed by, for example, X-ray crystallography, nuclear magnetic resonance or homology modeling, all of which are well-known methods. The use of RANK structural information in molecular modeling software systems to assist in inhibitor design is also encompassed by the invention. Such computer-assisted modeling and drug design may utilize information such as chemical conformational analysis, electrostatic potential of the molecules, protein folding, etc. A particular method of the invention comprises analyzing the three dimensional structure of RANK for likely binding sites of substrates, synthesizing a new molecule that incorporates a predictive reactive site, and assaying the new molecule as described above.

Expression of Recombinant RANK

The proteins of the present invention are preferably produced by recombinant DNA methods by inserting a DNA sequence encoding RANK protein or an analog thereof into a recombinant expression vector and expressing the DNA sequence in a recombinant expression system under conditions promoting expression. DNA sequences encoding the proteins provided by this invention can be assembled from cDNA fragments and short oligonucleotide linkers, or from a series of oligonucleotides, to provide a synthetic gene which is capable of being inserted in a recombinant expression vector and expressed in a recombinant transcriptional unit.

Recombinant expression vectors include synthetic or cDNA-derived DNA fragments encoding RANK, or homologs, muteins or bioequivalent analogs thereof, operably linked to suitable transcriptional or translational regulatory elements derived from mammalian, microbial, viral or insect genes. Such regulatory elements include a transcriptional promoter, an optional operator sequence to control transcription, a sequence encoding suitable mRNA ribosomal binding sites, and sequences which control the termination of transcription and translation, as described in detail below. The ability

to replicate in a host, usually conferred by an origin of replication, and a selection gene to facilitate recognition of transformants may additionally be incorporated.

DNA regions are operably linked when they are functionally related to each other. For example, DNA for a signal peptide (secretory leader) is operably linked to DNA for a polypeptide if it is expressed as a precursor which participates in the secretion of the polypeptide; a promoter is operably linked to a coding sequence if it controls the transcription of the sequence; or a ribosome binding site is operably linked to a coding sequence if it is positioned so as to permit translation. Generally, operably linked means contiguous and, in the case of secretory leaders, contiguous and in reading frame. DNA sequences encoding RANK, or homologs or analogs thereof, which are to be expressed in a microorganism, will preferably contain no introns that could prematurely terminate transcription of DNA into mRNA.

Useful expression vectors for bacterial use can comprise a selectable marker and bacterial origin of replication derived from commercially available plasmids comprising genetic elements of the well known cloning vector pBR322 (ATCC 37017). Such commercial vectors include, for example, pKK223-3 (Pharmacia Fine Chemicals, Uppsala, Sweden) and pGEM1 (Promega Biotec, Madison, WI, USA). These pBR322 "backbone" sections are combined with an appropriate promoter and the structural sequence to be expressed. *E. coli* is typically transformed using derivatives of pBR322, a plasmid derived from an *E. coli* species (Bolivar et al., *Gene 2*:95, 1977). pBR322 contains genes for ampicillin and tetracycline resistance and thus provides simple means for identifying transformed cells.

Promoters commonly used in recombinant microbial expression vectors include the β-lactamase (penicillinase) and lactose promoter system (Chang et al., *Nature 275*:615, 1978; and Goeddel et al., *Nature 281*:544, 1979), the tryptophan (trp) promoter system (Goeddel et al., *Nucl. Acids Res. 8*:4057, 1980; and EPA 36,776) and tac promoter (Maniatis, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, p. 412, 1982). A particularly useful bacterial expression system employs the phage λ P_L promoter and cI857ts thermolabile repressor. Plasmid vectors available from the American Type Culture Collection which incorporate derivatives of the λ-P_L promoter include plasmid pHUB2, resident in *E. coli* strain JMB9 (ATCC 37092) and pPLc28, resident in *E. coli* RR1 (ATCC 53082).

Suitable promoter sequences in yeast vectors include the promoters for metallothionein, 3-phosphoglycerate kinase (Hitzeman et al., *J. Biol. Chem. 255*:2073, 1980) or other glycolytic enzymes (Hess et al., *J. Adv. Enzyme Reg. 7*:149, 1968; and Holland et al., *Biochem. 17*:4900, 1978), such as enolase, glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate

isomerase, phosphoglucose isomerase, and glucokinase. Suitable vectors and promoters for use in yeast expression are further described in R. Hitzeman et al., EPA 73,657.

Preferred yeast vectors can be assembled using DNA sequences from pBR322 for selection and replication in *E. coli* (Amp^r gene and origin of replication) and yeast DNA sequences including a glucose-repressible ADH2 promoter and α-factor secretion leader. The ADH2 promoter has been described by Russell et al. (*J. Biol. Chem. 258*:2674, 1982) and Beier et al. (*Nature 300*:724, 1982). The yeast α-factor leader, which directs secretion of heterologous proteins, can be inserted between the promoter and the structural gene to be expressed. *See, e.g.,* Kurjan et al., *Cell 30*:933, 1982; and Bitter et al., *Proc. Natl. Acad. Sci. USA 81*:5330, 1984. The leader sequence may be modified to contain, near its 3' end, one or more useful restriction sites to facilitate fusion of the leader sequence to foreign genes.

The transcriptional and translational control sequences in expression vectors to be used in transforming vertebrate cells may be provided by viral sources. For example, commonly used promoters and enhancers are derived from Polyoma, Adenovirus 2, Simian Virus 40 (SV40), and human cytomegalovirus. DNA sequences derived from the SV40 viral genome, for example, SV40 origin, early and late promoter, enhancer, splice, and polyadenylation sites may be used to provide the other genetic elements required for expression of a heterologous DNA sequence. The early and late promoters are particularly useful because both are obtained easily from the virus as a fragment which also contains the SV40 viral origin of replication (Fiers et al., *Nature 273*:113, 1978). Smaller or larger SV40 fragments may also be used, provided the approximately 250 bp sequence extending from the *Hind* III site toward the *BglI* site located in the viral origin of replication is included. Further, viral genomic promoter, control and/or signal sequences may be utilized, provided such control sequences are compatible with the host cell chosen. Exemplary vectors can be constructed as disclosed by Okayama and Berg (*Mol. Cell. Biol. 3*:280, 1983).

A useful system for stable high level expression of mammalian receptor cDNAs in C127 murine mammary epithelial cells can be constructed substantially as described by Cosman et al. (*Mol. Immunol. 23*:935, 1986). A preferred eukaryotic vector for expression of RANK DNA is referred to as pDC406 (McMahan et al., *EMBO J.* 10:2821, 1991), and includes regulatory sequences derived from SV40, human immunodeficiency virus (HIV), and Epstein-Barr virus (EBV). Other preferred vectors include pDC409 and pDC410, which are derived from pDC406. pDC410 was derived from pDC406 by substituting the EBV origin of replication with sequences encoding the SV40 large T antigen. pDC409 differs from pDC406 in that a *Bgl* II restriction site outside of the multiple cloning site has been deleted, making the *Bgl* II site within the multiple cloning site unique.

A useful cell line that allows for episomal replication of expression vectors, such as pDC406 and pDC409, which contain the EBV origin of replication, is CV-1/EBNA (ATCC CRL 10478). The CV-1/EBNA cell line was derived by transfection of the CV-1 cell line with a gene encoding Epstein-Barr virus nuclear antigen-1 (EBNA-1) and constitutively express EBNA-1 driven from human CMV immediate-early enhancer/promoter.

Host Cells

Transformed host cells are cells that have been transformed or transfected with expression vectors constructed using recombinant DNA techniques and which contain sequences encoding the proteins of the present invention. Transformed host cells may express the desired protein (RANK, or homologs or analogs thereof), but host cells transformed for purposes of cloning or amplifying the inventive DNA do not need to express the protein. Expressed proteins will preferably be secreted into the culture supernatant, depending on the DNA selected, but may be deposited in the cell membrane.

Suitable host cells for expression of proteins include prokaryotes, yeast or higher eukaryotic cells under the control of appropriate promoters. Prokaryotes include gram negative or gram positive organisms, for example *E. coli* or *Bacillus* spp. Higher eukaryotic cells include established cell lines of mammalian origin as described below. Cell-free translation systems could also be employed to produce proteins using RNAs derived from the DNA constructs disclosed herein. Appropriate cloning and expression vectors for use with bacterial, fungal, yeast, and mammalian cellular hosts are described by Pouwels et al. (*Cloning Vectors: A Laboratory Manual*, Elsevier, New York, 1985), the relevant disclosure of which is hereby incorporated by reference.

Prokaryotic expression hosts may be used for expression of RANK, or homologs or analogs thereof that do not require extensive proteolytic and disulfide processing. Prokaryotic expression vectors generally comprise one or more phenotypic selectable markers, for example a gene encoding proteins conferring antibiotic resistance or supplying an autotrophic requirement, and an origin of replication recognized by the host to ensure amplification within the host. Suitable prokaryotic hosts for transformation include *E. coli, Bacillus subtilis*, *Salmonella typhimurium*, and various species within the genera *Pseudomonas*, *Streptomyces*, and *Staphylococcus*, although others may also be employed as a matter of choice.

Recombinant RANK may also be expressed in yeast hosts, preferably from the *Saccharomyces* species, such as *S. cerevisiae*. Yeast of other genera, such as *Pichia* or *Kluyveromyces* may also be employed. Yeast vectors will generally contain an origin of replication from the 2μ yeast plasmid or an autonomously replicating sequence (ARS), promoter, DNA encoding the protein, sequences for polyadenylation and transcription

termination and a selection gene. Preferably, yeast vectors will include an origin of replication and selectable marker permitting transformation of both yeast and *E. coli*, e.g., the ampicillin resistance gene of *E. coli* and *S. cerevisiae* trp1 gene, which provides a selection marker for a mutant strain of yeast lacking the ability to grow in tryptophan, and a promoter derived from a highly expressed yeast gene to induce transcription of a structural sequence downstream. The presence of the trp1 lesion in the yeast host cell genome then provides an effective environment for detecting transformation by growth in the absence of tryptophan.

Suitable yeast transformation protocols are known to those of skill in the art; an exemplary technique is described by Hinnen et al., *Proc. Natl. Acad. Sci. USA 75*:1929, 1978, selecting for Trp^+ transformants in a selective medium consisting of 0.67% yeast nitrogen base, 0.5% casamino acids, 2% glucose, 10 μg/ml adenine and 20 μg/ml uracil. Host strains transformed by vectors comprising the ADH2 promoter may be grown for expression in a rich medium consisting of 1% yeast extract, 2% peptone, and 1% glucose supplemented with 80 μg/ml adenine and 80 μg/ml uracil. Derepression of the ADH2 promoter occurs upon exhaustion of medium glucose. Crude yeast supernatants are harvested by filtration and held at 4°C prior to further purification.

Various mammalian or insect cell culture systems can be employed to express recombinant protein. Baculovirus systems for production of heterologous proteins in insect cells are reviewed by Luckow and Summers, *Bio/Technology 6*:47 (1988). Examples of suitable mammalian host cell lines include the COS-7 lines of monkey kidney cells, described by Gluzman (*Cell 23*:175, 1981), and other cell lines capable of expressing an appropriate vector including, for example, CV-1/EBNA (ATCC CRL 10478), L cells, C127, 3T3, Chinese hamster ovary (CHO), HeLa and BHK cell lines. Mammalian expression vectors may comprise nontranscribed elements such as an origin of replication, a suitable promoter and enhancer linked to the gene to be expressed, and other 5' or 3' flanking nontranscribed sequences, and 5' or 3' nontranslated sequences, such as necessary ribosome binding sites, a polyadenylation site, splice donor and acceptor sites, and transcriptional termination sequences.

Purification of Recombinant RANK

Purified RANK, and homologs or analogs thereof are prepared by culturing suitable host/vector systems to express the recombinant translation products of the DNAs of the present invention, which are then purified from culture media or cell extracts. For example, supernatants from systems that secrete recombinant protein into culture media can be first concentrated using a commercially available protein concentration filter, for example, an Amicon or Millipore Pellicon ultrafiltration unit.

Following the concentration step, the concentrate can be applied to a suitable purification matrix. For example, a suitable affinity matrix can comprise a counter structure protein or lectin or antibody molecule bound to a suitable support. Alternatively, an anion exchange resin can be employed, for example, a matrix or substrate having pendant diethylaminoethyl (DEAE) groups. The matrices can be acrylamide, agarose, dextran, cellulose or other types commonly employed in protein purification. Alternatively, a cation exchange step can be employed. Suitable cation exchangers include various insoluble matrices comprising sulfopropyl or carboxymethyl groups. Sulfopropyl groups are preferred. Gel filtration chromatography also provides a means of purifying the inventive proteins.

Affinity chromatography is a particularly preferred method of purifying RANK and homologs thereof. For example, a RANK expressed as a fusion protein comprising an immunoglobulin Fc region can be purified using Protein A or Protein G affinity chromatography. Moreover, a RANK protein comprising an oligomerizing zipper domain may be purified on a resin comprising an antibody specific to the oligomerizing zipper domain. Monoclonal antibodies against the RANK protein may also be useful in affinity chromatography purification, by utilizing methods that are well-known in the art. A ligand may also be used to prepare an affinity matrix for affinity purification of RANK.

Finally, one or more reversed-phase high performance liquid chromatography (RP-HPLC) steps employing hydrophobic RP-HPLC media, e.g., silica gel having pendant methyl or other aliphatic groups, can be employed to further purify a RANK composition. Some or all of the foregoing purification steps, in various combinations, can also be employed to provide a homogeneous recombinant protein.

Recombinant protein produced in bacterial culture is usually isolated by initial extraction from cell pellets, followed by one or more concentration, salting-out, aqueous ion exchange or size exclusion chromatography steps. Finally, high performance liquid chromatography (HPLC) can be employed for final purification steps. Microbial cells employed in expression of recombinant protein can be disrupted by any convenient method, including freeze-thaw cycling, sonication, mechanical disruption, or use of cell lysing agents.

Fermentation of yeast which express the inventive protein as a secreted protein greatly simplifies purification. Secreted recombinant protein resulting from a large-scale fermentation can be purified by methods analogous to those disclosed by Urdal et al. (*J. Chromatog.* *296*:171, 1984). This reference describes two sequential, reversed-phase HPLC steps for purification of recombinant human GM-CSF on a preparative HPLC column.

Protein synthesized in recombinant culture is characterized by the presence of cell components, including proteins, in amounts and of a character which depend upon the

purification steps taken to recover the inventive protein from the culture. These components ordinarily will be of yeast, prokaryotic or non-human higher eukaryotic origin and preferably are present in innocuous contaminant quantities, on the order of less than about 1 percent by weight. Further, recombinant cell culture enables the production of the inventive proteins free of other proteins which may be normally associated with the proteins as they are found in nature in the species of origin.

Uses and Administration of RANK Compositions

The present invention provides methods of using therapeutic compositions comprising an effective amount of a protein and a suitable diluent and carrier, and methods for regulating an immune or inflammatory response. The use of RANK in conjunction with soluble cytokine receptors or cytokines, or other immunoregulatory molecules is also contemplated.

For therapeutic use, purified protein is administered to a patient, preferably a human, for treatment in a manner appropriate to the indication. Thus, for example, RANK protein compositions administered to regulate immune function can be given by bolus injection, continuous infusion, sustained release from implants, or other suitable technique. Typically, a therapeutic agent will be administered in the form of a composition comprising purified RANK, in conjunction with physiologically acceptable carriers, excipients or diluents. Such carriers will be nontoxic to recipients at the dosages and concentrations employed.

Ordinarily, the preparation of such protein compositions entails combining the inventive protein with buffers, antioxidants such as ascorbic acid, low molecular weight (less than about 10 residues) polypeptides, proteins, amino acids, carbohydrates including glucose, sucrose or dextrins, chelating agents such as EDTA, glutathione and other stabilizers and excipients. Neutral buffered saline or saline mixed with conspecific serum albumin are exemplary appropriate diluents. Preferably, product is formulated as a lyophilizate using appropriate excipient solutions (e.g., sucrose) as diluents. Appropriate dosages can be determined in trials. The amount and frequency of administration will depend, of course, on such factors as the nature and severity of the indication being treated, the desired response, the condition of the patient, and so forth.

Soluble forms of RANK and other RANK antagonists such as antagonistic monoclonal antibodies can be administered for the purpose of inhibiting RANK-induced induction of NF-κB activity. NF-κB is a transcription factor that is utilized extensively by cells of the immune system, and plays a role in the inflammatory response. Thus, inhibitors of RANK signaling will be useful in treating conditions in which signaling through RANK has given rise to negative consequences, for example, toxic or septic shock, or graft-versus-host reactions. They may also be useful in interfering with the role

of NF-κB in cellular transformation. Tumor cells are more responsive to radiation when their NF-κB is blocked; thus, soluble RANK (or other antagonists of RANK signaling) will be useful as an adjunct therapy for disease characterized by neoplastic cells that express RANK.

The following examples are offered by way of illustration, and not by way of limitation. Those skilled in the art will recognize that variations of the invention embodied in the examples can be made, especially in light of the teachings of the various references cited herein, the disclosures of which are incorporated by reference.

EXAMPLE 1

The example describes the identification and isolation of a DNA encoding a novel member of the TNF receptor superfamily. A partial cDNA insert with a predicted open reading frame having some similarity to CD40 (a cell-surface antigen present on the surface of both normal and neoplastic human B cells that has been shown to play an important role in B-cell proliferation and differentiation; Stamenkovic et al., *EMBO J.* 8:1403, 1989), was identified in a database containing sequence information from cDNAs generated from human bone marrow-derived dendritic cells (DC). The insert was excised from the vector by restriction endonuclease digestion, gel purified. labeled with ^{32}P, and used to hybridize to colony blots generated from a DC cDNA library containing larger cDNA inserts using high stringency hybridization and washing techniques (hybridization in 5xSSC, 50% formamide at 42°C overnight, washing in 0.5xSSC at 63°C); other suitable high stringency conditions are disclosed in Sambrook et al. in Molecular Cloning: A Laboratory Manual, 2nd ed. (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY; 1989), 9.52-9.55. Initial experiments yielded a clone referred to as 9D-8A (SEQ ID NO:1); subsequent analysis indicated that this clone contained all but the extreme 5' end of a novel cDNA, with predicted intron sequence at the extreme 5' end (nucleotides 1-92 of SEQ ID NO:1). Additional colony hybridizations were performed, and a second clone was isolated. The second clone, referred to as 9D-15C (SEQ ID NO:3), contained the 5' end without intron interruption but not the full 3'end. SEQ ID NO:5 shows the nucleotide and amino acid sequence of a predicted full-length protein based on alignment of the overlapping sequences of SEQ ID NOs:1 and 3.

The encoded protein was designated RANK, for receptor activator of NF-κB. The cDNA encodes a predicted Type 1 transmembrane protein having 616 amino acid residues, with a predicted 24 amino acid signal sequence (the computer predicted cleavage site is after Leu24), a 188 amino acid extracellular domain, a 21 amino acid transmembrane domain, and a 383 amino acid cytoplasmic tail. The extracellular region

of RANK displayed significant amino acid homology (38.5% identity, 52.3% similarity) to CD40. A cloning vector (pBluescriptSK-) containing human RANK sequence, designated pBluescript:huRANK (in *E. coli* DH10B), was deposited with the American Type Culture Collection, Rockville, MD (ATCC) on December 20, 1996, under terms of the Budapest Treaty, and given accession number 98285.

EXAMPLE 2

This example describes construction of a RANK DNA construct to express a RANK/Fc fusion protein. A soluble form of RANK fused to the Fc region of human IgG_1 was constructed in the mammalian expression vector pDC409 (USSN 08/571,579). This expression vector encodes the leader sequence of the Cytomegalovirus (CMV) open reading frame R27080 (SEQ ID NO:9), followed by amino acids 33-213 of RANK, followed by a mutated form of the constant domain of human IgG_1 that exhibits reduced affinity for Fc receptors (SEQ ID NO:8; for the fusion protein, the Fc portion of the construct consisted of Arg3 through Lys232). An alternative expression vector encompassing amino acids 1-213 of RANK (using the native leader sequence) followed by the IgG_1 mutein was also prepared. Both expression vectors were found to induce high levels of expression of the RANK/Fc fusion protein in transfected cells.

To obtain RANK/Fc protein, a RANK/Fc expression plasmid is transfected into CV-1/EBNA cells, and supernatants are collected for about one week. The RANK/Fc fusion protein is purified by means well-known in the art for purification of Fc fusion proteins, for example, by protein A sepharose column chromatography according to manufacturer's recommendations (i.e., Pharmacia, Uppsala, Sweden). SDS-polyacrylamide gel electrophoresis analysis indicted that the purified RANK/Fc protein migrated with a molecular weight of ~55kDa in the presence of a reducing agent, and at a molecular weight of ~110kDa in the absence of a reducing agent.

N-terminal amino acid sequencing of the purified protein made using the CMV R27080 leader showed 60% cleavage after Ala20, 20% cleavage after Pro22 and 20% cleavage after Arg28 (which is the Furin cleavage site; amino acid residues are relative to SEQ ID NO:9); N-terminal amino acid analysis of the fusion protein expressed with the native leader showed cleavage predominantly after Gln25 (80% after Gln25 and 20% after Arg23; amino acid residues are relative to SEQ ID NO:6, full-length RANK). Both fusion proteins were able to bind a ligand for RANK is a specific manner (i.e., they bound to the surface of various cell lines such as a murine thymoma cell line, EL4), indicating that the presence of additional amino acids at the N-terminus of RANK does not interfere with its ability to bind RANKL. Moreover, the construct comprising the CMV leader encoded RANK beginning at amino acid 33; thus, a RANK peptide having an N-terminus at an

amino acid between Arg23 and Pro33, inclusive, is expected to be able to bind a ligand for RANK in a specific manner.

Other members of the TNF receptor superfamily have a region of amino acids between the transmembrane domain and the ligand binding domain that is referred to as a 'spacer' region, which is not necessary for ligand binding. In RANK, the amino acids between 196 and 213 are predicted to form such a spacer region. Accordingly, a soluble form of RANK that terminates with an amino acid in this region is expected to retain the ability to bind a ligand for RANK in a specific manner. Preferred C-terminal amino acids for soluble RANK peptides are selected from the group consisting of amino acids 213 and 196 of SEQ ID NO:6, although other amino acids in the spacer region may be utilized as a C-terminus.

EXAMPLE 3

This example illustrates the preparation of monoclonal antibodies against RANK. Preparations of purified recombinant RANK, for example, or transfected cells expressing high levels of RANK, are employed to generate monoclonal antibodies against RANK using conventional techniques, such as those disclosed in U.S. Patent 4,411,993. DNA encoding RANK can also be used as an immunogen, for example, as reviewed by Pardoll and Beckerleg in *Immunity* 3:165, 1995. Such antibodies are likely to be useful in interfering with RANK-induced signaling (antagonistic or blocking antibodies) or in inducing a signal by cross-linking RANK (agonistic antibodies), as components of diagnostic or research assays for RANK or RANK activity, or in affinity purification of RANK.

To immunize rodents, RANK immunogen is emulsified in an adjuvant (such as complete or incomplete Freund's adjuvant, alum, or another adjuvant, such as Ribi adjuvant R700 (Ribi, Hamilton, MT), and injected in amounts ranging from 10-100 μg subcutaneously into a selected rodent, for example, BALB/c mice or Lewis rats. DNA may be given intradermally (Raz et al., *Proc. Natl. Acad. Sci. USA* 91:9519, 1994) or intamuscularly (Wang et al., *Proc. Natl. Acad. Sci. USA* 90:4156, 1993); saline has been found to be a suitable diluent for DNA-based antigens. Ten days to three weeks days later, the immunized animals are boosted with additional immunogen and periodically boosted thereafter on a weekly, biweekly or every third week immunization schedule.

Serum samples are periodically taken by retro-orbital bleeding or tail-tip excision for testing by dot-blot assay (antibody sandwich), ELISA (enzyme-linked immunosorbent assay), immunoprecipitation, or other suitable assays, including FACS analysis. Following detection of an appropriate antibody titer, positive animals are given an intravenous injection of antigen in saline. Three to four days later, the animals are sacrificed, splenocytes harvested, and fused to a murine myeloma cell line (e.g., NS1 or

preferably Ag 8.653 [ATCC CRL 1580]). Hybridoma cell lines generated by this procedure are plated in multiple microtiter plates in a selective medium (for example, one containing hypoxanthine, aminopterin, and thymidine, or HAT) to inhibit proliferation of non-fused cells, myeloma-myeloma hybrids, and splenocyte-splenocyte hybrids.

Hybridoma clones thus generated can be screened by ELISA for reactivity with RANK, for example, by adaptations of the techniques disclosed by Engvall et al., *Immunochem.* 8:871 (1971) and in U.S. Patent 4,703,004. A preferred screening technique is the antibody capture technique described by Beckman et al., *J. Immunol.* 144:4212 (1990). Positive clones are then injected into the peritoneal cavities of syngeneic rodents to produce ascites containing high concentrations (>1 mg/ml) of anti-RANK monoclonal antibody. The resulting monoclonal antibody can be purified by ammonium sulfate precipitation followed by gel exclusion chromatography. Alternatively, affinity chromatography based upon binding of antibody to protein A or protein G can also be used, as can affinity chromatography based upon binding to RANK protein.

Monoclonal antibodies were generated using RANK/Fc fusion protein as the immunogen. These reagents were screened to confirm reactivity against the RANK protein. Using the methods described herein to monitor the activity of the mAbs, both blocking (i.e., antibodies that bind RANK and inhibit binding of a ligand to RANK) and non-blocking (i.e., antibodies that bind RANK and do not inhibit ligand binding) were isolated.

EXAMPLE 4

This example illustrates the induction of NF-κB activity by RANK in 293/EBNA cells (cell line was derived by transfection of the 293 cell line with a gene encoding Epstein-Barr virus nuclear antigen-1 (EBNA-1) that constitutively express EBNA-1 driven from human CMV immediate-early enhancer/promoter). Activation of NF-κB activity was measured in 293/EBNA cells essentially as described by Yao et al. (*Immunity* 3:811, 1995). Nuclear extracts were prepared and analyzed for NF-κB activity by a gel retardation assay using a 25 base pair oligonucleotide spanning the NF-κB binding sites. Two million cells were seeded into 10 cm dishes two days prior to DNA transfection and cultured in DMEM-F12 media containing 2.5% FBS (fetal bovine serum). DNA transfections were performed as described herein for the IL-8 promoter/reporter assays.

Nuclear extracts were prepared by solubilization of isolated nuclei with 400 mM NaCl (Yao et al., *supra*). Oligonucleotides containing an NF-κB binding site were annealed and endlabeled with ^{32}P using T4 DNA polynucleotide kinase. Mobility shift reactions contained 10 μg of nuclear extract, 4 μg of poly(dI-dC) and 15,000 cpm labeled double-stranded oligonucleotide and incubated at room temperature for 20 minutes.

Resulting protein-DNA complexes were resolved on a 6% native polyacrylamide gel in 0.25 X Tris-borate-EDTA buffer.

Overexpression of RANK resulted in induction of NFκB activity as shown by an appropriate shift in the mobility of the radioactive probe on the gel. Similar results were observed when RANK was triggered by a ligand that binds RANK and transduces a signal to cells expressing the receptor (i.e., by co-transfecting cells with human RANK and murine RANKL DNA; see Example 7 below), and would be expected to occur when triggering is done with agonistic antibodies.

EXAMPLE 5

This example describes a gene promoter/reporter system based on the human Interleukin-8 (IL-8) promoter used to analyze the activation of gene transcription in vivo. The induction of human IL-8 gene transcription by the cytokines Interleukin-1 (IL-1) or tumor necrosis factor-alpha (TNF-α) is known to be dependent upon intact NF-κB and NF-IL-6 transcription factor binding sites. Fusion of the cytokine-responsive IL-8 promoter with a cDNA encoding the murine IL-4 receptor (mIL-4R) allows measurement of promoter activation by detection of the heterologous reporter protein (mIL-4R) on the cell surface of transfected cells.

Human kidney epithelial cells (293/EBNA) are transfected (via the DEAE/DEXTRAN method) with plasmids encoding 1). the reporter/promoter construct (referred to as pIL-8rep), and 2). the cDNA(s) of interest. DNA concentrations are always kept constant by the addition of empty vector DNA. The 293/EBNA cells are plated at a density of 2.5 x 10^4 cells/ml (3 ml/ well) in a 6 well plate and incubated for two days prior to transfection. Two days after transfection, the mIL-4 receptor is detected by a radioimmunoassay (RIA) described below.

In one such experiment, the 293/EBNA cells were co-transfected with DNA encoding RANK and with DNA encoding RANKL (see Example 7 below). Co-expression of this receptor and its counterstructure by cells results in activation of the signaling process of RANK. For such co-transfection studies, the DNA concentration/well for the DEAE transfection were as follows: 40 ng of pIL-8rep [pBluescriptSK$^-$ vector (Stratagene)]; 0.4 ng CD40 (DNA encoding CD40, a control receptor; pCDM8 vector); 0.4 ng RANK (DNA encoding RANK; pDC409 vector), and either 1-50 ng CD40L (DNA encoding the ligand for CD40, which acts as a positive control when co-transfected with CD40 and as a negative control when co-transfected with RANK; in pDC304) or RANKL (DNA encoding a ligand for RANK; in pDC406). Similar experiments can be done using soluble RANKL or agonistic antibodies to RANK to trigger cells transfected with RANK.

For the mIL-4R-specific RIA, a monoclonal antibody reactive with mIL-4R is labeled with ^{125}I via a Chloramine T conjugation method; the resulting specific activity is typically 1.5 x 10^{16} cpm/nmol. After 48 hours, transfected cells are washed once with media (DMEM/F12 5% FBS). Non-specific binding sites are blocked by the addition of pre-warmed binding media containing 5% non-fat dry milk and incubation at 37°C/5% CO_2 in a tissue culture incubator for one hour. The blocking media is decanted and binding buffer containing ^{125}I anti-mIL-4R (clone M1; rat IgG1) is added to the cells and incubated with rocking at room temperature for 1 hour. After incubation of the cells with the radio-labeled antibody, cells are washed extensively with binding buffer (2X) and twice with phosphate-buffered saline (PBS). Cells are lysed in 1 ml of 0.5M NaOH, and total radioactivity is measured with a gamma counter.

Using this assay, 293/EBNA co-transfected with DNAs encoding RANK demonstrated transcriptional activation, as shown by detection of muIL-4R on the cell surface. Overexpression of RANK resulted in transcription of muIL-4R, as did triggering of the RANK by RANKL. Similar results are observed when RANK is triggered by agonistic antibodies.

EXAMPLE 6

This example illustrates the association of RANK with TRAF proteins. Interaction of RANK with cytoplasmic TRAF proteins was demonstrated by co-immunoprecipitation assays essentially as described by Hsu et al. (*Cell* 84:299; 1996). Briefly, 293/EBNA cells were co-transfected with plasmids that direct the synthesis of RANK and epitope-tagged (FLAG®; SEQ ID NO:7) TRAF2 or TRAF3. Two days after transfection, surface proteins were labeled with biotin-ester, and cells were lysed in a buffer containing 0.5% NP-40. RANK and proteins associated with this receptor were immunoprecipitated with anti-RANK, washed extensively, resolved by electrophoretic separation on a 6-10% SDS polyacrylamide gel and electrophoretically transferred to a nitrocellulose membrane for Western blotting. The association of TRAF2 and TRAF3 proteins with RANK was visualized by probing the membrane with an antibody that specifically recognizes the FLAG® epitope. TRAFs 2 and 3 did not immunopreciptitate with anti-RANK in the absence of RANK expression.

EXAMPLE 7

This example describes isolation of a ligand for RANK, referred to as RANKL, by direct expression cloning. The ligand was cloned essentially as described in USSN 08/249,189, filed May 24, 1994 (the relevant disclosure of which is incorporated by reference herein), for CD40L. Briefly, a library was prepared from a clone of a mouse thymoma cell line EL-4 (ATCC TIB 39), called EL-40.5, derived by sorting five times

with biotinylated CD40/Fc fusion protein in a FACS (fluorescence activated cell sorter). The cDNA library was made using standard methodology; the plasmid DNA was isolated and transfected into sub-confluent CV1-EBNA cells using a DEAE-dextran method. Transfectants were screened by slide autoradiography for expression of RANKL using a two-step binding method with RANK/Fc fusion protein as prepared in Example 2 followed by radioiodinated goat anti-human IgG antibody.

A clone encoding a protein that specifically bound RANK was isolated and sequenced; the clone was referred to as 11H. An expression vector containing murine RANKL sequence, designated pDC406:muRANK-L (in *E. coli* DH10B), was deposited with the American Type Culture Collection, Rockville, MD (ATCC) on December 20, 1996, under terms of the Budapest Treaty, and given accession number 98284. The nucleotide sequence and predicted amino acid sequence of this clone are illustrated in SEQ ID NO:10. This clone did not contain an initiator methionine; additional, full-length clones were obtained from a 7B9 library (prepared substantially as described in US patent 5,599,905, issued February 4, 1997); the 5' region was found to be identical to that of human RANKL as shown in SEQ ID NO: 12, amino acids 1 through 22, except for substitution of a Gly for a Thr at residue 9.

This ligand is useful for assessing the ability of RANK to bind RANKL by a number of different assays. For example, transfected cells expressing RANKL can be used in a FACS assay (or similar assay) to evaluate the ability of soluble RANK to bind RANKL. Moreover, soluble forms of RANKL can be prepared and used in assays that are known in the art (i.e., ELISA or BIAcore assays essentially as described in USSN 08/249,189, filed May 24, 1994). RANKL is also useful in affinity purification of RANK, and as a reagent in methods to measure the levels of RANK in a sample. Soluble RANKL is also useful in inducing NF-κB activation and thus protecting cells that express RANK from apoptosis.

EXAMPLE 8

This example describes the isolation of a human RANK ligand (RANKL) using a PCR-based technique. Murine RANK ligand-specific oligonucleotide primers were used in PCR reactions using human cell line-derived first strand cDNAs as templates. Primers corresponded to nucleotides 478-497 and to the complement of nucleotides 858-878 of murine RANK ligand (SEQ ID NO:10). An amplified band approximately 400 bp in length from one reaction using the human epidermoid cell line KB (ATCC CCL-17) was gel purified, and its nucleotide sequence determined; the sequence was 85% identical to the corresponding region of murine RANK ligand, confirming that the fragment was from human RANKL.

To obtain full-length human RANKL cDNAs, two human RANKL-specific oligonucleotides derived from the KB PCR product nucleotide sequence were radiolabeled and used as hybridization probes to screen a human PBL cDNA library prepared in lambda gt10 (Stratagene, La Jolla, CA), substantially as described in US patent 5,599,905, issued February 4, 1997. Several positive hybridizing plaques were identified and purified, their inserts subcloned into pBluescript SK$^-$ (Stratagene, La Jolla, CA), and their nucleotide sequence determined. One isolate, PBL3, was found to encode most of the predicted human RANKL, but appeared to be missing approximately 200 bp of 5' coding region. A second isolate, PBL5 was found to encode much of the predicted human RANKL, including the entire 5' end and an additional 200 bp of 5' untranslated sequence.

The 5' end of PBL5 and the 3' end of PBL3 were ligated together to form a full length cDNA encoding human RANKL. The nucleotide and predicted amino acid sequence of the full-length human RANK ligand is shown in SEQ ID NO:12. Human RANK ligand shares 83% nucleotide and 84% amino acid identity with murine RANK ligand. A plasmid vector containing human RANKL sequence, designated pBluescript:huRANK-L (in *E. coli* DH10B), was deposited with the American Type Culture Collection, Rockville, MD (ATCC) on March 11, 1997 under terms of the Budapest Treaty, and given accession number 98354.

Murine and human RANKL are Type 2 transmembrane proteins. Murine RANKL contains a predicted 48 amino acid intracellular domain, 21 amino acid transmembrane domain and 247 amino acid extracellular domain. Human RANKL contains a predicted 47 amino acid intracellular domain, 21 amino acid transmembrane domain and 249 amino acid extracellular domain.

EXAMPLE 9

This example describes the chromosomal mapping of human RANK using PCR-based mapping strategies. Initial human chromosomal assignments were made using RANK and RANKL-specific PCR primers and a BIOS Somatic Cell Hybrid PCRable DNA kit from BIOS Laboratories (New Haven, CT), following the manufacturer's instructions. RANK mapped to human chromosome 18; RANK ligand mapped to human chromosome 13. More detailed mapping was performed using a radiation hybrid mapping panel Genebridge 4 Radiation Hybrid Panel (Research Genetics, Huntsville, AL; described in Walter, MA et al., *Nature Genetics* 7:22-28, 1994). Data from this analysis was then submitted electronically to the MIT Radiation Hybrid Mapper (URL: http://www-genome.wi.mit.edu/cgi-bin/contig/rhmapper.pl) following the instructions contained therein. This analysis yielded specific genetic marker names which, when submitted electronically to the NCBI Entrez browser (URL:

http://www3.ncbi.nlm.nih.gov/htbin-post/Entrez/query?db=c&form=0), yielded the specific map locations. RANK mapped to chromosome 18q22.1, and RANKL mapped to chromosome 13q14.

EXAMPLE 10

This example illustrates the preparation of monoclonal antibodies against RANKL. Preparations of purified recombinant RANKL, for example, or transfixed cells expressing high levels of RANKL, are employed to generate monoclonal antibodies against RANKL using conventional techniques, such as those disclosed in US Patent 4,411,993. DNA encoding RANKL can also be used as an immunogen, for example, as reviewed by Pardoll and Beckerleg in *Immunity* 3:165, 1995. Such antibodies are likely to be useful in interfering with RANKL signaling (antagonistic or blocking antibodies), as components of diagnostic or research assays for RANKL or RANKL activity, or in affinity purification of RANKL.

To immunize rodents, RANKL immunogen is emulsified in an adjuvant (such as complete or incomplete Freund's adjuvant, alum, or another adjuvant, such as Ribi adjuvant R700 (Ribi, Hamilton, MT), and injected in amounts ranging from 10-100 μg subcutaneously into a selected rodent, for example, BALB/c mice or Lewis rats. DNA may be given intradermally (Raz et al., *Proc. Natl. Acad. Sci. USA* 91:9519, 1994) or intamuscularly (Wang et al., *Proc. Natl. Acad. Sci. USA* 90:4156, 1993); saline has been found to be a suitable diluent for DNA-based antigens. Ten days to three weeks days later, the immunized animals are boosted with additional immunogen and periodically boosted thereafter on a weekly, biweekly or every third week immunization schedule.

Serum samples are periodically taken by retro-orbital bleeding or tail-tip excision for testing by dot-blot assay (antibody sandwich), ELISA (enzyme-linked immunosorbent assay), immunoprecipitation, or other suitable assays, including FACS analysis. Following detection of an appropriate antibody titer, positive animals are given an intravenous injection of antigen in saline. Three to four days later, the animals are sacrificed, splenocytes harvested, and fused to a murine myeloma cell line (e.g., NS1 or preferably Ag 8.653 [ATCC CRL 1580]). Hybridoma cell lines generated by this procedure are plated in multiple microtiter plates in a selective medium (for example, one containing hypoxanthine, aminopterin, and thymidine, or HAT) to inhibit proliferation of non-fused cells, myeloma-myeloma hybrids, and splenocyte-splenocyte hybrids.

Hybridoma clones thus generated can be screened by ELISA for reactivity with RANKL, for example, by adaptations of the techniques disclosed by Engvall et al., *Immunochem. 8*:871 (1971) and in US Patent 4,703,004. A preferred screening technique is the antibody capture technique described by Beckman et al., *J. Immunol.* 144:4212

(1990). Positive clones are then injected into the peritoneal cavities of syngeneic rodents to produce ascites containing high concentrations (>1 mg/ml) of anti-RANK monoclonal antibody. The resulting monoclonal antibody can be purified by ammonium sulfate precipitation followed by gel exclusion chromatography. Alternatively, affinity chromatography based upon binding of antibody to protein A or protein G can also be used, as can affinity chromatography based upon binding to RANKL protein. Using the methods described herein to monitor the activity of the mAbs, both blocking (i.e., antibodies that bind RANKL and inhibit binding to RANK) and non-blocking (i.e., antibodies that bind RANKL and do not inhibit binding) are isolated.

EXAMPLE 11

This example demonstrates that RANK expression can be up-regulated. Human peripheral blood T cells were purified by flow cytometry sorting or by negative selection using antibody coated beads, and activated with anti-CD3 (OKT3, Dako) coated plates or phytohemagglutinin in the presence or absence of various cytokines, including Interleukin-4 (IL-4), Transforming Growth Factor-ß (TGF-ß) and other commercially available cytokines (IL1-□, IL-2, IL-3, IL-6, IL-7, IL-8, IL-10, IL-12, IL-15, IFN-□, TNF-□□). Expression of RANK was evaluated by FACS in a time course experiment for day 2 to day 8, using a mouse monoclonal antibody mAb144 (prepared as described in Example 3), as shown in the table below. Results are expressed as '+' to '++++' referring to the relative increase in intensity of staining with anti-RANK. Double labeling experiments using both anti-RANK and anti-CD8 or anti-CD4 antibodies were also performed.

Table 1: Upregulation of RANK by Cytokines

Cytokine (concentration)	**Results:**
IL-4 (50 ng/ml)	+
TGF-ß (5 ng/ml)	+ to ++
IL-4 (50 ng/ml) +TGF-ß (5 ng/ml)	++++
IL1-□ (10ng/ml)	-
IL-2 (20ng/ml)	-
IL-3 (25ng/ml)	-
IL-7 (20ng/ml)	-
IL-8 (10ng/ml)	-
IL-10 (50ng/ml)	-
IL-12 (10ng/ml)	-

Of the cytokines tested, IL-4 and TGF-ß increased the level of RANK expression on both CD8+ cytotoxic and CD4+ helper T cells from day 4 to day 8. The combination of IL-4 and TGF-ß acted synergistically to upregulate expression of this receptor on activated T cells. This particular combination of cytokines is secreted by suppresser T cells, and is believed to be important in the generation of tolerance (reviewed in Mitchison and Sieper, *Z. Rheumatol.* 54:141, 1995), implicating the interaction of RANK in regulation of an immune response towards either tolerance or induction of an active immune response.

EXAMPLE 12

This example illustrates the influence of RANK.Fc and hRANKL on activated T cell growth. The addition of TGFß to anti-CD3 activated human peripheral blood T lymphocytes induces proliferation arrest and ultimately death of most lymphocytes within the first few days of culture. We tested the effect of RANK:RANKL interactions on TGFß-treated T cells by adding RANK.Fc or soluble human RANKL to T cell cultures.

Human peripheral blood T cells (7 x 10^5 PBT) were cultured for six days on anti-CD3 (OKT3, 5µg/ml) and anti-Flag (M1, 5µg/ml) coated 24 well plates in the presence of TGFß (1ng/ml) and IL-4 (10ng/ml), with or without recombinant FLAG-tagged soluble hRANKL (1µg/ml) or RANK.Fc (10µg/ml). Viable T cell recovery was determined by triplicate trypan blue countings.

The addition of RANK.Fc significantly reduced the number of viable T cells recovered after six days, whereas soluble RANKL greatly increased the recovery of viable T cells. Thus, endogenous or exogenous RANKL enhances the number of viable T cells generated in the presence of TGFß TGFß, along with IL-4, has been implicated in immune response regulation when secreted by the T_H3/regulatory T cell subset. These T cells are believed to mediate bystander suppression of effector T cells. Accordingly, RANK and its ligand may act in an auto/paracrine fashion to influence T cell tolerance. Moreover, TGFß is known to play a role in the evasion of the immune system effected by certain pathogenic or opportunistic organisms. In addition to playing a role in the development of tolerance, RANK may also play a role in immune system evasion by pathogens.

EXAMPLE 13

This example illustrates the influence of the interaction of RANK on CD1a+ dendritic cells (DC). Functionally mature dendritic cells (DC) were generated *in vitro* from CD34+ bone marrow (BM) progenitors. Briefly, human BM cells from normal healthy volunteers were density fractionated using Ficoll medium and CD34+ cells immunoaffinity isolated using an anti-CD34 matrix column (Ceprate, CellPro). The CD34+ BM cells were then cultured in human GM-CSF (20 ng/ml), human IL-4 (20 ng/ml), human TNF-□ (20 ng/ml), human CHO-derived Flt3L (FL; 100 ng/ml) in Super McCoy's medium supplemented with 10% fetal calf serum in a fully humidified 37°C incubator (5% CO_2) for 14 days. CD1a+, HLA-DR+ DC were then sorted using a FACStar Plus™, and used for biological evaluation of RANK.

On human CD1a+ DC derived from CD34+ bone marrow cells, only a subset (20-30%) of CD1a+ DC expressed RANK at the cell surface as assessed by flow cytometric analysis. However, addition of CD40L to the DC cultures resulted in RANK surface

expression on the majority of CD1a$^+$ DC. CD40L has been shown to activate DC by enhancing *in vitro* cluster formation, inducing DC morphological changes and upregulating HLA-DR, CD54, CD58, CD80 and CD86 expression.

Addition of RANKL to DC cultures significantly increased the degree of DC aggregation and cluster formation above control cultures, similar to the effects seen with CD40L. Sorted human CD1a$^+$ DC were cultured in a cytokine cocktail (GM-CSF, IL-4, TNF-α and FL), in cocktail plus CD40L (1μg/ml), in cocktail plus RANKL (1μg/ml), or in cocktail plus heat inactivated RANKL (1μg/ml) in 24-well flat bottomed culture plates in 1 ml culture media for 48-72 hours and then photographed using an inversion microscope. An increase in DC aggregation and cluster formation above control cultures was not evident when heat inactivated RANKL was used, indicating that this effect was dependent on biologically active protein. However, initial phenotypic analysis of adhesion molecule expression indicated that RANKL-induced clustering was not due to increased levels of CD2, CD11a, CD54 or CD58.

The addition of RANKL to CD1a$^+$ DC enhanced their allo-stimulatory capacity in a mixed lymphocyte reaction (MLR) by at least 3- to 10-fold, comparable to CD40L-cultured DC. Allogeneic T cells ($1x10^5$) were incubated with varying numbers of irradiated (2000 rad) DC cultured as indicated above in 96-well round bottomed culture plates in 0.2 ml culture medium for four days. The cultures were pulsed with 0.5 mCi [^{3}H]-thymidine for eight hours and the cells harvested onto glass fiber sheets for counting on a gas phase beta counter. The background counts for either T cells or DC cultured alone were <100 cpm. Values were plotted that represented the mean ± SD of triplicate cultures. Results indicated that heat inactivated RANKL had no effect. DC allo-stimulatory activity was not further enhanced when RANKL and CD40L were used in combination, possibly due to DC functional capacity having reached a maximal level with either cytokine alone. Neither RANKL nor CD40L enhanced the *in vitro* growth of DCs over the three day culture period. Unlike CD40L, RANKL did not significantly increase the levels of HLA-DR expression nor the expression of CD80 or CD86.

RANKL can enhance DC cluster formation and functional capacity without modulating known molecules involved in cell adhesion (CD18, CD54), antigen presentation (HLA-DR) or costimulation (CD86), all of which are regulated by CD40/CD40L signaling. The lack of an effect on the expression of these molecules suggests that RANKL may regulate DC function via an alternate pathway(s) distinct from CD40/CD40L. Given that CD40L regulates RANK surface expression on *in vitro*-generated DC and that CD40L is upregulated on activated T cells during DC-T cell interactions, RANK and its ligand may form an important part of the activation cascade that is induced during DC-mediated T cell expansion. Furthermore, culture of DC in

RANKL results in decreased levels of CD1b/c expression, and increased levels of CD83. Both of these molecules are similarly modulated during DC maturation by CD40L (Caux et al. *J. Exp. Med.* 180:1263; 1994), indicating that RANKL induces DC maturation.

Dendritic cells are referred to as "professional" antigen presenting cells, and have a high capacity for sensitizing MHC-restricted T cells. There is growing interest in using dendritic cells *ex vivo* as tumor or infectious disease vaccine adjuvants (see, for example, Romani, et al., *J. Exp. Med.*, 180:83, 1994). Therefore, an agent such as RANKL that induces DC maturation and enhances the ability of dendritic cells to stimulate an immune response is likely to be useful in immunotherapy of various diseases.

EXAMPLE 14

This example describes the isolation of the murine homolog of RANK, referred to as muRANK. MuRANK was isolated by a combination of cross-species PCR and colony hybridization. The conservation of Cys residues in the Cys-rich pseudorepeats of the extracellular domains of TNFR superfamily member proteins was exploited to design human RANK-based PCR primers to be used on murine first strand cDNAs from various sources. Both the sense upstream primer and the antisense downstream primer were designed to have their 3' ends terminate within Cys residues.

The upstream sense primer encoded nucleotides 272-295 of SEQ ID NO:5 (region encoding amino acids 79-86); the downstream antisense primer encoded the complement of nucleotides 409-427 (region encoding amino acids 124-130). Standard PCR reactions were set up and run, using these primers and first strand cDNAs from various murine cell line or tissue sources. Thirty reaction cycles of 94°C for 30 seconds, 50°C for 30 seconds, and 72°C for 20 seconds were run. PCR products were analyzed by electrophoresis, and specific bands were seen in several samples. The band from one sample was gel purified and DNA sequencing revealed that the sequence between the primers was approximately 85% identical to the corresponding human RANK nucleotide sequence.

A plasmid based cDNA library prepared from the murine fetal liver epithelium line FLE18 (one of the cell lines identified as positive in the PCR screen) was screened for full-length RANK cDNAs using murine RANK-specific oligonucleotide probes derived from the murine RANK sequence determined from sequencing the PCR product. Two cDNAs, one encoding the 5' end and one encoding the 3' end of full-length murine RANK (based on sequence comparison with the full-length human RANK) were recombined to generate a full-length murine RANK cDNA. The nucleotide and amino acid sequence of muRANK are shown in SEQ ID Nos:14 and 15.

The cDNA encodes a predicted Type 1 transmembrane protein having 625 amino acid residues, with a predicted 30 amino acid signal sequence, a 184 amino acid

extracellular domain, a 21 amino acid transmembrane domain, and a 390 amino acid cytoplasmic tail. The extracellular region of muRANK displayed significant amino acid homology (69.7% identity, 80.8% similarity) to huRANK. Those of skill in the art will recognize that the actual cleavage site can be different from that predicted by computer; accordingly, the N-terminal of RANK may be from amino acid 25 to amino acid 35.

Other members of the TNF receptor superfamily have a region of amino acids between the transmembrane domain and the ligand binding domain that is referred to as a 'spacer' region, which is not necessary for ligand binding. In muRANK, the amino acids between 197 and 214 are predicted to form such a spacer region. Accordingly, a soluble form of RANK that terminates with an amino acid in this region is expected to retain the ability to bind a ligand for RANK in a specific manner. Preferred C-terminal amino acids for soluble RANK peptides are selected from the group consisting of amino acids 214, and 197 of SEQ ID NO:14, although other amino acids in the spacer region may be utilized as a C-terminus.

EXAMPLE 15

This example illustrates the preparation of several different soluble forms of RANK and RANKL. Standard techniques of restriction enzyme cutting and ligation, in combination with PCR-based isolation of fragments for which no convenient restriction sites existed, were used. When PCR was utilized, PCR products were sequenced to ascertain whether any mutations had been introduced; no such mutations were found.

In addition to the huRANK/Fc described in Example 2, another RANK/Fc fusion protein was prepared by ligating DNA encoding amino acids 1-213 of SEQ ID NO:6, to DNA encoding amino acids 3-232 of the Fc mutein described previously (SEQ ID NO:8). A similar construct was prepared for murine RANK, ligating DNA encoding amino acids 1-213 of full-length murine RANK (SEQ ID NO:15) to DNA encoding amino acids 3-232 of the Fc mutein (SEQ ID NO:8).

A soluble, tagged, poly-His version of huRANKL was prepared by ligating DNA encoding the leader peptide from the immunoglobulin kappa chain (SEQ ID NO:16) to DNA encoding a short version of the FLAG™ tag (SEQ ID NO:17), followed by codons encoding Gly Ser, then a poly-His tag (SEQ ID NO:18), followed by codons encoding Gly Thr Ser, and DNA encoding amino acids 138-317 of SEQ ID NO:13. A soluble, poly-His tagged version of murine RANKL was prepared by ligating DNA encoding the CMV leader (SEQ ID NO:9) to codons encoding Arg Thr Ser, followed by DNA encoding poly-His (SEQ ID NO:18) followed by DNA encoding amino acids 119-294 of SEQ ID NO:11.

A soluble, oligomeric form of huRANKL was prepared by ligating DNA encoding the CMV leader (SEQ ID NO:9) to a codon encoding Asp followed by DNA ending a

trimer-former "leucine" zipper (SEQ ID NO:19), then by codons encoding Thr Arg Ser followed by amino acids 138-317 of SEQ ID NO:13.

These and other constructs are prepared by routine experimentation. The various DNAs are then inserted into a suitable expression vector, and expressed. Particularly preferred expression vectors are those which can be used in mammalian cells. For example, pDC409 and pDC304, described herein, are useful for transient expression. For stable transfection, the use of CHO cells is preferred; several useful vectors are described in USSN 08/785,150, now allowed, for example, one of the 2A5-3 □-derived expression vectors discussed therein.

EXAMPLE 16

This example demonstrates that RANKL expression can be up-regulated on murine T cells. Cells were obtained from mesenteric lymph nodes of C57BL/6 mice, and activated with anti-CD3 coated plates, Concanavalin A (ConA) or phorbol myristate acetate in combination with ionomycin (anti-CD3: 500A2; Immunex Corporation, Seattle WA; ConA, PMA, ionomycin, Sigma, St. Louis, MO) substantially as described herein, and cultured from about 2 to 5 days. Expression of RANKL was evaluated in a three color analysis by FACS, using antibodies to the T cell markers CD4, CD8 and CD45RB, and RANK/Fc, prepared as described herein.

RANKL was not expressed on unstimulated murine T cells. T cells stimulated with either anti-CD3, ConA, or PMA/ionomycin, showed differential expression of RANKL: $CD4^+/CD45RB^{Lo}$ and $CD4^+/CD45RB^{Hi}$ cells were positive for RANKL, but CD8+ cells were not. RANKL was not observed on B cells, similar to results observed with human cells.

EXAMPLE 17

This example illustrates the effects of murine RANKL on cell proliferation and activation. Various cells or cell lines representative of cells that play a role in an immune response (murine spleen, thymus and lymph node) were evaluated by culturing them under conditions promoting their viability, in the presence or absence of RANKL. RANKL did not stimulate any of the tested cells to proliferate. One cell line, a macrophage cell line referred to as RAW 264.7 (ATCC accession number TIB 71) exhibited some signs of activation.

RAW cells constitutively produce small amounts of TNF-α. Incubation with either human or murine RANKL enhanced production of TNF-α by these cells in a dose dependent manner. The results were not due to contamination of RANKL preparations

with endotoxin, since boiling RANKL for 10 minutes abrogated TNF-α production, whereas a similar treatment of purified endotoxin (LPS) did not affect the ability of the LPS to stimulate TNF-α production. Despite the fact that RANKL activated the macrophage cell line RAW T64.7 for TNF-α production, neither human RANKL nor murine RANKL stimulated nitric oxide production by these cells.

EXAMPLE 18

This example illustrates the effects of murine RANKL on growth and development of the thymus in fetal mice. Pregnant mice were injected with 1 mg of RANK/Fc or vehicle control protein (murine serum albumin; MSA) on days 13, 16 and 19 of gestation. After birth, the neonates continued to be injected with RANK/Fc intraperitoneally (IP) on a daily basis, beginning at a dose of 1 μg, and doubling the dose about every four days, for a final dosage of 4 μg. Neonates were taken at days 1, 8 and 15 post birth, their thymuses and spleens harvested and examined for size, cellularity and phenotypic composition.

A slight reduction in thymic size at day 1 was observed in the neonates born to the female injected with RANK/Fc; a similar decrease in size was not observed in the control neonates. At day 8, thymic size and cellularity were reduced by about 50% in the RANK/Fc-treated animals as compared to MSA treated mice. Phenotypic analysis demonstrated that the relative proportions of different T cell populations in the thymus were the same in the RANK/Fc mice as the control mice, indicating that the decreased cellularity was due to a global depression in the number of thymic T cells as opposed to a decrease in a specific population(s). The RANK/Fc-treated neonates were not significantly different from the control neonates at day 15 with respect to size, cellularity or phenotype of thymic cells. No significant differences were observed in spleen size, cellularity or composition at any of the time points evaluated. The difference in cellularity on day 8 and not on day 15 may suggest that RANK/Fc may assert its effect early in thymic development.

EXAMPLE 19

This example demonstrates that the C-terminal region of the cytoplasmic domain of RANK is important for binding of several different TRAF proteins. RANK contains at least two recognizable PXQX(X)T motifs that are likely TRAF docking sites. Accordingly, the importance of various regions of the cytoplasmic domain of RANK for TRAF binding was evaluated. A RANK/GST fusion protein was prepared substantially as described in Smith and Johnson, *Gene* 67:31 (1988), and used in the preparation of various truncations as described below.

Comparison of the nucleotide sequence of murine and human RANK indicated that there were several conserved regions that could be important for TRAF binding. Accordingly, a PCR-based technique was developed to facilitate preparation of various C-terminal truncations that would retain the conserved regions. PCR primers were designed to introduce a stop codon and restriction enzyme site at selected points, yielding the truncations described in Table 1 below. Sequencing confirmed that no undesired mutations had been introduced in the constructs.

Radio-labeled (^{35}S-Met, Cys) TRAF proteins were prepared by *in vitro* translation using a commercially available reticulocyte lysate kit according to manufacturer's instructions (Promega). Truncated GST fusion proteins were purified substantially as described in Smith and Johnson (supra). Briefly, *E. coli* were transfected with an expression vector encoding a fusion protein, and induced to express the protein. The bacteria were lysed, insoluble material removed, and the fusion protein isolated by precipitation with glutathione-coated beads (Sepahrose 4B, Pharmacia, Uppsala Sweden)

The beads were washed, and incubated with various radiolabeled TRAF proteins. After incubation and wash steps, the fusion protein/TRAF complexes were removed from the beads by boiling in 0.1% SDS + ß-mercaptoethanol, and loaded onto 12% SDS gels (Novex). The gels were subjected to autoradiography, and the presence or absence of radiolabeled material recorded. The results are shown in Table 2 below.

Table 2: Binding of Various TRAF Proteins to the Cytoplasmic Domain of RANK

C terminal Truncations:	E206-S339	E206-Y421	E206-M476	E206-G544	Full length
TRAF1	-	-	-	-	++
TRAF2	-	-	-	-	++
TRAF3	-	-	-	-	++
TRAF4	-	-	-	-	-
TRAF5	-	-	-	-	+
TRAF6	-	+	+	+	++

These results indicate that TRAF1, TRAF2, TRAF3, TRAF 5 and TRAF6 bind to the most distal portion of the RANK cytoplasmic domain (between amino-acid G544 and

A616). TRAF6 also has a binding site between S339 and Y421. In this experiment, TRAF5 also bound the cytoplasmic domain of RANK.

In another experiment using the same methods to prepare the RANK cytoplasmic domain fragment and test for TRAF6 binding, TRAF6 bound to the huRANK cytoplasmic domain fragment amino acids 339-362.

To confirm that the *in vitro* interaction of TRAFs with RANK also occur in cells, co-immunoprecipitation experiments were performed in 293 cells cotransfected with RANK, or RANK fragments, and tagged TRAFs. The results demonstrated that full length RANK associates with TRAF 1, 2, 3, 5 and 6. 293 cells containing the RANK G544 deletion (RANK lacking the C-terminal 72 amino acids) coprecipitated with TRAFS 2, 3 and 6. Since the *in vitro* binding experiments did not show TRAF2 and TRAF3 association with this RANK fragment, these results suggest that TRAF2/TRAF6 and TRAF3/TRAF6 form heterocomplexes. These results also support the conclusions reached in the experiment described in Example 6.

EXAMPLE 20

The following describes the functional effect of RANK cytoplasmic deletion mutants expressed in 293 cells using NF-κB reporter assays.

A NF-κB-responsive reporter plasmid was constructed in pGL2-Basic obtained from Promega with the human IL-8 promoter containing a NF-κB binding site fused to a luciferase reporter. RANK cytoplasmic deletions were subcloned into a full length RANK expression vector pDC304. 293 cells from Invitrogen, San Diego, CA were transiently transfected by the DEAE-Dextran method with the reporter plasmid either alone or in combination with the RANK cDNA in pDC304 and reporter activity was measured in 293 cells.

Transfection of 293 cells with the RANK deletion construct lacking the COOH-terminal 72 amino acids (RANKΔ544) resulted in reduced NF-κB reporter activity in the absence of RANKL activation. This contrasts with RANKL treatment of RANKΔ544-expressing cells which induced NF-κB activation to levels similar to that seen with full-length RANK. Cells transfected with RANK constructs having further deletion of COOH-terminal sequences had minimal effects on the constitutive and RANKL-mediated reporter activity until the removal of amino acids 339-422 (RANKΔ339) which completely abrogated both constitutive signaling and responsiveness to RANKL. These data indicate that RANK contains two domains (amino acids 339-422 and amino acids 544-616 of SEQ ID NO:6) within the cytoplasmic region that are important for NF-κB signaling. These two regions correspond with the two domains which affect binding TRAFS1, 2, 3, 5, and 6 (the domain characterized as amino acids 544-616 of SEQ ID NO:6) and TRAF6 (the domain characterized as amino acids 339-422 of SEQ ID NO:6).

These results also suggest that RANK's ability to directly bind TRAF6, in the absence of direct binding of other TRAFs, allows optimal RANK signaling. This is supported by the results obtained in Example 21 in which signaling through amino acids 339-442 results in partially inhibited JNK activity.

EXAMPLE 21

The following describes the functional effect of RANK cytoplasmic deletion mutants expressed in 293 cells using JNK activation assays. For the JNK assays, whole cell extracts were prepared from transfected 293 cells 24 hr after transfection. The cells were lysed in a buffer and clarified lysates were immunoprecipitated with anti-JNK (FL) and anti-JNK (C17). The complexes were washed and JNK activity was determined by an immune-complex assay using GST-cJun and [^{32}]-ATP as substrates. Reaction products were resolved on SDS/PAGE and visualized by autoradiography.

Transfection of full length RANK significantly induced JNK activity. Transfection of RANK having a truncated COOH-terminal 72 amino acids abrogated the majority of JNK activity. Residual JNK activity was completely inhibited in cells transfected with RANK having further truncation of amino acids 339-422 (SEQ ID NO:6) in the RANK cytoplasmic domain.

These results demonstrate that two distinct RANK cytoplasmic domains (residues 544-616 and 339-422 of SEQ ID NO:6) play functional roles in JNK activation similar to the domains necessary for NF-κB activation described in Example 20.

EXAMPLE 22

This example describes a plate binding assay useful in comparing the ability of various ligands or test compounds to bind receptors. The assay is performed essentially as described in Smith et al., Virology 236:316 (1997). Briefly, 96-well microtiter plates are coated with an antibody to human Fc (i.e., polyclonal goat anti human Fc). Receptor/Fc fusion proteins are then added, and after incubation, the plates are washed. Serial dilutions of the ligands are then added. The ligands may be directly labeled (i.e., with ^{125}I), or a detecting reagent that is radioactively labeled may be used. After incubation, the plates are washed, specifically bound ligands are released, and the amount of ligand bound quantified.

Using this method, RANK/Fc and OPG/Fc were bound to 96-well plates. In an indirect method, a RANKL/zipper fusion is detected using a labeled antibody to the zipper moiety. It was found that human OPG/Fc binds mRANKL at 0.05 nM, and human RANK/Fc binds mRANKL at 0.1 nM. These values indicate similar binding affinities of

OPG and RANK for RANKL, confirming the utility of RANK as an inhibitor of osteoclast activity in a manner similar to OPG.

EXAMPLE 23

The following describes the formation of osteoclast like cells from bone marrow cell cultures using a soluble RANKL in the form of soluble RANKL/leucine zipper fusion protein (RANKL LZ).

Using RANKL LZ at 1μg/ml, osteoclasts were generated from murine bone marrow (BM) in the presence of CSF-1. These osteoclasts are formed by the fusion of macrophage-like cells and are characterized by their TRAP (tartrate-resistant acid phosphatase) positivity. No $TRAP^+$ cells were seen in cultures containing CSF-1 alone or in cultures containing CSF-1 and TRAIL LZ (a control for the soluble RANKL LZ). Even though human and monkey bone marrow contains more contaminating fibroblasts than murine bone marrow, osteoclasts were generated from murine and monkey bone marrow with the combination of CSF-1 and soluble RANKL LZ. In a dose-response study using murine bone marrow and suboptimal amounts of CSF-1 (40ng/ml), the effects of soluble RANKL LZ plateau at about 100ng/ml.

The effect of soluble RANKL LZ on proliferation of cells was studied in the same cultures using Alamar Blue. After 5 days, the proliferative response was lower in cultures containing CSF-1 and RANKL LZ than in those containing CSF-1 alone. This supports the observation that soluble RANKL LZ is inducing osteoclast differentiation. When CSF-1 and RANKL LZ are washed out of murine BM cultures at day 7 or 8, cells do not survive if they are recultured in medium or in RANKL LZ alone. In contrast, cells do survive if recultured in CSF-1. When RANKL LZ was added to these cultures there was no added benefit. Thus, the combination of CSF-1 and RANKL are required for the generation of osteoclasts. Additionally, once formed, CSF-1 is sufficient to maintain their survival in culture.

Finally, using human bone marrow, soluble anti-human RANK mAb and immobilized anti-human RANK mAb were compared to RANKL LZ for the generation of osteoclasts in the presence of CSF-1. Immobilized M331 and RANKL LZ were found to be equally effective for osteoclast generation while soluble M331 was superior to both immobilized antibody and RANKL LZ. This confirms that the osteoclast differentiating activity of RANKL is mediated through RANK rather than via an alternative receptor.

Since osteoclasts cannot readily be harvested and analyzed by flow cytometry, ^{125}I-labeled calcitonin binding assays were used to identify osteoclasts (the calcitonin receptor is considered to be an osteoclast-specific marker). Osteoclasts generated from murine BM cultured with CSF-1 and RANKL LZ for 9 days showed binding of radiolabeled calcitonin confirming their osteoclast identity.

The above confirms that RANK/RANKL binding is important in myeloid cell and osteoclast differentiation. The results described in Example 2_ suggest that if the TRAF6 binding domain is absent in RANK, NF-κB transcription factor activity induced by RANK/RANKL interaction is non-existent. Since it is known that mice lacking two NF-κB subunits have osteoclast defects, there is strong suggestion that TRAF6 is especially important in mediating RANK signals in the osteoclast differentiation pathway.

EXAMPLE 24

In order to determine RANKL expression by either of two different squamous cell carcinomas, standard Western blot and RT-PCR studies were performed on MH-85 and OKK cells. One of these carcinoma cells, the MH-85 cells, is associated with hypercalcemia.

The results confirmed that MH-85 and OKK squamous cells express RANKL. MH-85 cells, in addition to being linked with hypercalcemia in patients inflicted with this carcinoma, also express M-CSF (CSF-1). It was also determined that CSF-1 upregulates RANK expression on osteoclast precursors. The enhanced amount of CSF-1 in MH-85 type squamous cell cancer patients can lead to an upregulation of RANK and increased RANK interaction with RANKL. Signals transduced by RANK and RANKL interaction result in increased numbers of mature osteoclasts and bone breakdown. Since soluble forms of RANK can inhibit the RANK/RANKL interaction, administering a soluble form of RANK (e.g. the extracellular region of RANK fused to an Fc) to a squamous cell cancer patient provides relief from adverse effects of this cancer, including hypercalcemia.

EXAMPLE 25

The following describes mutation detection and mutation analysis of family members previously diagnosed with familial expansile osteolysis (FEO). In one FEO family the disease was linked to markers on chromosome 18q. In a separate family, linkage to the FEO locus in 18q was established although the disease allele was transmitted on a different haplotype.

The RANK intron-exon organization and the RANK gene promoter region were characterized by directly sequencing bacterial artificial chromosomes (BACs) , genomic subclones and PCT products using vectorette PCR on P1 artificial chromosomes (PACs) containing genomic DNA. Results support localization of the RANK gene between D18S383 and D18S51. Further analysis of clones in the Genebridge 4 radiation hybrid panel suggests a RANK location in the region of D18S60 between WI-9823 and SGC33768, placing the RANK gene within the critical region for the FEO causative gene.

Normal members and members diagnosed with FEO of two separate FEO families were screened for mutations in all RANK exons, RANK intron-exon junctions, 5' and 3' UTRs and the RANK gene promoter region by directly sequencing PCR products. The PCR products were obtained by performing PCR in 20 μL reactions using *Taq* DNA polymerase (0.5U;Qiagen). The PCT conditions and reagents included 1x buffer and solution Q, dNTPs (200 μM), primers (0.3 μM) and 50 ng of DNA. The initial denaturation was at 97°C for 3 minutes, followed by 10 temperature cycles at 97°C for 1 minute each. The DNA was allowed to anneal for 1 minute, followed by a 72°C cycle for 1-3 minutes and 30 similar cycles using the lower designated annealing temperature, before a final period of 10 minutes at 72°C.

The PCR products were purified on Wizard columns (Promega) and sequenced with an ABI377 instrument using BigDye™ terminator cycle sequencing chemistry. 5% DMSO was included in sequencing reactions for exon 1. Mutations were confirmed by size variation after electrophoresis on 5 or 7% polyacrylamide gels run in 1xTBE buffer and stained with ethidium bromide. Individual alleles were excised and re-amplified by PCR, as described above, prior to their sequencing.

Sequencing results confirmed identical tandem duplication of bases 84 to 101 in all tested FEO individuals (RANK 84dup18 (See SEQ ID NO:20) which segregated with the disease in both families. The insertion was confirmed further by observing an allele of increased size, clearly visible after polyacrylamide gel electrophoresis of PCR products from FEO diagnosed individuals. No insertions were detected in non-FEO members of the families. Similarly, no insertions were detected in 30 individuals with sporadic Paget's disease of bone, nor in 64 age-matched controls. An identical 84dup18 duplication was also found in DNA from the only available affected member of a second family available for study. Similarly, analysis of exon 1 in members of four Pagetic families with possible evidence of linkage to 18q, resulted in the identification, in one family characterized as having early onset Paget's disease of bone (EP), of a slightly larger duplication involving bases 75 to 101 (75dup27) that co-segregated with the disease (SEQ ID NO:22).

In wild-type RANK, the amino acids between 196 and 213 are predicted to form a spacer region between the transmembrane domain and the receptor binding domain that is not necessary for receptor binding. In FEO RANK, which contains an 18 nucleotide duplication, this spacer thus is displaced by 6 amino acids, and therefore corresponds to amino acids 202-219 in SEQ ID NO:21. In EP RANK, which contains a 27 nucleotide duplication, the spacer thus corresponds to amino acids 205 through 222 in SEQ ID NO:23. Accordingly, the invention encompasses soluble forms of FEO and EP RANK that terminate with an amino acid in these spacer regions, and these soluble forms are

expected to retain the ability to bind specifically with a ligand for RANK, such as RANKL.

The two identified duplications are likely to have arisen by a common mechanism of reverse slippage during DNA replication. They share the same 3'endpoint at base 101, and bases 92 to 101 show 90% homology with bases 74 to 83 (to produce 84dup18) and 80% homology with bases 65 to 74 (to produce 75dup27). In both cases a region of 70% homology extends over the relevant 20 bases at the 3' end when mispairing during mutagenesis. This observation anticipates finding a 90dup12 mutation because bases 95 to 101 show perfect homology with 83 to 89; however, the preceding region is of low homology.

Several polymorphisms, were detected in the RANK gene. These include –1G/A close to the start position of transcription and 30T/C within the 5' UTR of the RNA sited 9 bases before the initiation of coding. An IVS1+5G/A polymorphism was found in the 5' splice site of intron 1. The rarer AG/gtaa**a** variant segregated with one family FEO duplication, whereas the identical North American mutation was found on homozygous AG/gtaa**g** background. This implies that these FEO mutations arose independently. The similarity of the phenotypes in the families suggests that splicing is unlikely to be affected significantly by the IVS1+5G/A polymorphism. A variant V/A192 was identified in exon 6 which codes within a predicted cysteine-rich pseudorepeat in the extracellular region of RANK. Valine (which shows homology at this position with mouse RANK) was found more commonly than alanine. V/A192 was in strong linkage disequilibrium with three intronic polymorphisms, IVS5-17T/C, IVS6+79A/G and IVS6+166A/G. Intron 6 contained another cluster of polymorphisms at its 3' end: IVS6-151A/G, IVS6-245A/G and IVS6-258A/C. Variation was also found in the 3' UTR, eg 2212delAT and length variation in the polyG stretch starting at 2198 and polyC starting at 2518.

EXAMPLE 26

The following describes transfecting mammalian cells with mutant RANK and wild type RANK expression plasmids and analyzing the transfected cells for RANK and mutant RANK expression levels.

Mammalian expression plasmids encoding full length wild type RANK, FEO RANK and EP RANK were prepared by ligating synthetic oligonucleotide pairs encoding the corresponding 5' regions of the different RANK species to a DNA fragment encoding the downstream portion of the wild type RANK coding region.

The appropriate combinations of oligonucleotide pairs were phosphorylated with ATP and T4 polynucleotide kinase and allowed to anneal following heat inactivation of the kinase. Oligonucleotide pairs were ligated to the downstream coding fragment of

human RANK and a mammalian expression vector backbone to generate pWTRANK, pFEORANK and pEPRANK. All constructs were verified by dye termination sequencing of both DNA strands.

Subconfluent 293EBNA cells were transfected in 6-well plates with 2 micrograms/well of empty vector, pWTRANK, pFEORANK or pEPRANK. The transfected 293EBNA cells were metabolically labeled 48 hr following transfection for 15 minutes with 50 microcuries/ml [^{35}S]-cysteine/methionine in cysteine-free, methionine-free DMEM. The pulse-labeled cells were washed twice with PBS and harvested with PBS/0.5% NP40, and cell lysates immunoprecipitated with 10 μgm/mL monoclonal anti-RANK antibodyand protein A and G sepharose (Pharmacia).

Immune complexes were resolved by PAGE on a 4-20% gradient Tris-glycine-SDS gel and radiolabeled proteins detected by autoradiography. The relative expression levels of the three RANK cDNAs were compared and there was significantly less protein expressed by pFEORANK (0.25 times wild type level) and pEPRANK (0.5 times wild type) transfectants compared to pWTRANK transfectants. Further, both FEO RANK and EP RANK protein bands migrated at approximately 83 kD compared to the approximately 80 kD position of wild type RANK, suggesting that unlike wild type RANK, the approximately 3 kD signal peptides of FEO and EP RANK were retained on the proteins.

EXAMPLE 27

The following describes evaluating cells transfected with plasmids expressing mutant RANK and cells transfected with wild type RANK for their relative ability to activate an NF-κB reporter. The transcription factor NF-κB is known to be activated as a consequence of RANK signal transduction.

A nuclear factor kappa B (NF-κB)-responsive reporter plasmid utilizing an NF-κB responsive promoter fused to a luciferase reporter was transfected together with varying amounts of pWTRANK, pFEORANK or pEPRANK (see Example 26) into 293EBNA cells (purchased from Invitrogen, San Diego, CA) using the DEAE-dextran transfection method. A plasmid expressing β-galactosidase (present at 25 ng/well) was included as an internal transfection efficiency control. The total amount of plasmid DNA for each transfection was equalized by adding empty vector. Cell lysates from transfected cultures were harvested after incubating the cells for 48 hours using reported lysis buffer (purchased from Promega). Luciferase activity was measured in cell lysates using luciferase substrate (purchased from Promega) and an EG&G/Berthold Luminometer. Relative luciferase activity was normalized to the β-galactosidase activity in each sample.

Cells co-transfected with pFEORANK or pEPRANK and the reporter plasmid consistently showed a higher level of luciferase activity when compared to pWTRANK. This result clearly shows that the FEO and EP mutations causes higher constitutive

RANK activity. Thus, RANK DNA mutations and encoded polypeptide mutations that are responsible for FEO or EP are useful in therapeutic and other applications in which increased RANK activity is desirable, for example, the treatment of conditions where insufficient bone remodeling occurs, e.g., osteopetrosis.

CLAIMS

What is claimed is:

1. An isolated DNA selected from the group consisting of:
   (a) a DNA capable of encoding a polypeptide comprising the amino acid sequence of SEQ ID NO:21;
   (b) a DNA capable of encoding a polypeptide comprising the amino acid sequence x through y of SEQ ID NO:21, wherein x is amino acid 1 of SEQ ID NO:21 and y is selected from the group consisting of any one of amino acids 202 through 219;
   (c) a DNA capable of encoding a polypeptide comprising the amino acid sequence of SEQ ID NO:23;
   (d) a DNA capable of encoding a polypeptide comprising amino acids x through y of SEQ ID NO:23, wherein x is amino acid 1 and y is selected from the group consisting of any one of amino acids 205 through 222; and
   (e) a DNA capable of encoding a polypeptide capable of binding RANKL or a TRAF, said polypeptide comprising a duplication encompassing between 1 and 10 consecutive amino acids located within residues 1-34 of SEQ ID NO:6, said DNA being capable of hybridizing to a DNA of (a)-(d) under stringent conditions, wherein the stringent conditions comprise hybridizing in 6 X SSC at 63°C and washing in 3 X SSC at 55°C.

2. An isolated DNA according to claim 1, wherein the polypeptide encoded by the DNA further comprises an amino acid sequence selected from the group consisting of an immunoglobulin Fc domain, an immunoglobulin Fc mutein, a FLAG™ tag, a peptide comprising at least about 6 His residues, a leucine zipper, and combinations thereof.

3. A recombinant expression vector comprising a DNA according to claim 1.

4. A host cell transformed or transfected with an expression vector according to claim 3.

5. A process for preparing a polypeptide, comprising culturing a host cell according to claim 4 under conditions promoting expression.

6. An isolated polypeptide prepared according to the process of claim 5.

7. A recombinant expression vector comprising a DNA according to claim 2.

8. A host cell transformed or transfected with an expression vector according to claim 7.

9. A process for preparing a polypeptide, comprising culturing a host cell according to claim 8 under conditions promoting expression.

10. An isolated polypeptide prepared according to the process of claim 9.

11. An isolated DNA comprising a nucleotide sequence selected from the group consisting of nucleotides 39-1904 of SEQ ID NO:20 and nucleotides 39-1913 of SEQ ID NO:22.

12. A recombinant expression vector comprising a DNA according to claim 11

13. A host cell transformed or transfected with the recombinant expression vector of claim 12.

14. A process for preparing a polypeptide comprising culturing a host cell according to claim 13 under conditions promoting expression.

15. An isolated polypeptide prepared according to the process of claim 14.

SEQUENCE LISTING

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Hughes, Anne E.

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cag cag gga gca tgt gaa ggt gtc tta ctg ctg act ctg gag gag aag      917
Gln Gln Gly Ala Cys Glu Gly Val Leu Leu Leu Thr Leu Glu Glu Lys
        280                 285                 290

aca ttt cca gaa gat atg tgc tac cca gat caa ggt ggt gtc tgt cag      965
Thr Phe Pro Glu Asp Met Cys Tyr Pro Asp Gln Gly Gly Val Cys Gln
    295                 300                 305

ggc acg tgt gta gga ggt ggt ccc tac gca caa ggc gaa gat gcc agg     1013
Gly Thr Cys Val Gly Gly Gly Pro Tyr Ala Gln Gly Glu Asp Ala Arg
310                 315                 320                 325

atg ctc tca ttg gtc agc aag acc gag ata gag gaa gac agc ttc aga     1061
Met Leu Ser Leu Val Ser Lys Thr Glu Ile Glu Glu Asp Ser Phe Arg
                330                 335                 340
```

```
cag atg ccc aca gaa gat gaa tac atg gac agg ccc tcc cag ccc aca      1109
Gln Met Pro Thr Glu Asp Glu Tyr Met Asp Arg Pro Ser Gln Pro Thr
            345                 350                 355

gac cag tta ctg ttc ctc act gag cct gga agc aaa tcc aca cct cct      1157
Asp Gln Leu Leu Phe Leu Thr Glu Pro Gly Ser Lys Ser Thr Pro Pro
        360                 365                 370

ttc tct gaa ccc ctg gag gtg ggg gag aat gac agt tta agc cag tgc      1205
Phe Ser Glu Pro Leu Glu Val Gly Glu Asn Asp Ser Leu Ser Gln Cys
    375                 380                 385

ttc acg ggg aca cag agc aca gtg ggt tca gaa agc tgc aac tgc act      1253
Phe Thr Gly Thr Gln Ser Thr Val Gly Ser Glu Ser Cys Asn Cys Thr
390                 395                 400                 405

gag ccc ctg tgc agg act gat tgg act ccc atg tcc tct gaa aac tac      1301
Glu Pro Leu Cys Arg Thr Asp Trp Thr Pro Met Ser Ser Glu Asn Tyr
                410                 415                 420

ttg caa aaa gag gtg gac agt ggc cat tgc ccg cac tgg gca gcc agc      1349
Leu Gln Lys Glu Val Asp Ser Gly His Cys Pro His Trp Ala Ala Ser
            425                 430                 435

ccc agc ccc aac tgg gca gat gtc tgc aca ggc tgc cgg aac              1391
Pro Ser Pro Asn Trp Ala Asp Val Cys Thr Gly Cys Arg Asn
        440                 445                 450
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<210> 4
<211> 451
<212> PRT
<213> Human

<400> 4

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Met Ala Pro Arg Ala Arg Arg Arg Arg Pro Leu Phe Ala Leu Leu Leu
  1               5                  10                  15

Leu Cys Ala Leu Leu Ala Arg Leu Gln Val Ala Leu Gln Ile Ala Pro
             20                  25                  30

Pro Cys Thr Ser Glu Lys His Tyr Glu His Leu Gly Arg Cys Cys Asn
         35                  40                  45

Lys Cys Glu Pro Gly Lys Tyr Met Ser Ser Lys Cys Thr Thr Thr Ser
     50                  55                  60

Asp Ser Val Cys Leu Pro Cys Gly Pro Asp Glu Tyr Leu Asp Ser Trp
 65                  70                  75                  80

Asn Glu Glu Asp Lys Cys Leu Leu His Lys Val Cys Asp Thr Gly Lys
                 85                  90                  95

Ala Leu Val Ala Val Val Ala Gly Asn Ser Thr Thr Pro Arg Arg Cys
            100                 105                 110

Ala Cys Thr Ala Gly Tyr His Trp Ser Gln Asp Cys Glu Cys Cys Arg
        115                 120                 125

Arg Asn Thr Glu Cys Ala Pro Gly Leu Gly Ala Gln His Pro Leu Gln
    130                 135                 140

Leu Asn Lys Asp Thr Val Cys Lys Pro Cys Leu Ala Gly Tyr Phe Ser
145                 150                 155                 160
```

```
Asp Ala Phe Ser Ser Thr Asp Lys Cys Arg Pro Trp Thr Asn Cys Thr
                165                 170                 175

Phe Leu Gly Lys Arg Val Glu His His Gly Thr Glu Lys Ser Asp Ala
            180                 185                 190

Val Cys Ser Ser Ser Leu Pro Ala Arg Lys Pro Pro Asn Glu Pro His
        195                 200                 205

Val Tyr Leu Pro Gly Leu Ile Ile Leu Leu Leu Phe Ala Ser Val Ala
    210                 215                 220

Leu Val Ala Ala Ile Ile Phe Gly Val Cys Tyr Arg Lys Lys Gly Lys
225                 230                 235                 240

Ala Leu Thr Ala Asn Leu Trp His Trp Ile Asn Glu Ala Cys Gly Arg
                245                 250                 255

Leu Ser Gly Asp Lys Glu Ser Ser Gly Asp Ser Cys Val Ser Thr His
            260                 265                 270

Thr Ala Asn Phe Gly Gln Gln Gly Ala Cys Glu Gly Val Leu Leu Leu
        275                 280                 285

Thr Leu Glu Glu Lys Thr Phe Pro Glu Asp Met Cys Tyr Pro Asp Gln
    290                 295                 300

Gly Gly Val Cys Gln Gly Thr Cys Val Gly Gly Gly Pro Tyr Ala Gln
305                 310                 315                 320

Gly Glu Asp Ala Arg Met Leu Ser Leu Val Ser Lys Thr Glu Ile Glu
                325                 330                 335

Glu Asp Ser Phe Arg Gln Met Pro Thr Glu Asp Glu Tyr Met Asp Arg
            340                 345                 350

Pro Ser Gln Pro Thr Asp Gln Leu Leu Phe Leu Thr Glu Pro Gly Ser
        355                 360                 365

Lys Ser Thr Pro Pro Phe Ser Glu Pro Leu Glu Val Gly Glu Asn Asp
    370                 375                 380

Ser Leu Ser Gln Cys Phe Thr Gly Thr Gln Ser Thr Val Gly Ser Glu
385                 390                 395                 400

Ser Cys Asn Cys Thr Glu Pro Leu Cys Arg Thr Asp Trp Thr Pro Met
                405                 410                 415

Ser Ser Glu Asn Tyr Leu Gln Lys Glu Val Asp Ser Gly His Cys Pro
            420                 425                 430

His Trp Ala Ala Ser Pro Ser Pro Asn Trp Ala Asp Val Cys Thr Gly
        435                 440                 445

Cys Arg Asn
    450
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<210> 5
<211> 3136
<212> cDNA
<213> Human

<220>
<221> CDS
<222> (39)..(1886)

<400> 5

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ccgctgaggc cgcggcgccc gccagcctgt cccgcgcc atg gcc ccg cgc gcc          53
                                          Met Ala Pro Arg Ala
                                            1               5

cgg cgg cgc cgc ccg ctg ttc gcg ctg ctg ctg ctc tgc gcg ctg ctc       101
Arg Arg Arg Arg Pro Leu Phe Ala Leu Leu Leu Leu Cys Ala Leu Leu
                10                  15                  20

gcc cgg ctg cag gtg gct ttg cag atc gct cct cca tgt acc agt gag       149
Ala Arg Leu Gln Val Ala Leu Gln Ile Ala Pro Pro Cys Thr Ser Glu
            25                  30                  35

aag cat tat gag cat ctg gga cgg tgc tgt aac aaa tgt gaa cca gga       197
Lys His Tyr Glu His Leu Gly Arg Cys Cys Asn Lys Cys Glu Pro Gly
        40                  45                  50

aag tac atg tct tct aaa tgc act act acc tct gac agt gta tgt ctg       245
Lys Tyr Met Ser Ser Lys Cys Thr Thr Thr Ser Asp Ser Val Cys Leu
    55                  60                  65

ccc tgt ggc ccg gat gaa tac ttg gat agc tgg aat gaa gaa gat aaa       293
Pro Cys Gly Pro Asp Glu Tyr Leu Asp Ser Trp Asn Glu Glu Asp Lys
70                  75                  80                  85

tgc ttg ctg cat aaa gtt tgt gat aca ggc aag gcc ctg gtg gcc gtg       341
Cys Leu Leu His Lys Val Cys Asp Thr Gly Lys Ala Leu Val Ala Val
                90                  95                  100

gtc gcc ggc aac agc acg acc ccc cgg cgc tgc gcg tgc acg gct ggg       389
Val Ala Gly Asn Ser Thr Thr Pro Arg Arg Cys Ala Cys Thr Ala Gly
            105                 110                 115

tac cac tgg agc cag gac tgc gag tgc tgc cgc cgc aac acc gag tgc       437
Tyr His Trp Ser Gln Asp Cys Glu Cys Cys Arg Arg Asn Thr Glu Cys
        120                 125                 130

gcg ccg ggc ctg ggc gcc cag cac ccg ttg cag ctc aac aag gac aca       485
Ala Pro Gly Leu Gly Ala Gln His Pro Leu Gln Leu Asn Lys Asp Thr
    135                 140                 145

gtg tgc aaa cct tgc ctt gca ggc tac ttc tct gat gcc ttt tcc tcc       533
Val Cys Lys Pro Cys Leu Ala Gly Tyr Phe Ser Asp Ala Phe Ser Ser
150                 155                 160                 165

acg gac aaa tgc aga ccc tgg acc aac tgt acc ttc ctt gga aag aga       581
Thr Asp Lys Cys Arg Pro Trp Thr Asn Cys Thr Phe Leu Gly Lys Arg
                170                 175                 180

gta gaa cat cat ggg aca gag aaa tcc gat gcg gtt tgc agt tct tct       629
Val Glu His His Gly Thr Glu Lys Ser Asp Ala Val Cys Ser Ser Ser
            185                 190                 195

ctg cca gct aga aaa cca cca aat gaa ccc cat gtt tac ttg ccc ggt       677
Leu Pro Ala Arg Lys Pro Pro Asn Glu Pro His Val Tyr Leu Pro Gly
        200                 205                 210

tta ata att ctg ctt ctc ttc gcg tct gtg gcc ctg gtg gct gcc atc       725
Leu Ile Ile Leu Leu Leu Phe Ala Ser Val Ala Leu Val Ala Ala Ile
    215                 220                 225
```

```
atc ttt ggc gtt tgc tat agg aaa aaa ggg aaa gca ctc aca gct aat       773
Ile Phe Gly Val Cys Tyr Arg Lys Lys Gly Lys Ala Leu Thr Ala Asn
230                 235                 240                 245

ttg tgg cac tgg atc aat gag gct tgt ggc cgc cta agt gga gat aag       821
Leu Trp His Trp Ile Asn Glu Ala Cys Gly Arg Leu Ser Gly Asp Lys
                250                 255                 260

gag tcc tca ggt gac agt tgt gtc agt aca cac acg gca aac ttt ggt       869
Glu Ser Ser Gly Asp Ser Cys Val Ser Thr His Thr Ala Asn Phe Gly
            265                 270                 275

cag cag gga gca tgt gaa ggt gtc tta ctg ctg act ctg gag gag aag       917
Gln Gln Gly Ala Cys Glu Gly Val Leu Leu Leu Thr Leu Glu Glu Lys
        280                 285                 290

aca ttt cca gaa gat atg tgc tac cca gat caa ggt ggt gtc tgt cag       965
Thr Phe Pro Glu Asp Met Cys Tyr Pro Asp Gln Gly Gly Val Cys Gln
    295                 300                 305

ggc acg tgt gta gga ggt ggt ccc tac gca caa ggc gaa gat gcc agg      1013
Gly Thr Cys Val Gly Gly Gly Pro Tyr Ala Gln Gly Glu Asp Ala Arg
310                 315                 320                 325

atg ctc tca ttg gtc agc aag acc gag ata gag gaa gac agc ttc aga      1061
Met Leu Ser Leu Val Ser Lys Thr Glu Ile Glu Glu Asp Ser Phe Arg
                330                 335                 340

cag atg ccc aca gaa gat gaa tac atg gac agg ccc tcc cag ccc aca      1109
Gln Met Pro Thr Glu Asp Glu Tyr Met Asp Arg Pro Ser Gln Pro Thr
            345                 350                 355

gac cag tta ctg ttc ctc act gag cct gga agc aaa tcc aca cct cct      1157
Asp Gln Leu Leu Phe Leu Thr Glu Pro Gly Ser Lys Ser Thr Pro Pro
        360                 365                 370

ttc tct gaa ccc ctg gag gtg ggg gag aat gac agt tta agc cag tgc      1205
Phe Ser Glu Pro Leu Glu Val Gly Glu Asn Asp Ser Leu Ser Gln Cys
    375                 380                 385

ttc acg ggg aca cag agc aca gtg ggt tca gaa agc tgc aac tgc act      1253
Phe Thr Gly Thr Gln Ser Thr Val Gly Ser Glu Ser Cys Asn Cys Thr
390                 395                 400                 405

gag ccc ctg tgc agg act gat tgg act ccc atg tcc tct gaa aac tac      1301
Glu Pro Leu Cys Arg Thr Asp Trp Thr Pro Met Ser Ser Glu Asn Tyr
                410                 415                 420

ttg caa aaa gag gtg gac agt ggc cat tgc ccg cac tgg gca gcc agc      1349
Leu Gln Lys Glu Val Asp Ser Gly His Cys Pro His Trp Ala Ala Ser
            425                 430                 435

ccc agc ccc aac tgg gca gat gtc tgc aca ggc tgc cgg aac cct cct      1397
Pro Ser Pro Asn Trp Ala Asp Val Cys Thr Gly Cys Arg Asn Pro Pro
        440                 445                 450

ggg gag gac tgt gaa ccc ctc gtg ggt tcc cca aaa cgt gga ccc ttg      1445
Gly Glu Asp Cys Glu Pro Leu Val Gly Ser Pro Lys Arg Gly Pro Leu
    455                 460                 465

ccc cag tgc gcc tat ggc atg ggc ctt ccc cct gaa gaa gaa gcc agc      1493
Pro Gln Cys Ala Tyr Gly Met Gly Leu Pro Pro Glu Glu Glu Ala Ser
470                 475                 480                 485
```

```
agg acg gag gcc aga gac cag ccc gag gat ggg gct gat ggg agg ctc      1541
Arg Thr Glu Ala Arg Asp Gln Pro Glu Asp Gly Ala Asp Gly Arg Leu
                490                 495                 500

cca agc tca gcg agg gca ggt gcc ggg tct gga agc tcc cct ggt ggc      1589
Pro Ser Ser Ala Arg Ala Gly Ala Gly Ser Gly Ser Ser Pro Gly Gly
            505                 510                 515

cag tcc cct gca tct gga aat gtg act gga aac agt aac tcc acg ttc      1637
Gln Ser Pro Ala Ser Gly Asn Val Thr Gly Asn Ser Asn Ser Thr Phe
        520                 525                 530

atc tcc agc ggg cag gtg atg aac ttc aag ggc gac atc atc gtg gtc      1685
Ile Ser Ser Gly Gln Val Met Asn Phe Lys Gly Asp Ile Ile Val Val
    535                 540                 545

tac gtc agc cag acc tcg cag gag ggc gcg gcg gcg gct gcg gag ccc      1733
Tyr Val Ser Gln Thr Ser Gln Glu Gly Ala Ala Ala Ala Ala Glu Pro
550                 555                 560                 565

atg ggc cgc ccg gtg cag gag gag acc ctg gcg cgc cga gac tcc ttc      1781
Met Gly Arg Pro Val Gln Glu Glu Thr Leu Ala Arg Arg Asp Ser Phe
                570                 575                 580

gcg ggg aac ggc ccg cgc ttc ccg gac ccg tgc ggc ggc ccc gag ggg      1829
Ala Gly Asn Gly Pro Arg Phe Pro Asp Pro Cys Gly Gly Pro Glu Gly
            585                 590                 595

ctg cgg gag ccg gag aag gcc tcg agg ccg gtg cag gag caa ggc ggg      1877
Leu Arg Glu Pro Glu Lys Ala Ser Arg Pro Val Gln Glu Gln Gly Gly
        600                 605                 610

gcc aag gct tgagcgcccc ccatggctgg gagcccgaag ctcggagcca              1926
Ala Lys Ala
    615

gggctcgcga gggcagcacc gcagcctctg ccccagcccc ggccacccag ggatcgatcg    1986

gtacagtcga ggaagaccac ccggcattct ctgcccactt tgccttccag gaaatgggct    2046

tttcaggaag tgaattgatg aggactgtcc ccatgcccac ggatgctcag cagcccgccg    2106

cactggggca gatgtctccc ctgccactcc tcaaactcgc agcagtaatt tgtggcacta    2166

tgacagctat ttttatgact atcctgttct gtgggggggg ggtctatgtt ttcccccccat    2226

atttgtattc cttttcataa cttttcttga tatctttcct ccctcttttt taatgtaaag    2286

gttttctcaa aaattctcct aaaggtgagg gtctctttct tttctctttt cctttttttt    2346

ttctttttt ggcaacctgg ctctggccca ggctagagtg cagtggtgcg attatagccc    2406

ggtgcagcct ctaactcctg ggctcaagca atccaagtga tcctcccacc tcaaccttcg    2466

gagtagctgg gatcacagct gcaggccacg cccagcttcc tccccccgac tccccccccc    2526

cagagacacg gtcccaccat gttacccagc ctggtctcaa actccccagc taaagcagtc    2586

ctccagcctc ggcctcccaa agtactggga ttacaggcgt gagcccccac gctggcctgc    2646

tttacgtatt ttcttttgtg cccctgctca cagtgtttta gagatggctt tcccagtgtg    2706

tgttcattgt aaacactttt gggaaagggc taaacatgtg aggcctggag atagttgcta    2766

agttgctagg aacatgtggt gggactttca tattctgaaa aatgttctat attctcattt    2826
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ttctaaaaga aagaaaaaag gaaacccgat ttatttctcc tgaatctttt taagtttgtg 2886

tcgttcctta agcagaacta agctcagtat gtgaccttac ccgctaggtg gttaatttat 2946

ccatgctggc agaggcactc aggtacttgg taagcaaatt tctaaaactc caagttgctg 3006

cagcttggca ttcttcttat tctagaggtc tctctggaaa agatggagaa aatgaacagg 3066

acatggggct cctggaaaga aagggcccgg gaagttcaag gaagaataaa gttgaaattt 3126

taaaaaaaaa 3136

<210> 6
<211> 616
<212> PRT
<213> Human

<400> 6

Met Ala Pro Arg Ala Arg Arg Arg Arg Pro Leu Phe Ala Leu Leu Leu
1 5 10 15

Leu Cys Ala Leu Leu Ala Arg Leu Gln Val Ala Leu Gln Ile Ala Pro
20 25 30

Pro Cys Thr Ser Glu Lys His Tyr Glu His Leu Gly Arg Cys Cys Asn
35 40 45

Lys Cys Glu Pro Gly Lys Tyr Met Ser Ser Lys Cys Thr Thr Thr Ser
50 55 60

Asp Ser Val Cys Leu Pro Cys Gly Pro Asp Glu Tyr Leu Asp Ser Trp
65 70 75 80

Asn Glu Glu Asp Lys Cys Leu Leu His Lys Val Cys Asp Thr Gly Lys
85 90 95

Ala Leu Val Ala Val Val Ala Gly Asn Ser Thr Thr Pro Arg Arg Cys
100 105 110

Ala Cys Thr Ala Gly Tyr His Trp Ser Gln Asp Cys Glu Cys Cys Arg
115 120 125

Arg Asn Thr Glu Cys Ala Pro Gly Leu Gly Ala Gln His Pro Leu Gln
130 135 140

Leu Asn Lys Asp Thr Val Cys Lys Pro Cys Leu Ala Gly Tyr Phe Ser
145 150 155 160

Asp Ala Phe Ser Ser Thr Asp Lys Cys Arg Pro Trp Thr Asn Cys Thr
165 170 175

Phe Leu Gly Lys Arg Val Glu His His Gly Thr Glu Lys Ser Asp Ala
180 185 190

Val Cys Ser Ser Ser Leu Pro Ala Arg Lys Pro Pro Asn Glu Pro His
195 200 205

Val Tyr Leu Pro Gly Leu Ile Ile Leu Leu Leu Phe Ala Ser Val Ala
210 215 220

Leu Val Ala Ala Ile Ile Phe Gly Val Cys Tyr Arg Lys Lys Gly Lys
225 230 235 240

```
Ala Leu Thr Ala Asn Leu Trp His Trp Ile Asn Glu Ala Cys Gly Arg
                245                 250                 255

Leu Ser Gly Asp Lys Glu Ser Ser Gly Asp Ser Cys Val Ser Thr His
            260                 265                 270

Thr Ala Asn Phe Gly Gln Gln Gly Ala Cys Glu Gly Val Leu Leu Leu
        275                 280                 285

Thr Leu Glu Glu Lys Thr Phe Pro Glu Asp Met Cys Tyr Pro Asp Gln
    290                 295                 300

Gly Gly Val Cys Gln Gly Thr Cys Val Gly Gly Gly Pro Tyr Ala Gln
305                 310                 315                 320

Gly Glu Asp Ala Arg Met Leu Ser Leu Val Ser Lys Thr Glu Ile Glu
                325                 330                 335

Glu Asp Ser Phe Arg Gln Met Pro Thr Glu Asp Glu Tyr Met Asp Arg
            340                 345                 350

Pro Ser Gln Pro Thr Asp Gln Leu Leu Phe Leu Thr Glu Pro Gly Ser
        355                 360                 365

Lys Ser Thr Pro Pro Phe Ser Glu Pro Leu Glu Val Gly Glu Asn Asp
    370                 375                 380

Ser Leu Ser Gln Cys Phe Thr Gly Thr Gln Ser Thr Val Gly Ser Glu
385                 390                 395                 400

Ser Cys Asn Cys Thr Glu Pro Leu Cys Arg Thr Asp Trp Thr Pro Met
                405                 410                 415

Ser Ser Glu Asn Tyr Leu Gln Lys Glu Val Asp Ser Gly His Cys Pro
            420                 425                 430

His Trp Ala Ala Ser Pro Ser Pro Asn Trp Ala Asp Val Cys Thr Gly
        435                 440                 445

Cys Arg Asn Pro Pro Gly Glu Asp Cys Glu Pro Leu Val Gly Ser Pro
    450                 455                 460

Lys Arg Gly Pro Leu Pro Gln Cys Ala Tyr Gly Met Gly Leu Pro Pro
465                 470                 475                 480

Glu Glu Glu Ala Ser Arg Thr Glu Ala Arg Asp Gln Pro Glu Asp Gly
                485                 490                 495

Ala Asp Gly Arg Leu Pro Ser Ser Ala Arg Ala Gly Ala Gly Ser Gly
            500                 505                 510

Ser Ser Pro Gly Gly Gln Ser Pro Ala Ser Gly Asn Val Thr Gly Asn
        515                 520                 525

Ser Asn Ser Thr Phe Ile Ser Ser Gly Gln Val Met Asn Phe Lys Gly
    530                 535                 540

Asp Ile Ile Val Val Tyr Val Ser Gln Thr Ser Gln Glu Gly Ala Ala
545                 550                 555                 560

Ala Ala Ala Glu Pro Met Gly Arg Pro Val Gln Glu Glu Thr Leu Ala
                565                 570                 575

Arg Arg Asp Ser Phe Ala Gly Asn Gly Pro Arg Phe Pro Asp Pro Cys
            580                 585                 590
```

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Gly Gly Pro Glu Gly Leu Arg Glu Pro Glu Lys Ala Ser Arg Pro Val
        595                 600                 605

Gln Glu Gln Gly Gly Ala Lys Ala
    610                 615


<210>  7
<211>  8
<212>  Peptide
<213>  synthetic


<400>  7

Asp Tyr Lys Asp Asp Asp Asp Lys
  1               5


<210>  8
<211>  232
<212>  PRT
<213>  Human


<400>  8

Glu Pro Arg Ser Cys Asp Lys Thr His Thr Cys Pro Pro Cys Pro Ala
  1               5                  10                  15

Pro Glu Ala Glu Gly Ala Pro Ser Val Phe Leu Phe Pro Pro Lys Pro
             20                  25                  30

Lys Asp Thr Leu Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val
         35                  40                  45

Val Asp Val Ser His Glu Asp Pro Glu Val Lys Phe Asn Trp Tyr Val
     50                  55                  60

Asp Gly Val Glu Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln
 65                  70                  75                  80

Tyr Asn Ser Thr Tyr Arg Val Val Ser Val Leu Thr Val Leu His Gln
                 85                  90                  95

Asp Trp Leu Asn Gly Lys Asp Tyr Lys Cys Lys Val Ser Asn Lys Ala
            100                 105                 110

Leu Pro Ala Pro Met Gln Lys Thr Ile Ser Lys Ala Lys Gly Gln Pro
        115                 120                 125

Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser Arg Asp Glu Leu Thr
    130                 135                 140

Lys Asn Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Arg
145                 150                 155                 160

His Ile Ala Val Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr
                165                 170                 175

Lys Thr Thr Pro Pro Val Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr
            180                 185                 190

Ser Lys Leu Thr Val Asp Lys Ser Arg Trp Gln Gln Gly Asn Val Phe
        195                 200                 205
```

```
Ser Cys Ser Val Met His Glu Ala Leu His Asn His Tyr Thr Gln Lys
    210                 215                 220

Ser Leu Ser Leu Ser Pro Gly Lys
225                 230
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<210> 9
<211> 31
<212> Peptide
<213> synthetic

<400> 9

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     Met Ala Arg Arg Leu Trp Ile Leu Ser Leu Leu Ala Val Thr Leu Thr
     1               5                   10                  15

     Val Ala Leu Ala Ala Pro Ser Gln Lys Ser Lys Arg Arg Thr Ser
                 20                  25                  30
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<211> 1630
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<220>
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<222> (3)..(884)

<400> 10

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cc ggc gtc cca cac gag ggt ccg ctg cac ccc gcg cct tct gca ccg          47
   Gly Val Pro His Glu Gly Pro Leu His Pro Ala Pro Ser Ala Pro
     1               5                   10                  15

gct ccg gcg ccg cca ccc gcc gcc tcc cgc tcc atg ttc ctg gcc ctc         95
Ala Pro Ala Pro Pro Pro Ala Ala Ser Arg Ser Met Phe Leu Ala Leu
                 20                  25                  30

ctg ggg ctg gga ctg ggc cag gtg gtc tgc agc atc gct ctg ttc ctg        143
Leu Gly Leu Gly Leu Gly Gln Val Val Cys Ser Ile Ala Leu Phe Leu
             35                  40                  45

tac ttt cga gcg cag atg gat cct aac aga ata tca gaa gac agc act        191
Tyr Phe Arg Ala Gln Met Asp Pro Asn Arg Ile Ser Glu Asp Ser Thr
         50                  55                  60

cac tgc ttt tat aga atc ctg aga ctc cat gaa aac gca gat ttg cag        239
His Cys Phe Tyr Arg Ile Leu Arg Leu His Glu Asn Ala Asp Leu Gln
     65                  70                  75

gac tcg act ctg gag agt gaa gac aca cta cct gac tcc tgc agg agg        287
Asp Ser Thr Leu Glu Ser Glu Asp Thr Leu Pro Asp Ser Cys Arg Arg
 80                  85                  90                  95

atg aaa caa gcc ttt cag ggg gcc gtg cag aag gaa ctg caa cac att        335
Met Lys Gln Ala Phe Gln Gly Ala Val Gln Lys Glu Leu Gln His Ile
                100                 105                 110

gtg ggg cca cag cgc ttc tca gga gct cca gct atg atg gaa ggc tca        383
Val Gly Pro Gln Arg Phe Ser Gly Ala Pro Ala Met Met Glu Gly Ser
            115                 120                 125
```

```
tgg ttg gat gtg gcc cag cga ggc aag cct gag gcc cag cca ttt gca       431
Trp Leu Asp Val Ala Gln Arg Gly Lys Pro Glu Ala Gln Pro Phe Ala
        130                 135                 140

cac ctc acc atc aat gct gcc agc atc cca tcg ggt tcc cat aaa gtc       479
His Leu Thr Ile Asn Ala Ala Ser Ile Pro Ser Gly Ser His Lys Val
    145                 150                 155

act ctg tcc tct tgg tac cac gat cga ggc tgg gcc aag atc tct aac       527
Thr Leu Ser Ser Trp Tyr His Asp Arg Gly Trp Ala Lys Ile Ser Asn
160                 165                 170                 175

atg acg tta agc aac gga aaa cta agg gtt aac caa gat ggc ttc tat       575
Met Thr Leu Ser Asn Gly Lys Leu Arg Val Asn Gln Asp Gly Phe Tyr
                180                 185                 190

tac ctg tac gcc aac att tgc ttt cgg cat cat gaa aca tcg gga agc       623
Tyr Leu Tyr Ala Asn Ile Cys Phe Arg His His Glu Thr Ser Gly Ser
            195                 200                 205

gta cct aca gac tat ctt cag ctg atg gtg tat gtc gtt aaa acc agc       671
Val Pro Thr Asp Tyr Leu Gln Leu Met Val Tyr Val Val Lys Thr Ser
        210                 215                 220

atc aaa atc cca agt tct cat aac ctg atg aaa gga ggg agc acg aaa       719
Ile Lys Ile Pro Ser Ser His Asn Leu Met Lys Gly Gly Ser Thr Lys
    225                 230                 235

aac tgg tcg ggc aat tct gaa ttc cac ttt tat tcc ata aat gtt ggg       767
Asn Trp Ser Gly Asn Ser Glu Phe His Phe Tyr Ser Ile Asn Val Gly
240                 245                 250                 255

gga ttt ttc aag ctc cga gct ggt gaa gaa att agc att cag gtg tcc       815
Gly Phe Phe Lys Leu Arg Ala Gly Glu Glu Ile Ser Ile Gln Val Ser
                260                 265                 270

aac cct tcc ctg ctg gat ccg gat caa gat gcg acg tac ttt ggg gct       863
Asn Pro Ser Leu Leu Asp Pro Asp Gln Asp Ala Thr Tyr Phe Gly Ala
            275                 280                 285

ttc aaa gtt cag gac ata gac tgagactcat ttcgtggaac attagcatgg          914
Phe Lys Val Gln Asp Ile Asp
        290

atgtcctaga tgtttggaaa cttcttaaaa aatggatgat gtctatacat gtgtaagact     974

actaagagac atggcccacg gtgtatgaaa ctcacagccc tctctcttga gcctgtacag    1034

gttgtgtata tgtaaagtcc ataggtgatg ttagattcat ggtgattaca caacggtttt    1094

acaatttttgt aatgatttcc tagaattgaa ccagattggg agaggtattc cgatgcttat    1154

gaaaaactta cacgtgagct atggaagggg gtcacagtct ctgggtctaa cccctggaca    1214

tgtgccactg agaaccttga aattaagagg atgccatgtc attgcaaaga aatgatagtg    1274

tgaagggtta agttcttttg aattgttaca ttgcgctggg acctgcaaat aagttctttt    1334

tttctaatga ggagagaaaa atatatgtat ttttatataa tgtctaaagt tatatttcag    1394

gtgtaatgtt ttctgtgcaa agttttgtaa attatatttg tgctatagta tttgattcaa    1454

aatatttaaa aatgtctcac tgttgacata tttaatgttt taaatgtaca gatgtattta    1514
```

actggtgcac tttgtaattc ccctgaaggt actcgtagct aaggggggcag aatactgttt 1574

ctggtgacca catgtagttt atttctttat tctttttaac ttaatagagt cttcag 1630

<210> 11
<211> 294
<212> PRT
<213> Mus Musculus

<400> 11

Gly Val Pro His Glu Gly Pro Leu His Pro Ala Pro Ser Ala Pro Ala
1 5 10 15

Pro Ala Pro Pro Pro Ala Ala Ser Arg Ser Met Phe Leu Ala Leu Leu
20 25 30

Gly Leu Gly Leu Gly Gln Val Val Cys Ser Ile Ala Leu Phe Leu Tyr
35 40 45

Phe Arg Ala Gln Met Asp Pro Asn Arg Ile Ser Glu Asp Ser Thr His
50 55 60

Cys Phe Tyr Arg Ile Leu Arg Leu His Glu Asn Ala Asp Leu Gln Asp
65 70 75 80

Ser Thr Leu Glu Ser Glu Asp Thr Leu Pro Asp Ser Cys Arg Arg Met
85 90 95

Lys Gln Ala Phe Gln Gly Ala Val Gln Lys Glu Leu Gln His Ile Val
100 105 110

Gly Pro Gln Arg Phe Ser Gly Ala Pro Ala Met Met Glu Gly Ser Trp
115 120 125

Leu Asp Val Ala Gln Arg Gly Lys Pro Glu Ala Gln Pro Phe Ala His
130 135 140

Leu Thr Ile Asn Ala Ala Ser Ile Pro Ser Gly Ser His Lys Val Thr
145 150 155 160

Leu Ser Ser Trp Tyr His Asp Arg Gly Trp Ala Lys Ile Ser Asn Met
165 170 175

Thr Leu Ser Asn Gly Lys Leu Arg Val Asn Gln Asp Gly Phe Tyr Tyr
180 185 190

Leu Tyr Ala Asn Ile Cys Phe Arg His His Glu Thr Ser Gly Ser Val
195 200 205

Pro Thr Asp Tyr Leu Gln Leu Met Val Tyr Val Val Lys Thr Ser Ile
210 215 220

Lys Ile Pro Ser Ser His Asn Leu Met Lys Gly Gly Ser Thr Lys Asn
225 230 235 240

Trp Ser Gly Asn Ser Glu Phe His Phe Tyr Ser Ile Asn Val Gly Gly
245 250 255

Phe Phe Lys Leu Arg Ala Gly Glu Glu Ile Ser Ile Gln Val Ser Asn
260 265 270

Pro Ser Leu Leu Asp Pro Asp Gln Asp Ala Thr Tyr Phe Gly Ala Phe
275 280 285

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Lys Val Gln Asp Ile Asp
    290


<210>  12
<211>  954
<212>  cDNA
<213>  Human

<220>
<221>  CDS
<222>  (1)..(951)


<400>  12

atg cgc cgc gcc agc aga gac tac acc aag tac ctg cgt ggc tcg gag        48
Met Arg Arg Ala Ser Arg Asp Tyr Thr Lys Tyr Leu Arg Gly Ser Glu
  1               5                  10                  15

gag atg ggc ggc ggc ccc gga gcc ccg cac gag ggc ccc ctg cac gcc        96
Glu Met Gly Gly Gly Pro Gly Ala Pro His Glu Gly Pro Leu His Ala
             20                  25                  30

ccg ccg ccg cct gcg ccg cac cag ccc ccc gcc gcc tcc cgc tcc atg       144
Pro Pro Pro Pro Ala Pro His Gln Pro Pro Ala Ala Ser Arg Ser Met
         35                  40                  45

ttc gtg gcc ctc ctg ggg ctg ggg ctg ggc cag gtt gtc tgc agc gtc       192
Phe Val Ala Leu Leu Gly Leu Gly Leu Gly Gln Val Val Cys Ser Val
     50                  55                  60

gcc ctg ttc ttc tat ttc aga gcg cag atg gat cct aat aga ata tca       240
Ala Leu Phe Phe Tyr Phe Arg Ala Gln Met Asp Pro Asn Arg Ile Ser
 65                  70                  75                  80

gaa gat ggc act cac tgc att tat aga att ttg aga ctc cat gaa aat       288
Glu Asp Gly Thr His Cys Ile Tyr Arg Ile Leu Arg Leu His Glu Asn
                 85                  90                  95

gca gat ttt caa gac aca act ctg gag agt caa gat aca aaa tta ata       336
Ala Asp Phe Gln Asp Thr Thr Leu Glu Ser Gln Asp Thr Lys Leu Ile
            100                 105                 110

cct gat tca tgt agg aga att aaa cag gcc ttt caa gga gct gtg caa       384
Pro Asp Ser Cys Arg Arg Ile Lys Gln Ala Phe Gln Gly Ala Val Gln
        115                 120                 125

aag gaa tta caa cat atc gtt gga tca cag cac atc aga gca gag aaa       432
Lys Glu Leu Gln His Ile Val Gly Ser Gln His Ile Arg Ala Glu Lys
    130                 135                 140

gcg atg gtg gat ggc tca tgg tta gat ctg gcc aag agg agc aag ctt       480
Ala Met Val Asp Gly Ser Trp Leu Asp Leu Ala Lys Arg Ser Lys Leu
145                 150                 155                 160

gaa gct cag cct ttt gct cat ctc act att aat gcc acc gac atc cca       528
Glu Ala Gln Pro Phe Ala His Leu Thr Ile Asn Ala Thr Asp Ile Pro
                165                 170                 175

tct ggt tcc cat aaa gtg agt ctg tcc tct tgg tac cat gat cgg ggt       576
Ser Gly Ser His Lys Val Ser Leu Ser Ser Trp Tyr His Asp Arg Gly
            180                 185                 190
```

```
tgg gcc aag atc tcc aac atg act ttt agc aat gga aaa cta ata gtt       624
Trp Ala Lys Ile Ser Asn Met Thr Phe Ser Asn Gly Lys Leu Ile Val
        195                 200                 205

aat cag gat ggc ttt tat tac ctg tat gcc aac att tgc ttt cga cat       672
Asn Gln Asp Gly Phe Tyr Tyr Leu Tyr Ala Asn Ile Cys Phe Arg His
    210                 215                 220

cat gaa act tca gga gac cta gct aca gag tat ctt caa cta atg gtg       720
His Glu Thr Ser Gly Asp Leu Ala Thr Glu Tyr Leu Gln Leu Met Val
225                 230                 235                 240

tac gtc act aaa acc agc atc aaa atc cca agt tct cat acc ctg atg       768
Tyr Val Thr Lys Thr Ser Ile Lys Ile Pro Ser Ser His Thr Leu Met
                245                 250                 255

aaa gga gga agc acc aag tat tgg tca ggg aat tct gaa ttc cat ttt       816
Lys Gly Gly Ser Thr Lys Tyr Trp Ser Gly Asn Ser Glu Phe His Phe
            260                 265                 270

tat tcc ata aac gtt ggt gga ttt ttt aag tta cgg tct gga gag gaa       864
Tyr Ser Ile Asn Val Gly Gly Phe Phe Lys Leu Arg Ser Gly Glu Glu
        275                 280                 285

atc agc atc gag gtc tcc aac ccc tcc tta ctg gat ccg gat cag gat       912
Ile Ser Ile Glu Val Ser Asn Pro Ser Leu Leu Asp Pro Asp Gln Asp
    290                 295                 300

gca aca tac ttt ggg gct ttt aaa gtt cga gat ata gat tga               954
Ala Thr Tyr Phe Gly Ala Phe Lys Val Arg Asp Ile Asp
305                 310                 315
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<210> 13
<211> 317
<212> PRT
<213> Human

<400> 13

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Met Arg Arg Ala Ser Arg Asp Tyr Thr Lys Tyr Leu Arg Gly Ser Glu
  1               5                  10                  15

Glu Met Gly Gly Gly Pro Gly Ala Pro His Glu Gly Pro Leu His Ala
             20                  25                  30

Pro Pro Pro Pro Ala Pro His Gln Pro Pro Ala Ala Ser Arg Ser Met
         35                  40                  45

Phe Val Ala Leu Leu Gly Leu Gly Leu Gly Gln Val Val Cys Ser Val
     50                  55                  60

Ala Leu Phe Phe Tyr Phe Arg Ala Gln Met Asp Pro Asn Arg Ile Ser
 65                  70                  75                  80

Glu Asp Gly Thr His Cys Ile Tyr Arg Ile Leu Arg Leu His Glu Asn
                 85                  90                  95

Ala Asp Phe Gln Asp Thr Thr Leu Glu Ser Gln Asp Thr Lys Leu Ile
            100                 105                 110

Pro Asp Ser Cys Arg Arg Ile Lys Gln Ala Phe Gln Gly Ala Val Gln
        115                 120                 125

Lys Glu Leu Gln His Ile Val Gly Ser Gln His Ile Arg Ala Glu Lys
    130                 135                 140
```

```
Ala Met Val Asp Gly Ser Trp Leu Asp Leu Ala Lys Arg Ser Lys Leu
145                 150                 155                 160

Glu Ala Gln Pro Phe Ala His Leu Thr Ile Asn Ala Thr Asp Ile Pro
                165                 170                 175

Ser Gly Ser His Lys Val Ser Leu Ser Ser Trp Tyr His Asp Arg Gly
            180                 185                 190

Trp Ala Lys Ile Ser Asn Met Thr Phe Ser Asn Gly Lys Leu Ile Val
        195                 200                 205

Asn Gln Asp Gly Phe Tyr Tyr Leu Tyr Ala Asn Ile Cys Phe Arg His
    210                 215                 220

His Glu Thr Ser Gly Asp Leu Ala Thr Glu Tyr Leu Gln Leu Met Val
225                 230                 235                 240

Tyr Val Thr Lys Thr Ser Ile Lys Ile Pro Ser Ser His Thr Leu Met
                245                 250                 255

Lys Gly Gly Ser Thr Lys Tyr Trp Ser Gly Asn Ser Glu Phe His Phe
            260                 265                 270

Tyr Ser Ile Asn Val Gly Gly Phe Phe Lys Leu Arg Ser Gly Glu Glu
        275                 280                 285

Ile Ser Ile Glu Val Ser Asn Pro Ser Leu Leu Asp Pro Asp Gln Asp
    290                 295                 300

Ala Thr Tyr Phe Gly Ala Phe Lys Val Arg Asp Ile Asp
305                 310                 315
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<210> 14
<211> 1878
<212> cDNA
<213> Murine

<220>
<221> CDS
<222> (1)..(1875)

<400> 14

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atg gcc ccg cgc gcc cgg cgg cgc cgc cag ctg ccc gcg ccg ctg ctg   48
Met Ala Pro Arg Ala Arg Arg Arg Arg Gln Leu Pro Ala Pro Leu Leu
  1               5                  10                  15

gcg ctc tgc gtg ctg ctc gtt cca ctg cag gtg act ctc cag gtc act   96
Ala Leu Cys Val Leu Leu Val Pro Leu Gln Val Thr Leu Gln Val Thr
             20                  25                  30

cct cca tgc acc cag gag agg cat tat gag cat ctc gga cgg tgt tgc  144
Pro Pro Cys Thr Gln Glu Arg His Tyr Glu His Leu Gly Arg Cys Cys
         35                  40                  45

agc aga tgc gaa cca gga aag tac ctg tcc tct aag tgc act cct acc  192
Ser Arg Cys Glu Pro Gly Lys Tyr Leu Ser Ser Lys Cys Thr Pro Thr
     50                  55                  60

tcc gac agt gtg tgt ctg ccc tgt ggc ccc gat gag tac ttg gac acc  240
Ser Asp Ser Val Cys Leu Pro Cys Gly Pro Asp Glu Tyr Leu Asp Thr
 65                  70                  75                  80
```

```
tgg aat gaa gaa gat aaa tgc ttg ctg cat aaa gtc tgt gat gca ggc  288
Trp Asn Glu Glu Asp Lys Cys Leu Leu His Lys Val Cys Asp Ala Gly
                 85                  90                  95

aag gcc ctg gtg gcg gtg gat cct ggc aac cac acg gcc ccg cgt cgc  336
Lys Ala Leu Val Ala Val Asp Pro Gly Asn His Thr Ala Pro Arg Arg
            100                 105                 110

tgt gct tgc acg gct ggc tac cac tgg aac tca gac tgc gag tgc tgc  384
Cys Ala Cys Thr Ala Gly Tyr His Trp Asn Ser Asp Cys Glu Cys Cys
        115                 120                 125

cgc agg aac acg gag tgt gca cct ggc ttc gga gct cag cat ccc ttg  432
Arg Arg Asn Thr Glu Cys Ala Pro Gly Phe Gly Ala Gln His Pro Leu
    130                 135                 140

cag ctc aac aag gat acg gtg tgc aca ccc tgc ctc ctg ggc ttc ttc  480
Gln Leu Asn Lys Asp Thr Val Cys Thr Pro Cys Leu Leu Gly Phe Phe
145                 150                 155                 160

tca gat gtc ttt tcg tcc aca gac aaa tgc aaa cct tgg acc aac tgc  528
Ser Asp Val Phe Ser Ser Thr Asp Lys Cys Lys Pro Trp Thr Asn Cys
                165                 170                 175

acc ctc ctt gga aag cta gaa gca cac cag ggg aca acg gaa tca gat  576
Thr Leu Leu Gly Lys Leu Glu Ala His Gln Gly Thr Thr Glu Ser Asp
            180                 185                 190

gtg gtc tgc agc tct tcc atg aca ctg agg aga cca ccc aag gag gcc  624
Val Val Cys Ser Ser Ser Met Thr Leu Arg Arg Pro Pro Lys Glu Ala
        195                 200                 205

cag gct tac ctg ccc agt ctc atc gtt ctg ctc ctc ttc atc tct gtg  672
Gln Ala Tyr Leu Pro Ser Leu Ile Val Leu Leu Leu Phe Ile Ser Val
    210                 215                 220

gta gta gtg gct gcc atc atc ttc ggc gtt tac tac agg aag gga ggg  720
Val Val Val Ala Ala Ile Ile Phe Gly Val Tyr Tyr Arg Lys Gly Gly
225                 230                 235                 240

aaa gcg ctg aca gct aat ttg tgg aat tgg gtc aat gat gct tgc agt  768
Lys Ala Leu Thr Ala Asn Leu Trp Asn Trp Val Asn Asp Ala Cys Ser
                245                 250                 255

agt cta agt gga aat aag gag tcc tca ggg gac cgt tgt gct ggt tcc  816
Ser Leu Ser Gly Asn Lys Glu Ser Ser Gly Asp Arg Cys Ala Gly Ser
            260                 265                 270

cac tcg gca acc tcc agt cag caa gaa gtg tgt gaa ggt atc tta cta  864
His Ser Ala Thr Ser Ser Gln Gln Glu Val Cys Glu Gly Ile Leu Leu
        275                 280                 285

atg act cgg gag gag aag atg gtt cca gaa gac ggt gct gga gtc tgt  912
Met Thr Arg Glu Glu Lys Met Val Pro Glu Asp Gly Ala Gly Val Cys
    290                 295                 300

ggg cct gtg tgt gcg gca ggt ggg ccc tgg gca gaa gtc aga gat tct  960
Gly Pro Val Cys Ala Ala Gly Gly Pro Trp Ala Glu Val Arg Asp Ser
305                 310                 315                 320

agg acg ttc aca ctg gtc agc gag gtt gag acg caa gga gac ctc tcg 1008
Arg Thr Phe Thr Leu Val Ser Glu Val Glu Thr Gln Gly Asp Leu Ser
                325                 330                 335
```

```
agg aag att ccc aca gag gat gag tac acg gac cgg ccc tcg cag cct  1056
Arg Lys Ile Pro Thr Glu Asp Glu Tyr Thr Asp Arg Pro Ser Gln Pro
            340                 345                 350

tcg act ggt tca ctg ctc cta atc cag cag gga agc aaa tct ata ccc  1104
Ser Thr Gly Ser Leu Leu Leu Ile Gln Gln Gly Ser Lys Ser Ile Pro
        355                 360                 365

cca ttc cag gag ccc ctg gaa gtg ggg gag aac gac agt tta agc cag  1152
Pro Phe Gln Glu Pro Leu Glu Val Gly Glu Asn Asp Ser Leu Ser Gln
    370                 375                 380

tgt ttc acc ggg act gaa agc acg gtg gat tct gag ggc tgt gac ttc  1200
Cys Phe Thr Gly Thr Glu Ser Thr Val Asp Ser Glu Gly Cys Asp Phe
385                 390                 395                 400

act gag cct ccg agc aga act gac tct atg ccc gtg tcc cct gaa aag  1248
Thr Glu Pro Pro Ser Arg Thr Asp Ser Met Pro Val Ser Pro Glu Lys
                405                 410                 415

cac ctg aca aaa gaa ata gaa ggt gac agt tgc ctc ccc tgg gtg gtc  1296
His Leu Thr Lys Glu Ile Glu Gly Asp Ser Cys Leu Pro Trp Val Val
            420                 425                 430

agc tcc aac tca aca gat ggc tac aca ggc agt ggg aac act cct ggg  1344
Ser Ser Asn Ser Thr Asp Gly Tyr Thr Gly Ser Gly Asn Thr Pro Gly
        435                 440                 445

gag gac cat gaa ccc ttt cca ggg tcc ctg aaa tgt gga cca ttg ccc  1392
Glu Asp His Glu Pro Phe Pro Gly Ser Leu Lys Cys Gly Pro Leu Pro
    450                 455                 460

cag tgt gcc tac agc atg ggc ttt ccc agt gaa gca gca gcc agc atg  1440
Gln Cys Ala Tyr Ser Met Gly Phe Pro Ser Glu Ala Ala Ala Ser Met
465                 470                 475                 480

gca gag gcg gga gta cgg ccc cag gac agg gct gat gag agg gga gcc  1488
Ala Glu Ala Gly Val Arg Pro Gln Asp Arg Ala Asp Glu Arg Gly Ala
                485                 490                 495

tca ggg tcc ggg agc tcc ccc agt gac cag cca cct gcc tct ggg aac  1536
Ser Gly Ser Gly Ser Ser Pro Ser Asp Gln Pro Pro Ala Ser Gly Asn
            500                 505                 510

gtg act gga aac agt aac tcc acg ttc atc tct agc ggg cag gtg atg  1584
Val Thr Gly Asn Ser Asn Ser Thr Phe Ile Ser Ser Gly Gln Val Met
        515                 520                 525

aac ttc aag ggt gac atc atc gtg gtg tat gtc agc cag acc tcg cag  1632
Asn Phe Lys Gly Asp Ile Ile Val Val Tyr Val Ser Gln Thr Ser Gln
    530                 535                 540

gag ggc ccg ggt tcc gca gag ccc gag tcg gag ccc gtg ggc cgc cct  1680
Glu Gly Pro Gly Ser Ala Glu Pro Glu Ser Glu Pro Val Gly Arg Pro
545                 550                 555                 560

gtg cag gag gag acg ctg gca cac aga gac tcc ttt gcg ggc acc gcg  1728
Val Gln Glu Glu Thr Leu Ala His Arg Asp Ser Phe Ala Gly Thr Ala
                565                 570                 575

ccg cgc ttc ccc gac gtc tgt gcc acc ggg gct ggg ctg cag gag cag  1776
Pro Arg Phe Pro Asp Val Cys Ala Thr Gly Ala Gly Leu Gln Glu Gln
            580                 585                 590
```

```
ggg gca ccc cgg cag aag gac ggg aca tcg cgg ccg gtg cag gag cag  1824
Gly Ala Pro Arg Gln Lys Asp Gly Thr Ser Arg Pro Val Gln Glu Gln
        595                 600                 605

ggt ggg gcg cag act tca ctc cat acc cag ggg tcc gga caa tgt gca  1872
Gly Gly Ala Gln Thr Ser Leu His Thr Gln Gly Ser Gly Gln Cys Ala
    610                 615                 620

gaa tga                                                          1878
Glu
625
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<210> 15
<211> 625
<212> PRT
<213> Murine

<400> 15

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Met Ala Pro Arg Ala Arg Arg Arg Arg Gln Leu Pro Ala Pro Leu Leu
  1               5                  10                  15

Ala Leu Cys Val Leu Leu Val Pro Leu Gln Val Thr Leu Gln Val Thr
            20                  25                  30

Pro Pro Cys Thr Gln Glu Arg His Tyr Glu His Leu Gly Arg Cys Cys
        35                  40                  45

Ser Arg Cys Glu Pro Gly Lys Tyr Leu Ser Ser Lys Cys Thr Pro Thr
    50                  55                  60

Ser Asp Ser Val Cys Leu Pro Cys Gly Pro Asp Glu Tyr Leu Asp Thr
65                  70                  75                  80

Trp Asn Glu Glu Asp Lys Cys Leu Leu His Lys Val Cys Asp Ala Gly
                85                  90                  95

Lys Ala Leu Val Ala Val Asp Pro Gly Asn His Thr Ala Pro Arg Arg
            100                 105                 110

Cys Ala Cys Thr Ala Gly Tyr His Trp Asn Ser Asp Cys Glu Cys Cys
        115                 120                 125

Arg Arg Asn Thr Glu Cys Ala Pro Gly Phe Gly Ala Gln His Pro Leu
    130                 135                 140

Gln Leu Asn Lys Asp Thr Val Cys Thr Pro Cys Leu Leu Gly Phe Phe
145                 150                 155                 160

Ser Asp Val Phe Ser Ser Thr Asp Lys Cys Lys Pro Trp Thr Asn Cys
                165                 170                 175

Thr Leu Leu Gly Lys Leu Glu Ala His Gln Gly Thr Thr Glu Ser Asp
            180                 185                 190

Val Val Cys Ser Ser Ser Met Thr Leu Arg Arg Pro Pro Lys Glu Ala
        195                 200                 205

Gln Ala Tyr Leu Pro Ser Leu Ile Val Leu Leu Leu Phe Ile Ser Val
    210                 215                 220

Val Val Val Ala Ala Ile Ile Phe Gly Val Tyr Tyr Arg Lys Gly Gly
225                 230                 235                 240
```

```
Lys Ala Leu Thr Ala Asn Leu Trp Asn Trp Val Asn Asp Ala Cys Ser
                245                 250                 255

Ser Leu Ser Gly Asn Lys Glu Ser Ser Gly Asp Arg Cys Ala Gly Ser
            260                 265                 270

His Ser Ala Thr Ser Ser Gln Gln Glu Val Cys Glu Gly Ile Leu Leu
        275                 280                 285

Met Thr Arg Glu Glu Lys Met Val Pro Glu Asp Gly Ala Gly Val Cys
    290                 295                 300

Gly Pro Val Cys Ala Ala Gly Gly Pro Trp Ala Glu Val Arg Asp Ser
305                 310                 315                 320

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

Arg Lys Ile Pro Thr Glu Asp Glu Tyr Thr Asp Arg Pro Ser Gln Pro
            340                 345                 350

Ser Thr Gly Ser Leu Leu Leu Ile Gln Gln Gly Ser Lys Ser Ile Pro
        355                 360                 365

Pro Phe Gln Glu Pro Leu Glu Val Gly Glu Asn Asp Ser Leu Ser Gln
    370                 375                 380

Cys Phe Thr Gly Thr Glu Ser Thr Val Asp Ser Glu Gly Cys Asp Phe
385                 390                 395                 400

Thr Glu Pro Pro Ser Arg Thr Asp Ser Met Pro Val Ser Pro Glu Lys
                405                 410                 415

His Leu Thr Lys Glu Ile Glu Gly Asp Ser Cys Leu Pro Trp Val Val
            420                 425                 430

Ser Ser Asn Ser Thr Asp Gly Tyr Thr Gly Ser Gly Asn Thr Pro Gly
        435                 440                 445

Glu Asp His Glu Pro Phe Pro Gly Ser Leu Lys Cys Gly Pro Leu Pro
    450                 455                 460

Gln Cys Ala Tyr Ser Met Gly Phe Pro Ser Glu Ala Ala Ala Ser Met
465                 470                 475                 480

Ala Glu Ala Gly Val Arg Pro Gln Asp Arg Ala Asp Glu Arg Gly Ala
                485                 490                 495

Ser Gly Ser Gly Ser Ser Pro Ser Asp Gln Pro Pro Ala Ser Gly Asn
            500                 505                 510

Val Thr Gly Asn Ser Asn Ser Thr Phe Ile Ser Ser Gly Gln Val Met
        515                 520                 525

Asn Phe Lys Gly Asp Ile Ile Val Val Tyr Val Ser Gln Thr Ser Gln
    530                 535                 540

Glu Gly Pro Gly Ser Ala Glu Pro Glu Ser Glu Pro Val Gly Arg Pro
545                 550                 555                 560

Val Gln Glu Glu Thr Leu Ala His Arg Asp Ser Phe Ala Gly Thr Ala
                565                 570                 575

Pro Arg Phe Pro Asp Val Cys Ala Thr Gly Ala Gly Leu Gln Glu Gln
            580                 585                 590
```

Gly Ala Pro Arg Gln Lys Asp Gly Thr Ser Arg Pro Val Gln Glu Gln
595 600 605

Gly Gly Ala Gln Thr Ser Leu His Thr Gln Gly Ser Gly Gln Cys Ala
610 615 620

Glu
625

<210> 16
<211> 20
<212> Peptide
<213> synthetic

<400> 16

Met Glu Thr Asp Thr Leu Leu Leu Trp Val Leu Leu Leu Trp Val Pro
1 5 10 15

Gly Ser Thr Gly
20

<210> 17
<211> 5
<212> Peptide
<213> synthetic

<400> 17

Asp Tyr Lys Asp Glu
5

<210> 18
<211> 6
<212> Peptide
<213> synthetic

<400> 18

His His His His His His
5

<210> 19
<211> 33
<212> Peptide
<213> synthetic

<400> 19

Arg Met Lys Gln Ile Glu Asp Lys Ile Glu Glu Ile Leu Ser Lys Ile
1 5 10 15

Tyr His Ile Glu Asn Glu Ile Ala Arg Ile Lys Lys Leu Ile Gly Glu
20 25 30

Arg

<210> 20

<211> 3154
<212> DNA
<213> Human

<220>
<221> CDS
<222> (39)..(1904)

<400> 20
ccgctgaggc cgcggcgccc gccagcctgt cccgcgcc atg gcc ccg cgc gcc cgg 56
Met Ala Pro Arg Ala Arg
1 5

cgg cgc cgc ccg ctg ttc gcg ctg ctg ctg ctc tgc gcg ctg ctc ctg 104
Arg Arg Arg Pro Leu Phe Ala Leu Leu Leu Leu Cys Ala Leu Leu Leu
10 15 20

ctc tgc gcg ctg ctc gcc cgg ctg cag gtg gct ttg cag atc gct cct 152
Leu Cys Ala Leu Leu Ala Arg Leu Gln Val Ala Leu Gln Ile Ala Pro
25 30 35

cca tgt acc agt gag aag cat tat gag cat ctg gga cgg tgc tgt aac 200
Pro Cys Thr Ser Glu Lys His Tyr Glu His Leu Gly Arg Cys Cys Asn
40 45 50

aaa tgt gaa cca gga aag tac atg tct tct aaa tgc act act acc tct 248
Lys Cys Glu Pro Gly Lys Tyr Met Ser Ser Lys Cys Thr Thr Thr Ser
55 60 65 70

gac agt gta tgt ctg ccc tgt ggc ccg gat gaa tac ttg gat agc tgg 296
Asp Ser Val Cys Leu Pro Cys Gly Pro Asp Glu Tyr Leu Asp Ser Trp
75 80 85

aat gaa gaa gat aaa tgc ttg ctg cat aaa gtt tgt gat aca ggc aag 344
Asn Glu Glu Asp Lys Cys Leu Leu His Lys Val Cys Asp Thr Gly Lys
90 95 100

gcc ctg gtg gcc gtg gtc gcc ggc aac agc acg acc ccc cgg cgc tgc 392
Ala Leu Val Ala Val Val Ala Gly Asn Ser Thr Thr Pro Arg Arg Cys
105 110 115

gcg tgc acg gct ggg tac cac tgg agc cag gac tgc gag tgc tgc cgc 440
Ala Cys Thr Ala Gly Tyr His Trp Ser Gln Asp Cys Glu Cys Cys Arg
120 125 130

cgc aac acc gag tgc gcg ccg ggc ctg ggc gcc cag cac ccg ttg cag 488
Arg Asn Thr Glu Cys Ala Pro Gly Leu Gly Ala Gln His Pro Leu Gln
135 140 145 150

ctc aac aag gac aca gtg tgc aaa cct tgc ctt gca ggc tac ttc tct 536
Leu Asn Lys Asp Thr Val Cys Lys Pro Cys Leu Ala Gly Tyr Phe Ser
155 160 165

gat gcc ttt tcc tcc acg gac aaa tgc aga ccc tgg acc aac tgt acc 584
Asp Ala Phe Ser Ser Thr Asp Lys Cys Arg Pro Trp Thr Asn Cys Thr
170 175 180

ttc ctt gga aag aga gta gaa cat cat ggg aca gag aaa tcc gat gcg 632
Phe Leu Gly Lys Arg Val Glu His His Gly Thr Glu Lys Ser Asp Ala
185 190 195

gtt tgc agt tct tct ctg cca gct aga aaa cca cca aat gaa ccc cat 680
Val Cys Ser Ser Ser Leu Pro Ala Arg Lys Pro Pro Asn Glu Pro His
200 205 210

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gtt tac ttg ccc ggt tta ata att ctg ctt ctc ttc gcg tct gtg gcc   728
Val Tyr Leu Pro Gly Leu Ile Ile Leu Leu Leu Phe Ala Ser Val Ala
215                 220                 225                 230

ctg gtg gct gcc atc atc ttt ggc gtt tgc tat agg aaa aaa ggg aaa   776
Leu Val Ala Ala Ile Ile Phe Gly Val Cys Tyr Arg Lys Lys Gly Lys
                235                 240                 245

gca ctc aca gct aat ttg tgg cac tgg atc aat gag gct tgt ggc cgc   824
Ala Leu Thr Ala Asn Leu Trp His Trp Ile Asn Glu Ala Cys Gly Arg
            250                 255                 260

cta agt gga gat aag gag tcc tca ggt gac agt tgt gtc agt aca cac   872
Leu Ser Gly Asp Lys Glu Ser Ser Gly Asp Ser Cys Val Ser Thr His
        265                 270                 275

acg gca aac ttt ggt cag cag gga gca tgt gaa ggt gtc tta ctg ctg   920
Thr Ala Asn Phe Gly Gln Gln Gly Ala Cys Glu Gly Val Leu Leu Leu
    280                 285                 290

act ctg gag gag aag aca ttt cca gaa gat atg tgc tac cca gat caa   968
Thr Leu Glu Glu Lys Thr Phe Pro Glu Asp Met Cys Tyr Pro Asp Gln
295                 300                 305                 310

ggt ggt gtc tgt cag ggc acg tgt gta gga ggt ggt ccc tac gca caa   1016
Gly Gly Val Cys Gln Gly Thr Cys Val Gly Gly Gly Pro Tyr Ala Gln
                315                 320                 325

ggc gaa gat gcc agg atg ctc tca ttg gtc agc aag acc gag ata gag   1064
Gly Glu Asp Ala Arg Met Leu Ser Leu Val Ser Lys Thr Glu Ile Glu
            330                 335                 340

gaa gac agc ttc aga cag atg ccc aca gaa gat gaa tac atg gac agg   1112
Glu Asp Ser Phe Arg Gln Met Pro Thr Glu Asp Glu Tyr Met Asp Arg
        345                 350                 355

ccc tcc cag ccc aca gac cag tta ctg ttc ctc act gag cct gga agc   1160
Pro Ser Gln Pro Thr Asp Gln Leu Leu Phe Leu Thr Glu Pro Gly Ser
    360                 365                 370

aaa tcc aca cct cct ttc tct gaa ccc ctg gag gtg ggg gag aat gac   1208
Lys Ser Thr Pro Pro Phe Ser Glu Pro Leu Glu Val Gly Glu Asn Asp
375                 380                 385                 390

agt tta agc cag tgc ttc acg ggg aca cag agc aca gtg ggt tca gaa   1256
Ser Leu Ser Gln Cys Phe Thr Gly Thr Gln Ser Thr Val Gly Ser Glu
                395                 400                 405

agc tgc aac tgc act gag ccc ctg tgc agg act gat tgg act ccc atg   1304
Ser Cys Asn Cys Thr Glu Pro Leu Cys Arg Thr Asp Trp Thr Pro Met
            410                 415                 420

tcc tct gaa aac tac ttg caa aaa gag gtg gac agt ggc cat tgc ccg   1352
Ser Ser Glu Asn Tyr Leu Gln Lys Glu Val Asp Ser Gly His Cys Pro
        425                 430                 435

cac tgg gca gcc agc ccc agc ccc aac tgg gca gat gtc tgc aca ggc   1400
His Trp Ala Ala Ser Pro Ser Pro Asn Trp Ala Asp Val Cys Thr Gly
    440                 445                 450

tgc cgg aac cct cct ggg gag gac tgt gaa ccc ctc gtg ggt tcc cca   1448
Cys Arg Asn Pro Pro Gly Glu Asp Cys Glu Pro Leu Val Gly Ser Pro
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aaa cgt gga ccc ttg ccc cag tgc gcc tat ggc atg ggc ctt ccc cct   1496
Lys Arg Gly Pro Leu Pro Gln Cys Ala Tyr Gly Met Gly Leu Pro Pro
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gaa gaa gaa gcc agc agg acg gag gcc aga gac cag ccc gag gat ggg   1544
Glu Glu Glu Ala Ser Arg Thr Glu Ala Arg Asp Gln Pro Glu Asp Gly
            490                 495                 500

gct gat ggg agg ctc cca agc tca gcg agg gca ggt gcc ggg tct gga   1592
Ala Asp Gly Arg Leu Pro Ser Ser Ala Arg Ala Gly Ala Gly Ser Gly
        505                 510                 515

agc tcc cct ggt ggc cag tcc cct gca tct gga aat gtg act gga aac   1640
Ser Ser Pro Gly Gly Gln Ser Pro Ala Ser Gly Asn Val Thr Gly Asn
    520                 525                 530

agt aac tcc acg ttc atc tcc agc ggg cag gtg atg aac ttc aag ggc   1688
Ser Asn Ser Thr Phe Ile Ser Ser Gly Gln Val Met Asn Phe Lys Gly
535                 540                 545                 550

gac atc atc gtg gtc tac gtc agc cag acc tcg cag gag ggc gcg gcg   1736
Asp Ile Ile Val Val Tyr Val Ser Gln Thr Ser Gln Glu Gly Ala Ala
                555                 560                 565

gcg gct gcg gag ccc atg ggc cgc ccg gtg cag gag gag acc ctg gcg   1784
Ala Ala Ala Glu Pro Met Gly Arg Pro Val Gln Glu Glu Thr Leu Ala
            570                 575                 580

cgc cga gac tcc ttc gcg ggg aac ggc ccg cgc ttc ccg gac ccg tgc   1832
Arg Arg Asp Ser Phe Ala Gly Asn Gly Pro Arg Phe Pro Asp Pro Cys
        585                 590                 595

ggc ggc ccc gag ggg ctg cgg gag ccg gag aag gcc tcg agg ccg gtg   1880
Gly Gly Pro Glu Gly Leu Arg Glu Pro Glu Lys Ala Ser Arg Pro Val
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cag gag caa ggc ggg gcc aag gct tgagcgcccc ccatggctgg gagcccgaag  1934
Gln Glu Gln Gly Gly Ala Lys Ala
615                 620

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caagttgctg cagcttggca ttcttcttat tctagaggtc tctctggaaa agatggagaa 3074

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Ala Leu Gln Ile Ala Pro Pro Cys Thr Ser Glu Lys His Tyr Glu His
         35                  40                  45

Leu Gly Arg Cys Cys Asn Lys Cys Glu Pro Gly Lys Tyr Met Ser Ser
     50                  55                  60

Lys Cys Thr Thr Thr Ser Asp Ser Val Cys Leu Pro Cys Gly Pro Asp
 65                  70                  75                  80

Glu Tyr Leu Asp Ser Trp Asn Glu Glu Asp Lys Cys Leu Leu His Lys
                 85                  90                  95

Val Cys Asp Thr Gly Lys Ala Leu Val Ala Val Val Ala Gly Asn Ser
            100                 105                 110

Thr Thr Pro Arg Arg Cys Ala Cys Thr Ala Gly Tyr His Trp Ser Gln
        115                 120                 125

Asp Cys Glu Cys Cys Arg Arg Asn Thr Glu Cys Ala Pro Gly Leu Gly
    130                 135                 140

Ala Gln His Pro Leu Gln Leu Asn Lys Asp Thr Val Cys Lys Pro Cys
145                 150                 155                 160

Leu Ala Gly Tyr Phe Ser Asp Ala Phe Ser Ser Thr Asp Lys Cys Arg
                165                 170                 175

Pro Trp Thr Asn Cys Thr Phe Leu Gly Lys Arg Val Glu His His Gly
            180                 185                 190

Thr Glu Lys Ser Asp Ala Val Cys Ser Ser Ser Leu Pro Ala Arg Lys
        195                 200                 205

Pro Pro Asn Glu Pro His Val Tyr Leu Pro Gly Leu Ile Ile Leu Leu
    210                 215                 220
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Leu Phe Ala Ser Val Ala Leu Val Ala Ala Ile Ile Phe Gly Val Cys
225                 230                 235                 240

Tyr Arg Lys Lys Gly Lys Ala Leu Thr Ala Asn Leu Trp His Trp Ile
                245                 250                 255

Asn Glu Ala Cys Gly Arg Leu Ser Gly Asp Lys Glu Ser Ser Gly Asp
            260                 265                 270

Ser Cys Val Ser Thr His Thr Ala Asn Phe Gly Gln Gln Gly Ala Cys
        275                 280                 285

Glu Gly Val Leu Leu Leu Thr Leu Glu Glu Lys Thr Phe Pro Glu Asp
    290                 295                 300

Met Cys Tyr Pro Asp Gln Gly Gly Val Cys Gln Gly Thr Cys Val Gly
305                 310                 315                 320

Gly Gly Pro Tyr Ala Gln Gly Glu Asp Ala Arg Met Leu Ser Leu Val
                325                 330                 335

Ser Lys Thr Glu Ile Glu Glu Asp Ser Phe Arg Gln Met Pro Thr Glu
            340                 345                 350

Asp Glu Tyr Met Asp Arg Pro Ser Gln Pro Thr Asp Gln Leu Leu Phe
        355                 360                 365

Leu Thr Glu Pro Gly Ser Lys Ser Thr Pro Pro Phe Ser Glu Pro Leu
    370                 375                 380

Glu Val Gly Glu Asn Asp Ser Leu Ser Gln Cys Phe Thr Gly Thr Gln
385                 390                 395                 400

Ser Thr Val Gly Ser Glu Ser Cys Asn Cys Thr Glu Pro Leu Cys Arg
                405                 410                 415

Thr Asp Trp Thr Pro Met Ser Ser Glu Asn Tyr Leu Gln Lys Glu Val
            420                 425                 430

Asp Ser Gly His Cys Pro His Trp Ala Ala Ser Pro Ser Pro Asn Trp
        435                 440                 445

Ala Asp Val Cys Thr Gly Cys Arg Asn Pro Pro Gly Glu Asp Cys Glu
    450                 455                 460

Pro Leu Val Gly Ser Pro Lys Arg Gly Pro Leu Pro Gln Cys Ala Tyr
465                 470                 475                 480

Gly Met Gly Leu Pro Pro Glu Glu Glu Ala Ser Arg Thr Glu Ala Arg
                485                 490                 495

Asp Gln Pro Glu Asp Gly Ala Asp Gly Arg Leu Pro Ser Ser Ala Arg
            500                 505                 510

Ala Gly Ala Gly Ser Gly Ser Ser Pro Gly Gly Gln Ser Pro Ala Ser
        515                 520                 525

Gly Asn Val Thr Gly Asn Ser Asn Ser Thr Phe Ile Ser Ser Gly Gln
    530                 535                 540

Val Met Asn Phe Lys Gly Asp Ile Ile Val Val Tyr Val Ser Gln Thr
545                 550                 555                 560

Ser Gln Glu Gly Ala Ala Ala Ala Ala Glu Pro Met Gly Arg Pro Val
                565                 570                 575
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Gln Glu Glu Thr Leu Ala Arg Arg Asp Ser Phe Ala Gly Asn Gly Pro
            580                 585                 590

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Lys Ala Ser Arg Pro Val Gln Glu Gln Gly Gly Ala Lys Ala
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                                            1               5

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Arg Arg Arg Pro Leu Phe Ala Leu Leu Leu Leu Cys Ala Leu Leu Ala
              10                  15                  20

ctg ctg ctg ctc tgc gcg ctg ctc gcc cgg ctg cag gtg gct ttg cag   152
Leu Leu Leu Leu Cys Ala Leu Leu Ala Arg Leu Gln Val Ala Leu Gln
          25                  30                  35

atc gct cct cca tgt acc agt gag aag cat tat gag cat ctg gga cgg   200
Ile Ala Pro Pro Cys Thr Ser Glu Lys His Tyr Glu His Leu Gly Arg
      40                  45                  50

tgc tgt aac aaa tgt gaa cca gga aag tac atg tct tct aaa tgc act   248
Cys Cys Asn Lys Cys Glu Pro Gly Lys Tyr Met Ser Ser Lys Cys Thr
  55                  60                  65                  70

act acc tct gac agt gta tgt ctg ccc tgt ggc ccg gat gaa tac ttg   296
Thr Thr Ser Asp Ser Val Cys Leu Pro Cys Gly Pro Asp Glu Tyr Leu
                  75                  80                  85

gat agc tgg aat gaa gaa gat aaa tgc ttg ctg cat aaa gtt tgt gat   344
Asp Ser Trp Asn Glu Glu Asp Lys Cys Leu Leu His Lys Val Cys Asp
              90                  95                  100

aca ggc aag gcc ctg gtg gcc gtg gtc gcc ggc aac agc acg acc ccc   392
Thr Gly Lys Ala Leu Val Ala Val Val Ala Gly Asn Ser Thr Thr Pro
          105                 110                 115

cgg cgc tgc gcg tgc acg gct ggg tac cac tgg agc cag gac tgc gag   440
Arg Arg Cys Ala Cys Thr Ala Gly Tyr His Trp Ser Gln Asp Cys Glu
      120                 125                 130

tgc tgc cgc cgc aac acc gag tgc gcg ccg ggc ctg ggc gcc cag cac   488
Cys Cys Arg Arg Asn Thr Glu Cys Ala Pro Gly Leu Gly Ala Gln His
135                 140                 145                 150

ccg ttg cag ctc aac aag gac aca gtg tgc aaa cct tgc ctt gca ggc   536
Pro Leu Gln Leu Asn Lys Asp Thr Val Cys Lys Pro Cys Leu Ala Gly
                  155                 160                 165
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tac ttc tct gat gcc ttt tcc tcc acg gac aaa tgc aga ccc tgg acc   584
Tyr Phe Ser Asp Ala Phe Ser Ser Thr Asp Lys Cys Arg Pro Trp Thr
            170                 175                 180

aac tgt acc ttc ctt gga aag aga gta gaa cat cat ggg aca gag aaa   632
Asn Cys Thr Phe Leu Gly Lys Arg Val Glu His His Gly Thr Glu Lys
        185                 190                 195

tcc gat gcg gtt tgc agt tct tct ctg cca gct aga aaa cca cca aat   680
Ser Asp Ala Val Cys Ser Ser Ser Leu Pro Ala Arg Lys Pro Pro Asn
    200                 205                 210

gaa ccc cat gtt tac ttg ccc ggt tta ata att ctg ctt ctc ttc gcg   728
Glu Pro His Val Tyr Leu Pro Gly Leu Ile Ile Leu Leu Leu Phe Ala
215                 220                 225                 230

tct gtg gcc ctg gtg gct gcc atc atc ttt ggc gtt tgc tat agg aaa   776
Ser Val Ala Leu Val Ala Ala Ile Ile Phe Gly Val Cys Tyr Arg Lys
                235                 240                 245

aaa ggg aaa gca ctc aca gct aat ttg tgg cac tgg atc aat gag gct   824
Lys Gly Lys Ala Leu Thr Ala Asn Leu Trp His Trp Ile Asn Glu Ala
            250                 255                 260

tgt ggc cgc cta agt gga gat aag gag tcc tca ggt gac agt tgt gtc   872
Cys Gly Arg Leu Ser Gly Asp Lys Glu Ser Ser Gly Asp Ser Cys Val
        265                 270                 275

agt aca cac acg gca aac ttt ggt cag cag gga gca tgt gaa ggt gtc   920
Ser Thr His Thr Ala Asn Phe Gly Gln Gln Gly Ala Cys Glu Gly Val
    280                 285                 290

tta ctg ctg act ctg gag gag aag aca ttt cca gaa gat atg tgc tac   968
Leu Leu Leu Thr Leu Glu Glu Lys Thr Phe Pro Glu Asp Met Cys Tyr
295                 300                 305                 310

cca gat caa ggt ggt gtc tgt cag ggc acg tgt gta gga ggt ggt ccc  1016
Pro Asp Gln Gly Gly Val Cys Gln Gly Thr Cys Val Gly Gly Gly Pro
                315                 320                 325

tac gca caa ggc gaa gat gcc agg atg ctc tca ttg gtc agc aag acc  1064
Tyr Ala Gln Gly Glu Asp Ala Arg Met Leu Ser Leu Val Ser Lys Thr
            330                 335                 340

gag ata gag gaa gac agc ttc aga cag atg ccc aca gaa gat gaa tac  1112
Glu Ile Glu Glu Asp Ser Phe Arg Gln Met Pro Thr Glu Asp Glu Tyr
        345                 350                 355

atg gac agg ccc tcc cag ccc aca gac cag tta ctg ttc ctc act gag  1160
Met Asp Arg Pro Ser Gln Pro Thr Asp Gln Leu Leu Phe Leu Thr Glu
    360                 365                 370

cct gga agc aaa tcc aca cct cct ttc tct gaa ccc ctg gag gtg ggg  1208
Pro Gly Ser Lys Ser Thr Pro Pro Phe Ser Glu Pro Leu Glu Val Gly
375                 380                 385                 390

gag aat gac agt tta agc cag tgc ttc acg ggg aca cag agc aca gtg  1256
Glu Asn Asp Ser Leu Ser Gln Cys Phe Thr Gly Thr Gln Ser Thr Val
                395                 400                 405

ggt tca gaa agc tgc aac tgc act gag ccc ctg tgc agg act gat tgg  1304
Gly Ser Glu Ser Cys Asn Cys Thr Glu Pro Leu Cys Arg Thr Asp Trp
            410                 415                 420
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act ccc atg tcc tct gaa aac tac ttg caa aaa gag gtg gac agt ggc   1352
Thr Pro Met Ser Ser Glu Asn Tyr Leu Gln Lys Glu Val Asp Ser Gly
        425                 430                 435

cat tgc ccg cac tgg gca gcc agc ccc agc ccc aac tgg gca gat gtc   1400
His Cys Pro His Trp Ala Ala Ser Pro Ser Pro Asn Trp Ala Asp Val
    440                 445                 450

tgc aca ggc tgc cgg aac cct cct ggg gag gac tgt gaa ccc ctc gtg   1448
Cys Thr Gly Cys Arg Asn Pro Pro Gly Glu Asp Cys Glu Pro Leu Val
455                 460                 465                 470

ggt tcc cca aaa cgt gga ccc ttg ccc cag tgc gcc tat ggc atg ggc   1496
Gly Ser Pro Lys Arg Gly Pro Leu Pro Gln Cys Ala Tyr Gly Met Gly
                475                 480                 485

ctt ccc cct gaa gaa gaa gcc agc agg acg gag gcc aga gac cag ccc   1544
Leu Pro Pro Glu Glu Glu Ala Ser Arg Thr Glu Ala Arg Asp Gln Pro
            490                 495                 500

gag gat ggg gct gat ggg agg ctc cca agc tca gcg agg gca ggt gcc   1592
Glu Asp Gly Ala Asp Gly Arg Leu Pro Ser Ser Ala Arg Ala Gly Ala
        505                 510                 515

ggg tct gga agc tcc cct ggt ggc cag tcc cct gca tct gga aat gtg   1640
Gly Ser Gly Ser Ser Pro Gly Gly Gln Ser Pro Ala Ser Gly Asn Val
    520                 525                 530

act gga aac agt aac tcc acg ttc atc tcc agc ggg cag gtg atg aac   1688
Thr Gly Asn Ser Asn Ser Thr Phe Ile Ser Ser Gly Gln Val Met Asn
535                 540                 545                 550

ttc aag ggc gac atc atc gtg gtc tac gtc agc cag acc tcg cag gag   1736
Phe Lys Gly Asp Ile Ile Val Val Tyr Val Ser Gln Thr Ser Gln Glu
                555                 560                 565

ggc gcg gcg gcg gct gcg gag ccc atg ggc cgc ccg gtg cag gag gag   1784
Gly Ala Ala Ala Ala Ala Glu Pro Met Gly Arg Pro Val Gln Glu Glu
            570                 575                 580

acc ctg gcg cgc cga gac tcc ttc gcg ggg aac ggc ccg cgc ttc ccg   1832
Thr Leu Ala Arg Arg Asp Ser Phe Ala Gly Asn Gly Pro Arg Phe Pro
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gac ccg tgc ggc ggc ccc gag ggg ctg cgg gag ccg gag aag gcc tcg   1880
Asp Pro Cys Gly Gly Pro Glu Gly Leu Arg Glu Pro Glu Lys Ala Ser
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Arg Pro Val Gln Glu Gln Gly Gly Ala Lys Ala
615                 620                 625

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             20                  25                  30

Leu Gln Val Ala Leu Gln Ile Ala Pro Pro Cys Thr Ser Glu Lys His
         35                  40                  45

Tyr Glu His Leu Gly Arg Cys Cys Asn Lys Cys Glu Pro Gly Lys Tyr
     50                  55                  60

Met Ser Ser Lys Cys Thr Thr Thr Ser Asp Ser Val Cys Leu Pro Cys
 65                  70                  75                  80

Gly Pro Asp Glu Tyr Leu Asp Ser Trp Asn Glu Glu Asp Lys Cys Leu
                 85                  90                  95

Leu His Lys Val Cys Asp Thr Gly Lys Ala Leu Val Ala Val Val Ala
            100                 105                 110

Gly Asn Ser Thr Thr Pro Arg Arg Cys Ala Cys Thr Ala Gly Tyr His
        115                 120                 125

Trp Ser Gln Asp Cys Glu Cys Cys Arg Arg Asn Thr Glu Cys Ala Pro
    130                 135                 140

Gly Leu Gly Ala Gln His Pro Leu Gln Leu Asn Lys Asp Thr Val Cys
145                 150                 155                 160
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Lys Pro Cys Leu Ala Gly Tyr Phe Ser Asp Ala Phe Ser Ser Thr Asp
                165                 170                 175

Lys Cys Arg Pro Trp Thr Asn Cys Thr Phe Leu Gly Lys Arg Val Glu
            180                 185                 190

His His Gly Thr Glu Lys Ser Asp Ala Val Cys Ser Ser Ser Leu Pro
        195                 200                 205

Ala Arg Lys Pro Pro Asn Glu Pro His Val Tyr Leu Pro Gly Leu Ile
    210                 215                 220

Ile Leu Leu Leu Phe Ala Ser Val Ala Leu Val Ala Ala Ile Ile Phe
225                 230                 235                 240

Gly Val Cys Tyr Arg Lys Lys Gly Lys Ala Leu Thr Ala Asn Leu Trp
                245                 250                 255

His Trp Ile Asn Glu Ala Cys Gly Arg Leu Ser Gly Asp Lys Glu Ser
            260                 265                 270

Ser Gly Asp Ser Cys Val Ser Thr His Thr Ala Asn Phe Gly Gln Gln
        275                 280                 285

Gly Ala Cys Glu Gly Val Leu Leu Leu Thr Leu Glu Glu Lys Thr Phe
    290                 295                 300

Pro Glu Asp Met Cys Tyr Pro Asp Gln Gly Gly Val Cys Gln Gly Thr
305                 310                 315                 320

Cys Val Gly Gly Gly Pro Tyr Ala Gln Gly Glu Asp Ala Arg Met Leu
                325                 330                 335

Ser Leu Val Ser Lys Thr Glu Ile Glu Glu Asp Ser Phe Arg Gln Met
            340                 345                 350

Pro Thr Glu Asp Glu Tyr Met Asp Arg Pro Ser Gln Pro Thr Asp Gln
        355                 360                 365

Leu Leu Phe Leu Thr Glu Pro Gly Ser Lys Ser Thr Pro Pro Phe Ser
    370                 375                 380

Glu Pro Leu Glu Val Gly Glu Asn Asp Ser Leu Ser Gln Cys Phe Thr
385                 390                 395                 400

Gly Thr Gln Ser Thr Val Gly Ser Glu Ser Cys Asn Cys Thr Glu Pro
                405                 410                 415

Leu Cys Arg Thr Asp Trp Thr Pro Met Ser Ser Glu Asn Tyr Leu Gln
            420                 425                 430

Lys Glu Val Asp Ser Gly His Cys Pro His Trp Ala Ala Ser Pro Ser
        435                 440                 445

Pro Asn Trp Ala Asp Val Cys Thr Gly Cys Arg Asn Pro Pro Gly Glu
    450                 455                 460

Asp Cys Glu Pro Leu Val Gly Ser Pro Lys Arg Gly Pro Leu Pro Gln
465                 470                 475                 480

Cys Ala Tyr Gly Met Gly Leu Pro Pro Glu Glu Glu Ala Ser Arg Thr
                485                 490                 495

Glu Ala Arg Asp Gln Pro Glu Asp Gly Ala Asp Gly Arg Leu Pro Ser
            500                 505                 510
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Ser Ala Arg Ala Gly Ala Gly Ser Gly Ser Ser Pro Gly Gly Gln Ser
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Pro Ala Ser Gly Asn Val Thr Gly Asn Ser Asn Ser Thr Phe Ile Ser
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Ser Gly Gln Val Met Asn Phe Lys Gly Asp Ile Ile Val Val Tyr Val
545                 550                 555                 560

Ser Gln Thr Ser Gln Glu Gly Ala Ala Ala Ala Ala Glu Pro Met Gly
                565                 570                 575

Arg Pro Val Gln Glu Glu Thr Leu Ala Arg Arg Asp Ser Phe Ala Gly
            580                 585                 590

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Ala
625
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INTERNATIONAL SEARCH REPORT

Intern ıl Application No
PCT/US 00/31459

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/12 C12N15/62 C07K14/705

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EMBL, STRAND, EPO-Internal, WPI Data, PAJ, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	HUGHES ANNE E ET AL: "Mutations in TNFRSF11A, affecting the signal peptide of RANK, cause familial expansile osteolysis." NATURE GENETICS, vol. 24, no. 1, January 2000 (2000-01), pages 45-48, XP002164897 ISSN: 1061-4036 the whole document	1,3-6, 11-15
A	WO 98 28424 A (IMMUNEX CORP) 2 July 1998 (1998-07-02) cited in the application SEQ ID NO:5; SEQ ID NO:6 page 5, line 19 - line 23	1-15

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[X] Further documents are listed in the continuation of box C.

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Date of the actual completion of the international search	Date of mailing of the international search report
6 April 2001	04/05/2001

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Devijver, K

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	HUGHES ANNE E ET AL: "Genetic linkage of familial expansile osteolysis to chromosome 18q." HUMAN MOLECULAR GENETICS, vol. 3, no. 2, 1994, pages 359-361, XP002164898 ISSN: 0964-6906	

INTERNATIONAL SEARCH REPORT

Information on patent family members

Interr ıl Application No
PCT/US 00/31459

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9828424 A	02-07-1998	AU 713471 B	02-12-1999
		AU 5618098 A	17-07-1998
		AU 713473 B	02-12-1999
		AU 5718498 A	17-07-1998
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		WO 9828426 A	02-07-1998
		US 6017729 A	25-01-2000