



(11) **EP 1 344 821 B9**

(12) **CORRECTED EUROPEAN PATENT SPECIFICATION**

Note: Bibliography reflects the latest situation

(15) Correction information:

Corrected version no 1 (W1 B1)

Corrections, see

Description Paragraph(s) 118

(48) Corrigendum issued on:

27.02.2008 Bulletin 2008/09

(45) Date of publication and mention
of the grant of the patent:

01.08.2007 Bulletin 2007/31

(21) Application number: **01271116.4**

(22) Date of filing: **17.12.2001**

(51) Int Cl.:

C12N 9/64 (2006.01)

C07K 16/40 (2006.01)

C12N 1/19 (2006.01)

C12N 5/10 (2006.01)

G01N 33/15 (2006.01)

G01N 33/573 (2006.01)

C12N 15/57 (2006.01)

C12N 1/15 (2006.01)

C12N 1/21 (2006.01)

C12Q 1/37 (2006.01)

G01N 33/50 (2006.01)

C12R 1/91 (2006.01)

(86) International application number:

PCT/JP2001/011033

(87) International publication number:

WO 2002/050258 (27.06.2002 Gazette 2002/26)

(54) **NOVEL AGGRECANASE**

NEUE AGGRECANASE

NOUVELLE AGGRECANASE

(84) Designated Contracting States:

**AT BE CH CY DE DK ES FI FR GB GR IE IT LI LU
MC NL PT SE TR**

(30) Priority: **18.12.2000 JP 2000384300**

(43) Date of publication of application:

17.09.2003 Bulletin 2003/38

(73) Proprietor: **Astellas Pharma Inc.**

Tokyo 103-8411 (JP)

(72) Inventors:

- **YAMAJI, Noboru**
Chuo-ku -- Tokyo 103-8411 (JP)
- **NISHIMURA, Kouichi**
Chuo-ku --- Tokyo 103-8411 (JP)
- **ABE, Kunitake**
Chuo-ku --- Tokyo 103-8411 (JP)

(74) Representative: **HOFFMANN EITLE**

Patent- und Rechtsanwälte

Arabellastrasse 4

81925 München (DE)

(56) References cited:

WO-A-03/027282

WO-A2-99/05291

JP-A- 2003 144 154

- **TORTORELLA M.D. ET AL.:** 'Purification and cloning of aggrecanase-1: A member of the ADAMTS family of protein' **SCIENCE** vol. 284, 1999, pages 1664 - 1666, XP002937898
- **ABBASZADE I. ET AL.:** 'Cloning and characterization of ADAMTS11, an aggrecanase from the ADAMTS family' **J. BIOL. CHEM.** vol. 274, no. 33, 1999, pages 23443 - 23450, XP002937897

Note: Within nine months from the publication of the mention of the grant of the European patent, any person may give notice to the European Patent Office of opposition to the European patent granted. Notice of opposition shall be filed in a written reasoned statement. It shall not be deemed to have been filed until the opposition fee has been paid. (Art. 99(1) European Patent Convention).

EP 1 344 821 B9

Description

TECHNICAL FIELD

[0001] The present invention relates to a novel aggrecanase.

BACKGROUND ART

[0002] Joint diseases are diseases which show damage and degeneration of joint cartilage as the main morbid states. Though a disease having the most frequent number of patients among joint diseases is osteoarthritis (OA) (Elders M. J., J. Rheumatol., 27, Suppl., 60, 6-8, 2000), analgesic antiinflammatory drugs and hyaluronic acid preparations are used in the current therapeutic method merely as a symptomatic therapy for the purpose of alleviating pains accompanied by the degeneration of cartilage and the destruction of subchondral cartilage, so that it cannot be said that they are exerting sufficient therapeutic effects (Dieppe P., Scand. J. Rheumatol., 29, 279-281, 2000).

[0003] Joint cartilage is a tissue mainly composed of type II collagen and aggrecan which is a cartilage-specific proteoglycan, and degradation and degeneration of both of them are observed in the joint diseases. Because of this, it has been considered for a long time that control of the degradation and degeneration of these extracellular matrix components would lead to the treatment of joint diseases, so that attempts have been positively made to identify degradation-concerned proteases (collagenase and aggrecanase) and to screen their inhibitors and develop them as medicaments.

[0004] As proteases having collagenase activities, matrix metalloproteases (MMP1, MMP8, MMP13, MMP14 and the like) have been identified, and their selective inhibitors have been discovered. However, in spite of the attempts to develop a large number of MMP inhibitors having collagenase inhibition activities as therapeutic drugs for joint diseases including OA and rheumatic arthritis (RA), MMP inhibitors to be used in these diseases as the indication have not been put on the market (Greenwald R. A., Ann. New York Acad. Sci., 878, 413-419, 1999). Under such circumstances, attention has been directed toward aggrecanase which selectively degrades aggrecan which is another main constituting component of joint cartilage.

[0005] A joint disease-related role of an enzyme aggrecanase which cleaves aggrecan at the site between Glu373-Ala374 has been revealed by the reports of Sandy et al. and Lohmander et al. stating that all of the main digested aggrecan fragments found in the synovial fluid of human arthritis patients were generated by cleaving at the aggrecanase digestion site (Sandy J.D. et al., J. Clin. Invest., 89, 1512 - 1516, 1992; Lohmander L.S. et al., Arthritis Rheum., 36, 1214 - 1222, 1993). On the other hand, it has been known that, in an in vitro explant culture system of joint cartilage, degradation of aggrecan firstly occurs by IL-1 induction and then degradation of type II collagen is accelerated (Dingle L.T. et al., Ann. Rheum. Dis., 34, 303 - 311, 1975; Cawston T.E. et al., Biochem. Biophys. Res. Comm., 215, 377 - 385, 1995; Kozaci L.D. et al., Arthritis Rheum., 40, 164 - 174, 1997). It has been reported that the aggrecan degradation takes the precedence of the type II collagen degradation in a mouse arthritis model too (van Meurs J.B. et al., Arthritis Rheum., 42, 1128 - 1139, 1999). These reports suggest a possibility that the type II collagen degradation can be controlled by inhibiting the preceding aggrecan degradation.

[0006] However, although biochemical features show that it is a metalloprotease, that it is located on the outside of cells, that sugar chains contribute to a substrate recognition, and that an activity is induced by IL-1, TNF, or retinoic acid, aggrecanase as a cause of joint diseases has been unidentified for a long time. Recently, ADAMTS4 (aggrecanase-11; Tortorella M. D. et al., Science, 284, 1664-1666, 1999) and ADAMTS11 (aggrecanase-2; Abbaszade I. et al., J. Biol. Chem., 274, 23443-23450, 1999) were reported as the protease having the aggrecanase activity. However, the gene expressions of these proteases are not increased in human OA cartilage and not induced by IL-1, TNF, or retinoic acid (it is known that these compounds induce the aggrecanase activity in an explant culture system of human knee articular cartilage), and thus the presence of another protease relating to joint diseases was suggested (Flannery C. R. et al., Biochem. Biophys. Res. Commun., 260, 318-322, 1999).

[0007] In addition, recently, chromosomal loci in which genetic factors relating to OA are located (an OA-susceptibility locus) in accordance with a genetic research for patients suffering from OA or families having a strong family history of OA have been identified. For example, 11q (Chapman K. et al., Am. J. Hum. Genet., 65, 167-174, 1999), 4q12-4q21.2, 6q21.1-6q22.1, 16p13.1-16q12.1, and 16q21-16q23 (Loughlin J. et al., Am. J. Hum. Genet., 65, 1795-1798, 1999) are reported as the OA-susceptibility loci.

DISCLOSURE OF INVENTION

[0008] The object of the present invention is to provide a WO 03/027282 A discloses proteins belonging to the S Family, namely ADAMTS-15, -16, -17, -18 and -19. novel aggrecanase causative of joint diseases, which is useful as

a screening tool for an agent for treating joint diseases, a novel polynucleotide encoding the aggrecanase, and a convenient screening system for obtaining a substance useful as an agent for treating joint diseases.

[0009] With the aim of solving the aforementioned problems, the present inventors have conducted intensive studies and, as a result, obtained a gene encoding an aggrecanase [i.e., MDS8 (Metalloprotease and Disintegrin with Thrombospondin type-1 repeats 8)] causative of joint diseases, and expressed the aggrecanase protein. The inventors showed that the protein exhibits an aggrecanase activity, that the expression of the protein is induced by differentiation to cartilage cells, and that the chromosomal location of MDS8 is in the locus which is identified as an OA (one of joint diseases) susceptibility locus, and thus confirmed that the polypeptide of the present invention is an aggrecanase causative of joint diseases and useful as a screening tool for an agent for treating joint diseases. Further, the inventors established a method for detecting whether or not a compound to be tested inhibits an aggrecanase activity causative of joint diseases using the protein, and a method for screening an agent for treating joint diseases using the detecting method.

[0010] Accordingly, the present invention relates to:

[1] a polypeptide exhibiting an aggrecanase activity and comprising (1) an amino acid sequence of SEQ ID NO: 2 or (2) an amino acid sequence in which 1 to 10 amino acids are deleted, substituted, and/or inserted in an amino acid sequence of SEQ ID NO: 2;

[2] the polypeptide of the item [1], comprising an amino acid sequence of SEQ ID NO: 2, and exhibiting an aggrecanase activity;

[3] a polypeptide comprising an amino acid sequence having a 90% or more homology with an amino acid sequence of SEQ ID NO: 2, and exhibiting an aggrecanase activity;

[4] a polypeptide consisting of an amino acid sequence of SEQ ID NO: 2;

[5] a polynucleotide encoding the polypeptide of the items [1] to [4];

[6] an expression vector comprising the polynucleotide of the item [5];

[7] a cell transfected with the expression vector of the item [6];

[8] a method for detecting whether or not a compound to be tested inhibits an aggrecanase activity of a polypeptide of the items [1] to [4], comprising the steps of:

bringing into contact (1) said polypeptide, (2) a substrate polypeptide capable of digesting with aggrecanase, and (3) the compound to be tested; and

analyzing a digestion of the substrate polypeptide;

[9] a method for screening a substance which inhibits an aggrecanase activity of a polypeptide of the items [1] to [4], comprising the steps of:

detecting by the method of the item [10]; and
selecting a substance inhibiting an aggrecanase activity ;

[10] method for screening a substance for treating joint diseases by the method of the item [9]; and

[11] an antibody or a fragment thereof, which binds to the polypeptide of the items [1] to [4].

[0011] The term "aggrecan" as used herein means a proteoglycan which is located in an extracellular matrix of articular cartilage and consists of a core protein comprising two spherical domains (G1 and G2) at the N-terminus, glycosaminoglycan-binding region, and a spherical domain (G3) at the C-terminus, and chondroitin sulfate and glycosaminoglycan keratan sulfate which modify the core protein.

[0012] The term "aggrecanase activity" as used herein means an activity of selectively digesting human aggrecan present in articular cartilage between the 373rd glutamic acid residue and the 374th alanine residue (hereinafter referred to as "between Glu³⁷³-Ala³⁷⁴").

[0013] The term "aggrecanase" as used herein means a metalloprotease exhibiting the aggrecanase activity. The aggrecanase generally has a zinc-binding consensus sequence [i.e., His-Glu-Xaa-Xaa-His (Xaa means an arbitrary amino acid.); the sequence of SEQ ID NO: 24].

BEST MODE FOR CARRYING OUT THE INVENTION

[0014] The present invention will be explained in detail hereinafter.

[1] The polypeptide of the present invention

[0015] The polypeptide of the present invention includes

- (1) a polypeptide consisting of the amino acid sequence of SEQ ID NO: 2;
- (2) a polypeptide comprising the amino acid sequence of SEQ ID NO: 2, and exhibiting the aggrecanase activity;
- (3) a polypeptide comprising an amino acid sequence in which 1 to 10 amino acids are deleted, substituted, and/or inserted at one or plural positions in the amino acid sequence of SEQ ID NO: 2, and exhibiting the aggrecanase activity (hereinafter referred to as a variation functionally equivalent) ; and
- (4) a polypeptide comprising an amino acid sequence having a 90% or more homology with the amino acid sequence of SEQ ID NO: 2, and exhibiting the aggrecanase activity (hereinafter referred to as a homologous polypeptide).

[0016] Among the polypeptides (1) to (4) as the polypeptide of the present invention, a polypeptide comprising an amino acid sequence of which an expression is induced by differentiation to cartilage cells is preferable.

- (1) The polypeptide consisting of the amino acid sequence of SEQ ID NO: 2

As the polypeptide of the present invention, the polypeptide consisting of the amino acid sequence of SEQ ID NO: 2 is most preferable. The polypeptide consisting of the amino acid sequence of SEQ ID NO: 2 exhibits the aggrecanase activity and is a novel human aggrecanase consisting of 1221 amino acid residues. As shown in Example 5, the expression of the polypeptide is induced by differentiation to cartilage cells. Further, as shown in Example 6, the gene thereof is located in a chromosome relating to osteoarthritis (OA), and thus it is considered that the polypeptide consisting of the amino acid sequence of SEQ ID NO: 2 is an aggrecanase causative of joint diseases.

- (2) The polypeptide comprising the amino acid sequence of SEQ ID NO: 2, and exhibiting the aggrecanase activity
- The "polypeptide comprising the amino acid sequence of SEQ ID NO: 2 and exhibiting the aggrecanase activity" as the polypeptide of the present invention includes, for example, the polypeptide consisting of the amino acid sequence of SEQ ID NO: 2, and a polypeptide in which an appropriate marker sequence or the like is added to the N-terminus and/or the C-terminus of the polypeptide having the amino acid sequence of SEQ ID NO: 2 (i.e., a fusion polypeptide having an amino acid sequence in which an appropriate marker sequence or the like is added to the N-terminus and/or the C-terminus of the amino acid sequence of SEQ ID NO: 2).

As the marker sequence, a sequence for easily carrying out confirmation of polypeptide expression, confirmation of intracellular localization thereof, purification thereof, or the like may be used. As the sequence, there may be mentioned, for example, a FLAG tag, a hexa-histidine tag (i.e., a His tag), a hemagglutinin tag, or a myc epitope. A method for confirming whether or not a polypeptide to be tested exhibits the "aggrecanase activity" as used herein (hereinafter sometimes referred to as a "method for confirming the aggrecanase activity") is not particularly limited. It may be confirmed, for example, by bringing a polypeptide to be tested (such as cells, a tissue culture supernatant, or a tissue extract containing the test polypeptide, or a purified or partial purified preparation of the test polypeptide) into contact with a substrate of aggrecanase (such as human aggrecan) in an appropriate buffer [such as 50 mmol/L Tris-HCl (pH7.4)], and then analyzing whether or not the substrate is digested at the cleavage site (for example, between Glu³⁷³-Ala³⁷⁴ in human aggrecan), preferably by a method described in Example 4.

The substrate of aggrecanase is not particularly limited, so long as it is a polypeptide capable of being digested with aggrecanase (hereinafter referred to as a substrate polypeptide), but there may be mentioned, for example, aggrecan derived from human or other animals, a fragment of the aggrecan (a fragment capable of being digested with aggrecanase), or a fusion polypeptide of the aggrecan or the fragment thereof and an appropriate marker sequence (such as a FLAG tag, a His tag, a hemagglutinin tag, or a myc epitope, preferably a FLAG tag or a His tag) (a fusion polypeptide capable of being digested with aggrecanase). More particularly, for example, purified aggrecans from human or other animals cartilage tissue, recombinant aggrecans (such as recombinant aggrecan obtained by adding a FLAG tag to the N-terminus and a His tag to the C-terminus of aggrecan or a fragment thereof, respectively), commercially available aggrecans (SEIKAGAKU CORPORATION), or partial polypeptides of these aggrecans may be used.

As a method for analyzing whether or not the substrate polypeptide is digested by the aggrecanase activity, there may be mentioned, for example, a method for analyzing a polypeptide fragment generated by digesting the substrate polypeptide with the test polypeptide (such as a method for detecting the presence of the polypeptide fragment, or a method for measuring an amount of the generated polypeptide fragment), or a method for analyzing the substrate polypeptide decreased by the digestion (such as a method for detecting the presence of the substrate polypeptide, or a method for measuring an amount of the decreased substrate polypeptide).

For example, in the case of using human aggrecan as the substrate polypeptide, a method for analyzing a polypeptide fragment generated by digesting aggrecan between Glu³⁷³-Ala³⁷⁴ is not particularly limited, but, for example, a method for determining a N-terminus sequence or a C-terminus sequence of the digested fragment by a conventional

method, or more conveniently, immunological methods such as ELISA (Enzyme Linked Immuno Solvent Assay) or a western blotting method using an anti-neoepitope antibody (Hughes C. E. et al., Biochem. J., 305, 799-804, 1995) which specifically recognizes an Asn³⁶⁹-Ile³⁷⁰-Thr³⁷¹-Gly³⁷²-Glu³⁷³ sequence (the sequence of SEQ ID NO: 25) at the C-terminus or an Ala³⁷⁴-Arg³⁷⁵-Gly³⁷⁶-Ser³⁷⁷-Val³⁷⁸ sequence (the sequence of SEQ ID NO: 26) at the N-terminus generated by digesting aggrecan between Glu³⁷³-Ala³⁷⁴ may be used. The immunological methods such as ELISA or a western blotting method may be carried out in accordance with, for example, Migita, S., Konda, S., Honjo, T, Hamaoka, T., "Men-eki Jikken Sousa Hou (Methods in Immunological Experiments) I,II", Nanko-do, 1995. Further, in the case of using human aggrecan as the substrate polypeptide, the method for analyzing human aggrecan decreased by digesting aggrecan between Glu³⁷³-Ala³⁷⁴ is not particularly limited, but for example, a western blotting method using an anti-human aggrecan antibody may be used.

Furthermore, in the case of using the recombinant aggrecan obtained by adding a FLAG tag to the N-terminus and a His tag to the C-terminus of aggrecan or a fragment thereof, respectively, ELISA or a western blotting method using an anti-FLAG tag antibody and/or an anti-His tag antibody may be used as the method for analyzing the recombinant aggrecan decreased by digesting aggrecan between Glu³⁷³-Ala³⁷⁴.

(3) The variation functionally equivalent

The variation functionally equivalent of the present invention is not particularly limited, so long as it is a polypeptide comprising an amino acid sequence in which preferably 1 to 10, more preferably 1 to 7, most preferably 1 to 5 amino acids are deleted, substituted, and/or inserted at one or plural positions in the amino acid sequence of SEQ ID NO: 2 and exhibiting the aggrecanase activity (preferably further comprising an amino acid sequence of which an expression is induced by differentiation to cartilage cells). Further, an origin of the variation functionally equivalent is not limited to a human.

The variation functionally equivalent of the present invention includes, for example, human variations of the polypeptide having the amino acid sequence of SEQ ID NO: 2 and variations functionally equivalent derived from organisms other than a human (such as a mouse, a rat, a hamster, or a dog), and further polypeptides obtained by artificially modifying these native polypeptides (i.e., human variations or variations functionally equivalent derived from organisms other than a human) or the polypeptide having the amino acid sequence of SEQ ID NO: 2 by genetic engineering techniques. The term "variation" as used herein means an individual difference between the same polypeptides in the same species or a difference between homologous polypeptides in several species.

Human variations of the polypeptide consisting of the amino acid sequence of SEQ ID NO: 2 or variations functionally equivalent derived from organisms other than a human may be obtained by those skilled in the art in accordance with the information of a base sequence (for example, the base sequence of SEQ ID NO: 1) of a polynucleotide encoding the polypeptide consisting of the amino acid sequence of SEQ ID NO: 2. In this connection, genetic engineering techniques may be generally performed in accordance with known methods (for example, Sambrook, J. et al., "Molecular Cloning-A Laboratory Manual", Cold Spring Harbor Laboratory, NY, 1989).

For example, an appropriate probe or appropriate primers are designed in accordance with the information of a base sequence of a polynucleotide encoding the polypeptide consisting of the amino acid sequence of SEQ ID NO: 2. A polymerase chain reaction (PCR) method (Saiki, R. K. et al., Science, 239, 487-491, 1988) or a hybridization method is carried out using a sample (for example, total RNA or an mRNA fraction, a cDNA library, or a phage library) prepared from an organism (for example, a mammal such as a human, a mouse, a rat, a hamster, or a dog) of interest and the primers or the probe to obtain a polynucleotide encoding the polypeptide. A desired polypeptide may be obtained by expressing the resulting polynucleotide in an appropriate expression system and confirming that the expressed polypeptide exhibits the aggrecanase activity by, for example, the method described in Example 4. Further, the polypeptide artificially modified by genetic engineering techniques may be obtained by, for example, the following procedure. A gene encoding the polypeptide is obtained by a conventional method such as site-directed mutagenesis (Mark, D. F. et al., Proc. Natl. Acad. Sci. USA, 81, 5662-5666, 1984). A desired polypeptide may be obtained by expressing the resulting polynucleotide in an appropriate expression system and confirming that the expressed polypeptide exhibits the aggrecanase activity by, for example, the method described in Example 4.

The variation functionally equivalent of the present invention includes a fusion polypeptide exhibiting the aggrecanase activity and having an amino acid sequence in which one or plural amino acids are deleted, substituted, and/or inserted at one or plural positions in the amino acid sequence of SEQ ID NO: 2 and an appropriate marker sequence or the like is added to the N-terminus and/or the C-terminus thereof.

Further, as the variation functionally equivalent of the present invention, a polypeptide consisting of an amino acid sequence in which preferably 1 to 10, more preferably 1 to 7, most preferably 1 to 5 amino acids are deleted, substituted, and/or inserted at one or plural positions in the amino acid sequence of SEQ ID NO: 2, and exhibiting the aggrecanase activity is preferable, and a polypeptide further comprising an amino acid sequence of which an expression is induced by differentiation to cartilage cells is more preferable.

(4) The homologous polypeptide

The homologous polypeptide of the present invention is not particularly limited, so long as it is a polypeptide having

an amino acid sequence having a 90% or more homology with the amino acid sequence of SEQ ID NO: 2, and exhibiting the aggrecanase activity. The homologous polypeptide of the present invention may have an amino acid sequence having preferably a 95% or more homology, more preferably a 98% or more homology, most preferably a 99% or more homology, with respect to the amino acid sequence of SEQ ID NO: 2. As the homologous polypeptide of the present invention, a polypeptide consisting of an amino acid sequence having a 90% or more homology (preferably a 95% or more homology, more preferably a 98% or more homology, most preferably a 99% or more homology) and exhibiting the aggrecanase activity is preferable.

[0017] Further, as the homologous polypeptide of the present invention, a polypeptide exhibiting the aggrecanase activity and comprising an amino acid sequence of which an expression is induced by differentiation to cartilage cells is more preferable.

[0018] The term "homology" as used herein means a value obtained by a BLAST [Basic local alignment search tool; Altschul, S. F. et al., J. Mol. Biol., 215, 403-410, (1990)] search. The homology in the amino acid sequence may be calculated by a BLAST search algorithm. More particularly, it may be calculated using a bl2seq program (Tatiana A. Tatusova and Thomas L. Madden, FEMS Microbiol. Lett., 174, 247-250, 1999) in a BLAST package (sgi32bit edition, version 2.0.12; obtained from NCBI) in accordance with a default parameter. As a pairwise alignment parameter, a program "blastp" is used. Further, "0" as a Gap insertion cost value, "0" as a Gap elongation cost value, "SEG" as a filter for a Query sequence, and "BLOSUM62" as a Matrix are used, respectively.

[2] The polynucleotide of the present invention

[0019] The polynucleotide of the present invention is not particularly limited, so long as it encodes the polypeptide of the present invention. As the polynucleotide of the present invention, there may be mentioned, for example, a polynucleotide comprising the base sequence of SEQ ID NO: 1. Such polynucleotide consisting of the base sequence of SEQ ID NO: 1 is most preferable. In this connection, the term "polynucleotide" as used herein includes both DNA and RNA.

[0020] A method for producing the polynucleotide of the present invention is not particularly limited, but there may be mentioned, for example, (1) a method using PCR, (2) a method using conventional genetic engineering techniques (i.e., a method for selecting a transformant comprising a desired cDNA from strains transformed with a cDNA library), or (3) a chemical synthesis method. These methods will be explained in this order hereinafter.

[0021] In the method using PCR of the item (1), the polynucleotide of the present invention may be produced, for example, by the following procedure.

[0022] mRNA is extracted from human cells or tissue capable of producing the polypeptide of the present invention. A pair of primers, between which full-length mRNA corresponding to the polypeptide of the present invention or a partial region of the mRNA is located, is synthesized on the basis of the base sequence of a polynucleotide encoding the polynucleotide of the present invention. Full-length cDNA encoding the polypeptide of the present invention or a part of the cDNA may be obtained by performing a reverse transcriptase-polymerase chain reaction (RT-PCR) using the extracted mRNA as a template.

[0023] More particularly, total RNA containing mRNA encoding the polypeptide of the present invention is extracted by a known method from cells or tissue capable of producing the polypeptide of the present invention. As an extraction method, there may be mentioned, for example, a guanidine thiocyanate-hot phenol method, a guanidine thiocyanate-guanidine hydrochloride method, or a guanidine thiocyanate-caesium chloride method. The guanidine thiocyanate-caesium chloride method is preferably used. The cells or tissue capable of producing the polypeptide of the present invention may be identified, for example, by a northern blotting method using a polynucleotide or a part thereof encoding the polypeptide of the present invention or a western blotting method using an antibody specific for the polypeptide of the present invention.

[0024] Next, the extracted mRNA is purified. Purification of the mRNA may be made in accordance with a conventional method. For example, the mRNA may be purified by adsorption and elution using an oligo(dT)-cellulose column. The mRNA may be further fractionated by, for example, a sucrose density gradient centrifugation, if necessary. Alternatively, commercially available extracted and purified mRNA may be used without carrying out the extraction of the mRNA.

[0025] Next, the first-strand cDNA is synthesized by carrying out a reverse transcriptase reaction of the purified mRNA in the presence of a random primer, an oligo dT primer, and/or a custom primer. This synthesis may be carried out in accordance with a conventional method. The resulting first-strand cDNA is subjected to PCR using two primers between which a full-length or a partial region of the polynucleotide of interest is located, thereby amplifying the cDNA of interest. The resulting DNA is fractionated by, for example, an agarose gel electrophoresis. The DNA fragment of interest may be obtained by carrying out a digestion of the DNA with restriction enzymes and subsequent ligation, if necessary.

[0026] In the method using conventional genetic engineering techniques of the item (2), the polynucleotide of the present invention may be produced, for example, by the following procedure.

[0027] First, single-stranded cDNA is synthesized by using reverse transcriptase from mRNA prepared by the above-mentioned PCR method as a template, and then double-stranded cDNA is synthesized from the single-stranded cDNA. As this method, there may be mentioned, for example, an S1 nuclease method (Efstratiadis, A. et al., *Cell*, 7, 279-288, 1976), a Land method (Land, H. et al., *Nucleic Acids Res.*, 9, 2251-2266, 1981), an O. Joon Yoo method (Yoo, O. J. et al., *Proc. Natl. Acad. Sci. USA*, 79, 1049-1053, 1983), and an Okayama-Berg method (Okayama, H. and Berg, P., *Mol. Cell. Biol.*, 2, 161-170, 1982).

[0028] Next, a recombinant plasmid comprising the double-stranded cDNA is prepared and introduced into an *Escherichia coli* strain, such as DH 5 α , HB101, or JM109, thereby transforming the strain. A transformant is selected using a drug resistance against, for example, tetracycline, ampicillin, or kanamycin as a marker. When the host cell is *E. coli*, transformation of the host cell may be carried out, for example, by the method of Hanahan (Hanahan, D. J., *Mol. Biol.*, 166, 557-580, 1983); namely, a method in which the recombinant DNA is added to competent cells prepared in the presence of CaCl₂, MgCl₂, or RbCl. Further, as a vector other than a plasmid, a phage vector such as a lambda system may be used.

[0029] As a method for selecting a transformant containing the cDNA of interest from the resulting transformants, various methods such as (i) a method for screening a transformant using a synthetic oligonucleotide probe, (ii) a method for screening a transformant using a probe produced by PCR, (iii) a method for screening a transformant using an antibody against the polypeptide of the present invention, or (iv) a method for screening a transformant using a selective hybridization translation system, may be used.

[0030] In the method of the item (i) for screening a transformant using a synthetic oligonucleotide probe, the transformant containing the cDNA of interest may be selected, for example, by the following procedure.

[0031] An oligonucleotide which corresponds to the whole or a part of the polypeptide of the present invention is synthesized (in this case, it may be either a nucleotide sequence taking the codon usage into consideration or a plurality of nucleotide sequences as a combination of possible nucleotide sequences, and in the latter case, their numbers can be reduced by including inosine) and, using this oligonucleotide as a probe (labeled with ³²P or ³³P), hybridized with a nitrocellulose filter or a polyamide filter on which DNAs of the transformants are denatured and fixed, to screen and select resulting positive strains.

[0032] In the method of the item (ii) for screening a transformant using a probe produced by PCR, the transformant containing the cDNA of interest may be selected, for example, by the following procedure.

[0033] Oligonucleotides of a sense primer and an antisense primer corresponding to a part of the polypeptide of the present invention are synthesized, and a DNA fragment encoding the whole or a part of the polypeptide of interest is amplified by carrying out PCR using these primers in combination. As a template DNA used in this method, cDNA synthesized by a reverse transcription reaction from mRNA of cells capable of producing the polypeptide of the present invention, or genomic DNA, may be used. The resulting DNA fragment is labeled with ³²P or ³³P, and a transformant containing the cDNA of interest is selected by carrying out a colony hybridization or a plaque hybridization using this fragment as a probe.

[0034] In the method of the item (iii) for screening a transformant using an antibody against the polypeptide of the present invention, the transformant containing the cDNA of interest may be selected, for example, by the following procedure.

[0035] First, cDNA is integrated into an expression vector, and polypeptides are produced into a culture supernatant, inside the cells, or on the cell surface of transformants. A transformant containing the cDNA of interest is selected by detecting a strain producing the desired polypeptide using an antibody against the polypeptide of the present invention and a second antibody against the first antibody.

[0036] In the method of the item (iv) for screening a transformant using a selective hybridization translation system, the transformant containing the cDNA of interest may be selected, for example, by the following procedure.

[0037] First, cDNA obtained from each transformant is blotted on, for example, a nitrocellulose filter and hybridized with mRNA prepared from cells capable of producing the polypeptide of the present invention, and then the mRNA bound to the cDNA is dissociated and recovered. The recovered mRNA is translated into a polypeptide in an appropriate polypeptide translation system, for example, injection into *Xenopus* oocytes or a cell-free system such as a rabbit reticulocyte lysate or a wheat germ. A transformant containing the cDNA of interest is selected by detecting it with the use of an antibody against the polypeptide of the present invention.

[0038] A method for collecting the polynucleotide of the present invention from the resulting transformant of interest can be carried out in accordance with a known method (for example, Sambrook, J. et al., "Molecular Cloning-A Laboratory Manual", Cold Spring Harbor Laboratory, NY, 1989). For example, it may be carried out by separating a fraction corresponding to the plasmid DNA from cells and cutting out the cDNA region from the plasmid DNA.

[0039] In the chemical synthesis method of the item (3), the polynucleotide of the present invention may be produced, for example, by binding DNA fragments produced by a chemical synthesis method. Each DNA can be synthesized using a DNA synthesizer [for example, Oligo 1000M DNA Synthesizer (Beckman) or 394 DNA/RNA Synthesizer (Applied Biosystems)].

[0040] Further, the polynucleotide of the present invention may be produced by nucleic acid chemical synthesis in accordance with a conventional method such as a phosphite triester method (Hunkapiller, M. et al., *Nature*, 10, 105-111, 1984), based on the information on the polypeptide of the present invention. In this connection, codons for each amino acid are known and can be optionally selected and determined by the conventional method, for example, by taking a codon usage of each host to be used into consideration (Crantham, R. et al., *Nucleic Acids Res.*, 9, r43-r74, 1981). Further, a partial modification of codons of these base sequences can be carried out in accordance with a conventional method, such as site directed mutagenesis which uses a primer comprised of a synthetic oligonucleotide coding for a desired modification (Mark, D. F. et al., *Proc. Natl. Acad. Sci. USA*, 81, 5662-5666, 1984).

[0041] Determination of the DNA sequences obtained by the above-mentioned methods can be carried out by, for example, a Maxam-Gilbert chemical modification method (Maxam, A. M. and Gilbert, W., "*Methods in Enzymology*", 65, 499-559, 1980) or a dideoxynucleotide chain termination method (Messing, J. and Vieira, J., *Gene*, 19, 269-276, 1982).

[3] The expression vector and the cell of the present invention

[0042] An isolated polynucleotide of the present invention is re-integrated into an appropriate vector DNA and a eucaryotic or procaryotic host cell may be transfected by the resulting expression vector. Further, it is possible to express the polynucleotide in a desired host cell, by introducing an appropriate promoter and a sequence related to the gene expression into the vector.

[0043] The expression vector of the present invention is not particularly limited, so long as it comprises the polynucleotide of the present invention. As the expression vector, there may be mentioned, for example, an expression vector obtained by introducing the polynucleotide of the present invention into a known expression vector appropriately selected in accordance with a host cell to be used.

[0044] The cell of the present invention is not particularly limited, so long as it is transfected with the expression vector of the present invention and comprises the polynucleotide of the present invention. The cell of the present invention may be, for example, a cell in which the polynucleotide is integrated into a chromosome of a host cell, or a cell containing the polynucleotide as an expression vector comprising polynucleotide. Further, the cell of the present invention may be a cell expressing the polypeptide of the present invention, or a cell not expressing the polypeptide of the present invention. The cell of the present invention may be obtained by, for example, transfecting a desired host cell with the expression vector of the present invention.

[0045] In the eucaryotic host cells, for example, cells of vertebrates, insects, and yeast are included. As the vertebral cell, there may be mentioned, for example, a simian COS cell (Gluzman, Y., *Cell*, 23, 175-182, 1981), a dihydrofolate reductase defective strain of a Chinese hamster ovary cell (CHO) (Urlaub, G. and Chasin, L. A., *Proc. Natl. Acad. Sci. USA*, 77, 4216-4220, 1980), a human embryonic kidney derived HEK293 cell, or a 293-EBNA cell (Invitrogen) obtained by introducing an EBNA-1 gene of Epstein Barr Virus into HEK293 cell.

[0046] As an expression vector for a vertebral cell, a vector containing a promoter positioned upstream of the gene to be expressed, an RNA splicing site, a polyadenylation site, a transcription termination sequence, and the like may be generally used. The vector may further contain a replication origin, if necessary. As the expression vector, there may be mentioned, for example, pSV2dhfr containing an SV40 early promoter (Subramani, S. et al., *Mol. Cell. Biol.*, 1, 854-864, 1981), pEF-BOS containing a human elongation factor promoter (Mizushima, S. and Nagata, S., *Nucleic Acids Res.*, 18, 5322, 1990), or pCEP4 containing a cytomegalovirus promoter (Invitrogen).

[0047] When the 293-EBNA cell is used as the host cell, for example, pCEP4 (Invitrogen) containing a replication origin of Epstein Barr Virus and capable of performing an autonomous replication in the 293-EBNA cell may be used as the expression vector.

[0048] When the COS cell is used as the host cell, a vector which has an SV40 replication origin, can perform an autonomous replication in the COS cell, and has a transcription promoter, a transcription termination signal, and an RNA splicing site, may be used as the expression vector. As the vector, there may be mentioned, for example, pME18S (Maruyama, K. and Takebe, Y., *Med. Immunol.*, 20, 27-32, 1990), pEF-BOS (Mizushima, S. and Nagata, S., *Nucleic Acids Res.*, 18, 5322, 1990), or pCDM8 (Seed, B., *Nature*, 329, 840-842, 1987).

[0049] The expression vector may be incorporated into COS cells by, for example, a DEAE-dextran method (Luthman, H. and Magnusson, G., *Nucleic Acids Res.*, 11, 1295-1308, 1983), a calcium phosphate-DNA co-precipitation method (Graham, F. L. and van der Ed, A. J., *Virology*, 52, 456-457, 1973), a method using a commercially available transfection reagent (for example, FUGENE™ 6 Transfection Reagent; Boeringer Mannheim), or an electroporation method (Neumann, E. et al., *EMBO J.*, 1, 841-845, 1982).

[0050] When the CHO cell is used as the host cell, a transfected cell capable of stably producing the polypeptide of the present invention can be obtained by carrying out co-transfection of an expression vector comprising the polynucleotide encoding the polypeptide of the present invention, together with a vector capable of expressing a neo gene which functions as a G418 resistance marker, such as pRSVneo (Sambrook, J. et al., "*Molecular Cloning-A Laboratory Manual*", Cold Spring Harbor Laboratory, NY, 1989) or pSV2-neo (Southern, P. J. and Berg, P., *J. Mol. Appl. Genet.*, 1,

327-341,1982), and selecting a G418 resistant colony.

[0051] The cell of the present invention may be cultured in accordance with the conventional method [for example, "Shin Seikagaku Jikken Koza 18, Saibou Baiyou Gijyutsu (Japanese Biochemical Society)", Tokyo Kagaku Dojin, 1990], and the polypeptide of the present invention is produced outside the cells. As a medium to be used in the culturing, a medium commonly used in a desired host cell may be appropriately selected. In the case of the COS cell, for example, a medium such as an RPMI-1640 medium or a Dulbecco's modified Eagle's minimum essential medium (DMEM) may be used, by supplementing it with a serum component such as fetal bovine serum (FBS) if necessary. In the case of the 293-EBNA cell, a medium such as a Dulbecco's modified Eagle's minimum essential medium (DMEM) with a serum component such as fetal bovine serum (FBS) and G418 may be used.

[0052] The polypeptide of the present invention produced outside the cell of the present invention by culturing the cells may be separated and purified therefrom by various known separation techniques [for example, Okada, M. and Miyazaki K., "Kaitei, Tanpakushitsu Jikken Noto, Jyo.Ge (Revision, Notebook for Protein Experiments)", Yodo-sha 1999] making use of the physical properties, chemical properties and the like of the polypeptide. More particularly, the polypeptide of the present invention may be purified by treating a culture liquid containing the polypeptide of the present invention with a commonly used treatment, for example, a treatment with a protein precipitant, ultrafiltration, various liquid chromatography techniques such as molecular sieve chromatography (gel filtration), adsorption chromatography, ion exchange chromatography, affinity chromatography, or high performance liquid chromatography (HPLC), or dialysis, or a combination thereof.

[0053] When the polypeptide of the present invention is expressed as a fusion protein with a marker sequence in frame, identification of the expression of the polypeptide of the present invention, purification thereof, or the like may be easily carried out. As the marker sequence, there may be mentioned, for example, a FLAG tag, a hexa-histidine tag, a hemagglutinin tag, or a myc epitope. Further, by inserting a specific amino acid sequence recognized by a protease such as enterokinase, factor Xa, or thrombin between the marker sequence and the polypeptide of the present invention, the marker sequence may be removed by the protease.

[4] The detecting method and the screening method of the present invention

[0054] It is possible to detect whether or not a compound to be tested inhibits the aggrecanase activity of the polypeptide of the present invention, using the polypeptide of the present invention. Further, using this detecting method of the present invention, it is possible to screen a substance inhibiting the aggrecanase activity of the polypeptide of the present invention. The polypeptide of the present invention is a protein of which an expression is induced by differentiation to cartilage cells as described in Example 5, and the gene thereof is located in a chromosome relating to osteoarthritis (OA) as described in Example 6, and thus it is considered that the polypeptide of the present invention is an aggrecanase causative of joint diseases. Therefore, a substance inhibiting the aggrecanase activity of the polypeptide of the present invention is useful as a substance for treating joint diseases. Further, the polypeptide of the present invention per se may be used as a tool for screening a substance inhibiting the aggrecanase activity of the polypeptide of the present invention or a substance for treating joint diseases (particularly an agent for treating osteoarthritis).

[0055] Compounds to be tested which may be applied to the detecting method or screening method of the present invention are not particularly limited, but there may be mentioned, for example, various known compounds (including peptides) registered in chemical files, compounds obtained by combinatorial chemistry techniques (Terrett, N. K. et al., Tetrahedron, 51, 8135-8137, 1995) or conventional synthesis techniques, or random peptides prepared by employing a phage display method (Felici, F. et al., J. Mol. Biol., 222, 301-310, 1991) or the like. These known compounds include compounds (including peptides) known to exhibit an activity inhibiting metalloprotease but not known to inhibit the aggrecanase activity of the polypeptide of the present invention. In addition, culture supernatants of microorganisms, natural components derived from plants or marine organisms, or animal tissue extracts may be used as the test compounds for screening. Further, compounds (including peptides) obtained by chemically or biologically modifying compounds (including peptides) selected by the screening method of the present invention may be used.

[0056] The detecting method of the present invention may be carried out by a method similar to the above-mentioned method for confirming the aggrecanase activity, except that the polypeptide of the present invention, the substrate polypeptide, and the test compound are brought into contact with each other instead of bringing the test polypeptide into contact with the substrate polypeptide. Namely, in the detecting method of the present invention, it is possible to detect whether or not the test compound inhibits the aggrecanase activity of the polypeptide of the present invention by bringing the polypeptide of the present invention, the substrate polypeptide, and the test polypeptide into contact with each other and analyzing whether or not the substrate polypeptide is digested by the aggrecanase activity of the polypeptide of the present invention in the presence of the test compound (or analyzing the degree of the digestion). When the substrate polypeptide is not digested by the aggrecanase activity of the polypeptide of the present invention or the degree of the digestion is decreased, it is possible to confirm that the test compound inhibits the aggrecanase activity of the polypeptide of the present invention.

[0057] As the substrate polypeptide which may be used in the detecting method of the present invention, the substrate polypeptide previously described in the method for confirming the aggrecanase activity may be used.

[0058] In the detecting method of the present invention, as a method for analyzing whether or not the substrate polypeptide is digested by the aggrecanase activity of the polypeptide of the present invention, the methods previously described in the method for confirming the aggrecanase activity may be used. As these methods, there may be mentioned, for example, a method for analyzing a polypeptide fragment generated by digesting the substrate polypeptide by the aggrecanase activity of the polypeptide of the present invention (such as a method for detecting the presence of the polypeptide fragment, or a method for measuring an amount of the generated polypeptide fragment), or a method for analyzing the substrate polypeptide decreased by the digestion (such as a method for detecting the presence of the substrate polypeptide, or a method for measuring an amount of the decreased substrate polypeptide).

[0059] In the screening method of the present invention, it is possible to select a substance inhibiting the aggrecanase activity of the polypeptide of the present invention or a substance for treating joint diseases, on the basis of the results obtained by detecting whether or not the test compound inhibits the aggrecanase activity of the polypeptide of the present invention using the detecting method of the present invention. More particularly, for example, when the polypeptide of the present invention, the substrate polypeptide, and the test polypeptide are brought into contact with each other, a substance inhibiting the aggrecanase activity of the polypeptide of the present invention or a substance for treating joint diseases may be selected on the basis of the presence or degree of digestion of the substrate polypeptide in the presence of the test compound. When the substrate polypeptide is not digested by the aggrecanase activity of the polypeptide of the present invention or the degree of the digestion is decreased, it is possible to confirm that the test compound is a substance inhibiting the aggrecanase activity of the polypeptide of the present invention or a substance for treating joint diseases.

[5] The process for manufacturing a pharmaceutical composition for treating joint diseases of the present invention

[0060] The specification describes a pharmaceutical composition for treating joint diseases comprising, as an active ingredient, a substance inhibiting the aggrecanase activity of the polypeptide of the present invention selected by the screening method of the present invention.

[0061] The specification describes a process for manufacturing a pharmaceutical composition for treating joint diseases comprising the steps of detecting, in a quality control test of a pharmaceutical composition for treating joint diseases, whether or not the pharmaceutical composition inhibits the aggrecanase activity of the polypeptide of the present invention by the detecting method of the present invention, and preparing a medicament.

[0062] Further, the specification describes a process for manufacturing a pharmaceutical composition for treating joint diseases, consisting of the step of preparing a medicament using a substance obtained by the screening method of the present invention comprising the detecting step.

[0063] The preparation containing as an active ingredient a substance inhibiting the aggrecanase activity of the polypeptide of the present invention may be prepared using carriers, fillers, and/or other additives generally used in the preparation of medicaments, in accordance with the active ingredient.

[0064] Examples of administration include oral administration by tablets, pills, capsules, granules, fine granules, powders, oral solutions and the like, and parenteral administration by injections (e.g., intravenous, intramuscular, or the like), suppositories, transdermal preparations, transmucosal absorption preparations and the like. Particularly, in the case of peptides which are digested in the stomach, a parenteral administration such as intravenous injection or the like, or preparation techniques in which the polypeptide is not digested, such as a preparation technique disclosed in the WO95/28963 pamphlet, is preferable.

[0065] In the solid composition for use in the oral administration, one or more active substances may be mixed with at least one inert diluent such as lactose, mannitol, glucose, microcrystalline cellulose, hydroxypropylcellulose, starch, polyvinyl pyrrolidone, or aluminum magnesium silicate. In the usual way, the composition may contain additives other than the inert diluent, such as a lubricant, a disintegrating agent, a stabilizing agent, or a solubilizing or solubilization assisting agent. If necessary, tablets or pills may be coated with a sugar coating or a film of a gastric or enteric substance.

[0066] The liquid composition for oral administration may include, for example, emulsions, solutions, suspensions, syrups, and elixirs, and may contain a generally used inert diluent such as purified water or ethyl alcohol. The composition may contain additives other than the inert diluent, such as moistening agents, suspending agents, sweeteners, flavors, or antiseptics.

[0067] The injections for parenteral administration may include aseptic aqueous or non-aqueous solutions, suspensions, and emulsions. Examples of the diluent for use in the aqueous solutions and suspensions include distilled water for injection use and physiological saline. Examples of the diluent for use in the non-aqueous solutions and suspensions include propylene glycol, polyethylene glycol, plant oil (e.g., olive oil), alcohols (e.g., ethanol), polysorbate 80 and the like. Such a composition may further contain a moistening agent, an emulsifying agent, a dispersing agent, a stabilizing agent, a solubilizing or solubilization assisting agent, an antiseptic or the like. These compositions may be sterilized, for

example, by filtration through a bacteria retaining filter, blending of a germicide, or irradiation. Alternatively, they may be used by first making into sterile solid compositions and dissolving them in sterile water or other sterile solvent for injection use prior to their use.

[0068] The dose is optionally decided by taking into consideration the strength of each active ingredient selected by the aforementioned screening method, or symptoms, age, sex, or the like of each patient to be administered.

[0069] For example, in the case of oral administration, the usual dosage for an adult (60 kg in weight) is about 0.01 to 1000 mg, preferably 0.01 to 100 mg per day. In the case of parenteral administration, the usual dosage is about 0.01 to 1000 mg, preferably 0.01 to 100 mg per day in the form of an injection.

[6] The antibody and the fragment thereof of the present invention

[0070] An antibody, such as a polyclonal antibody or a monoclonal antibody, which reacts with the polypeptide of the present invention may be obtained by directly administering the polypeptide of the present invention or a fragment thereof to various animals. Alternatively, it may be obtained by a DNA vaccine method (Raz, E. et al., Proc. Natl. Acad. Sci. USA, 91, 9519-9523, 1994; or Donnelly, J. J. et al., J. Infect. Dis., 173, 314-320, 1996), using a plasmid into which a polynucleotide encoding the polypeptide of the present invention is inserted.

[0071] The polyclonal antibody may be produced from a serum or eggs of an animal such as a rabbit, a rat, a goat, or a chicken, in which the animal is immunized and sensitized by the polypeptide of the present invention or a fragment thereof emulsified in an appropriate adjuvant (for example, Freund's complete adjuvant) by intraperitoneal, subcutaneous, or intravenous administration. The polyclonal antibody may be separated and purified from the resulting serum or eggs in accordance with conventional methods for polypeptide isolation and purification. Examples of the separation and purification methods include, for example, centrifugal separation, dialysis, salting-out with ammonium sulfate, or a chromatographic technique using such as DEAE-cellulose, hydroxyapatite, protein A agarose, and the like.

[0072] The monoclonal antibody may be easily produced by those skilled in the art, according to, for example, a cell fusion method of Kohler and Milstein (Kohler, G. and Milstein, C., Nature, 256, 495-497, 1975).

[0073] A mouse is immunized intraperitoneally, subcutaneously, or intravenously several times at an interval of a few weeks by a repeated inoculation of emulsions in which the polypeptide of the present invention or a fragment thereof is emulsified into a suitable adjuvant such as Freund's complete adjuvant. Spleen cells are removed after the final immunization, and then fused with myeloma cells to prepare hybridomas.

[0074] As a myeloma cell for obtaining a hybridoma, a myeloma cell having a marker such as a deficiency in hypoxanthine-guanine phosphoribosyltransferase or thymidine kinase (for example, mouse myeloma cell line P3X63Ag8.U1) may be used. As a fusing agent, polyethylene glycol may be used. As a medium for preparation of hybridomas, for example, a commonly used medium such as an Eagle's minimum essential medium, a Dulbecco's modified minimum essential medium, or an RPMI-1640 medium may be used by adding properly 10 to 30% of a fetal bovine serum. The fused strains may be selected by a HAT selection method. A culture supernatant of the hybridomas is screened by a well-known method such as an ELISA method or an immunohistological method, to select hybridoma clones secreting the antibody of interest. The monoclonality of the selected hybridoma is guaranteed by repeating subcloning by a limiting dilution method. Antibodies in an amount which may be purified are produced by culturing the resulting hybridomas in a medium for 2 to 4 days, or in the peritoneal cavity of a pristane-pretreated BALB/c strain mouse for 10 to 20 days.

[0075] The resulting monoclonal antibodies in the culture supernatant or the ascites may be separated and purified by conventional polypeptide isolation and purification methods. Examples of the separation and purification methods include, for example, centrifugal separation, dialysis, salting-out with ammonium sulfate, or chromatographic technique using such as DEAE-cellulose, hydroxyapatite, protein A agarose, and the like.

[0076] Further, the monoclonal antibodies or the antibody fragments containing a part thereof may be produced by inserting the whole or a part of a gene encoding the monoclonal antibody into an expression vector and introducing the resulting expression vector into appropriate host cells (such as E. coli, yeast, or animal cells).

[0077] Antibody fragments comprising an active part of the antibody such as F(ab')₂, Fab, Fab', or Fv may be obtained by a conventional method, for example, by digesting the separated and purified antibodies (including polyclonal antibodies and monoclonal antibodies) with a protease such as pepsin or papain, and separating and purifying the resulting fragments by standard polypeptide isolation and purification methods.

[0078] Further, an antibody which reacts to the polypeptide of the present invention may be obtained in a form of single chain Fv or Fab in accordance with a method of Clackson et al. or a method of Zebedee et al. (Clackson, T. et al., Nature, 352, 624-628, 1991; or Zebedee, S. et al., Proc. Natl. Acad. Sci. USA, 89, 3175-3179, 1992). Furthermore, a humanized antibody may be obtained by immunizing a transgenic mouse in which mouse antibody genes are substituted with human antibody genes (Lonberg, N. et al., Nature, 368, 856-859, 1994).

EXAMPLES

[0079] The present invention now will be further illustrated by, but is by no means limited to, the following Examples. The procedures were performed in accordance with the known methods (Sambrook, J., et al., "Molecular Cloning - A Laboratory Manual", Cold Spring Harbor Laboratory, NY, 1989), unless otherwise specified.

Example 1: Preparation of expression vector having FLAG added to C-terminus

[0080] A expression vector pCEP4d wherein the Estein-Barr virus EBNA1 expression unit was removed is constructed by digesting plasmid pCEP4 (manufactured by Invitrogen) with restriction enzymes ClaI and NsiI, blunt-ending and then self-ligating. The resulting expression vector pCEP4d was digested with restriction enzymes, NheI and BamHI, and the resulting DNA fragment of approximately 7.7 kbp was extracted from agarose gel, then to which the double stranded oligonucleotide prepared by annealing the oligonucleotide consisting of the base sequence of SEQ ID NO: 7 and the oligonucleotide consisting of the base sequence of SEQ ID NO: 8 was inserted to construct the expression vector pCEP4d-FLAG. The base sequence of the resulting expression vector was analyzed to confirm that the desired sequence was included therein.

[0081] A PCR was carried out, using the expression vector pCEP4d-FLAG as a template, the oligonucleotide consisting of the base sequence of SEQ ID NO: 9 and the oligonucleotide consisting of the base sequence of SEQ ID NO: 10 as primers, and PyroBest DNA polymerase (PyroBest™; manufactured by Takara-shuzo). In the PCR, a thermal denaturing reaction was performed first at 94°C for 2 minutes. Then, a cycle reaction composed of treatments at 94°C for 30 seconds, at 55°C for 30 seconds and at 72°C for 30 seconds was repeated 15 times. Thereafter, an extension reaction was carried out at 72°C for 7 minutes.

[0082] A resulting DNA fragment of approximately 0.4 kbp was digested with a restriction enzyme, SpeI, and inserted into the expression vector pCEP4d-FLAG (about 7.7 kbp) which had been digested with XbaI, to obtain an expression vector pCEP4dE2-FLAG. In the resulting expression vector pCEP4dE2-FLAG, the XbaI recognition sequence, the NheI recognition sequence, the NotI recognition sequence, and the BamHI recognition sequence, and the FLAG tag were arranged from the promoter to the downstream thereof.

Example 2: Cloning of full-length ORF gene of novel aggrecanase gene MDTs8

[0083] A PCR was carried out, using a combination of the oligonucleotide consisting of the base sequence of SEQ ID NO: 3 (having an XbaI recognition sequence and a Kozak sequence added to the 5'-terminus) and the oligonucleotide consisting of the base sequence of SEQ ID NO: 4 (having an NotI recognition sequence added to the 5'-terminus) as the primers, a human placenta cDNA library (Marathon-Ready™ cDNA; manufactured by Clontech) as a template, and DNA polymerase (TaKaRa LA Taq™; manufactured by Takara-shuzo) as the DNA polymerase. In the PCR, a thermal denaturing reaction was first performed at 94°C for 2 minutes. Then, a cycle composed of treatments at 98°C for 10 seconds, and 68°C for 3 minutes was repeated 45 times. Thereafter, an extension reaction was carried out at 68°C for 7 minutes.

[0084] A resulting PCR product, a DNA fragment with approximately 2.3 kbp (having the XbaI recognition sequence and the Kozak sequence added to the 5'-terminus, and the NotI recognition sequence added to the 3'-terminus), was subcloned into a plasmid PCR2.1 (manufactured by Invitrogen) to obtain a clone pMDTs8Cys.

[0085] The resulting plasmid pMDTs8Cys was digested with restriction enzymes, XbaI and NotI, and a resulting DNA fragment of approximately 2.3 kbp was inserted into the XbaI and NotI site of the plasmid pCEP4dE2-FLAG constructed in Example 1 to obtain a plasmid pCEPdE2-MDTs8Cys-FLAG.

[0086] The resulting plasmid pCEPdE2-MDTs8Cys-FLAG was digested with restriction enzymes, KpnI and NotI, to obtain a DNA fragment of approximately 8.3 kbp (hereinafter referred to as DNA Fragment A). Further, the resulting plasmid pCEPdE2-MDTs8Cys-FLAG was digested with restriction enzymes, KpnI and SpeI, to obtain a DNA fragment of approximately 1.5 kbp (hereinafter referred to as DNA Fragment B).

[0087] The procedures described as above were repeated except that a combination of the oligonucleotide consisting of the base sequence of SEQ ID NO: 5 and the oligonucleotide consisting of the base sequence of SEQ ID NO: 6 (having an NotI recognition sequence added to the 5'-terminus) was used as the primers, instead of the combination of the oligonucleotide consisting of the base sequence of SEQ ID NO: 3 and the oligonucleotide consisting of the base sequence of SEQ ID NO: 4. A resulting PCR product, a DNA fragment of approximately 1.5 kbp (having the NotI recognition sequence added to the 3'-terminus), was subcloned into the plasmid PCR2.1 (manufactured by Invitrogen) to obtain a clone pMDTs8-3H. The resulting clone pMDTs8-3H was digested with restriction enzymes, SpeI and NotI, to obtain a DNA fragment with about 1.5 kbp (hereinafter referred to as DNA Fragment C).

[0088] The above DNA Fragment A, the above DNA Fragment B, and the above DNA Fragment C were ligated to prepare a plasmid pCEPdE2-MDTs8Full-FLAG. The resulting plasmid pCEPdE2-MDTs8Full-FLAG contains a gene

consisting of a 1st to 3663rd bases in the base sequence of SEQ ID NO: 1, i.e., the base sequence of the novel aggrecanase gene MDTs8. A polypeptide having a 1st to 1221st amino acid sequence in the amino acid sequence of SEQ ID NO: 2 and an amino acid sequence of SEQ ID NO: 23 added to the C-terminus thereof can be expressed from an animal cell as a host.

Example 3: Expression of MDTs8 full-length protein (MDTs8Full)

[0089] A commercially available transfection reagent (FUGENE™ 6 Transfection Reagent; manufactured by Boehringer Mannheim) was used, in accordance with a protocol attached thereto, to introduce the plasmid pCEPdE2-MDTs8Full-FLAG prepared in Example 2, or the plasmid pCEPdE2-FLAG as a control, into an HEK293-EBNA cell manufactured by Invitrogen.

[0090] An existence of the desired protein in a supernatant of a culture obtained 1 day to 2 days after the introduction of the plasmid was confirmed by a western blotting using an antibody (mouse anti-FLAG monoclonal antibody M2; manufactured by Sigma) against the FLAG tag added to the C-terminus. More particularly, the culture supernatant was electrophoresed on an SDS/10% - 20% acrylamide gel; manufactured by Daiichi Pure Chemicals, and transferred to a polyvinylidene difluoride (PVDF) membrane by a blotting apparatus. To the resulting PVDF membrane, a blocking agent (Block-ace manufactured by Dainippon Pharmaceutical) was added to perform a blocking. Then, the products on the membrane were reacted successively with the mouse anti-FLAG monoclonal antibody M2 and a rabbit anti-mouse IgG polyclonal antibody labeled with horseradish peroxidase (manufactured by Zymed or TAGO). Alternatively, after blocking, the products on the membrane were reacted successively with a biotinylated antibody M2 (manufactured by Sigma) and a streptavidin labeled with horseradish peroxidase (manufactured by Amersham Pharmacia Biotech). After the reaction, an expression of the desired protein was confirmed by a commercially available western blotting detecting system (ECL Western Blotting Detecting System; manufactured by Amersham Pharmacia Biotech).

[0091] An apparent molecular weight of the detected protein on the SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was about 120 to 140 kDa.

Example 4: Confirmation of aggrecanase activity of MDTs8 full-length protein

(1) Preparation of recombinant aggrecan G1G2

[0092] A PCR was carried out, using a combination of the oligonucleotide consisting of the base sequence of SEQ ID NO: 11 prepared on the basis of the gene sequence of a known human aggrecan (Doege K., et al., Biochem. Soc. Trans., 18, 200-202, 1990) and the oligonucleotide consisting of the base sequence of SEQ ID NO: 12 as the primers, a human placenta cDNA library (Marathon-Ready™ cDNA; manufactured by Clontech) as a template, and a PyroBest™ DNA polymerase (manufactured by Takara-shuzo) as the DNA polymerase. In the PCR, a thermal denaturing reaction was first performed at 94°C for 1 minute. Then, a cycle composed of treatments at 98°C for 10 seconds, and 68°C for 2 minutes was repeated 40 times. Thereafter, an extension reaction was carried out at 68°C for 7 minutes.

[0093] A resulting PCR product, a DNA fragment, was digested with a restriction enzyme, BamHI, and a resulting DNA fragment was inserted into the BamHI site of the plasmid pCEP-SigFla to prepare an expression plasmid pCEP-rAgg for expressing a protein (hereinafter referred to as a recombinant G1G2) containing a globular domain 1 - globular domain 2 of the human aggrecan and an FLAG tag added to the N-terminus of the globular domain 1 - the globular domain 2 and His tag added to the C-terminus of the globular domain 1 - the globular domain 2. The plasmid pCEP-SigFla was an expression vector prepared by inserting a double-stranded oligonucleotide which had been prepared by annealing the oligonucleotide consisting of the base sequence of SEQ ID NO: 13 and the oligonucleotide consisting of the base sequence of SEQ ID NO: 14, into the HindIII and XhoI site of the plasmid pCEP4d prepared in Example 1. The plasmid pCEP-SigFla contained a secretory signal sequence (Guan X-M., et al., J. Biol. Chem., 267, 21995-21998, 1992) stemmed from hemagglutinin of an influenza virus, a FLAG tag sequence, and a BamHI recognition sequence, in the order as above, from the promoter to the downstream thereof.

[0094] A resulting expression plasmid pCEP-rAgg was introduced into the HEK293-EBNA cell, and the transformant was cultured for 3 to 7 days to express the desired protein, i.e., the recombinant aggrecan G1G2. The desired protein was purified from the culture supernatant by an affinity chromatography, making use of the FLAG tag attached to the N-terminus. More particularly, the culture supernatant was applied to M2-agarose (manufactured by Sigma) packed in a column, washed with 20 mmol/L tris-HCl (pH 7.4)/150 mmol/L sodium chloride (hereinafter referred to as TBS), eluted by 0.1 mol/L glycine hydrochloride (pH 3.0) to fractionate, and immediately neutralized with 1 mmol/L tris-HCl (pH 8.0).

(2) Detection of aggrecanase activity

[0095] As described in Example 3, the plasmid pCEPdE2-MDTs8Full-FLAG prepared in Example 2 of the plasmid

pCEPdE2-FLAG prepared in Example 1 as a control was introduced into an HEK293-EBNA cell (manufactured by Invitrogen). Then, 16 hours after the plasmid introduction, a medium was replaced with a serum-free medium. The cultivation was continued for 34 hours, and thereafter a culture supernatant was recovered.

[0096] The resulting culture supernatant was mixed with the recombinant G1G2 prepared in Example 4(1), and reacted at 37°C overnight. Thereafter, as described in Example 3, an SDS-PAGE, a transfer to a PVDF membrane, and a blocking were carried out. Then, the products on the PVDF membrane were reacted successively with the mouse monoclonal antibody BC-3 (Hughes C.E., et al., Biochemical J., 305, 799-804, 1995) capable of recognizing an aggrecanase neo-epitope, and a goat anti-mouse IgG polyclonal antibody labeled with peroxidase (manufactured by TAGO). Then, a western blotting detecting system (ECL Western Blotting Detecting System; manufactured by Amersham Pharmacia) was used for detection.

[0097] It was revealed that, when the culture supernatant of the HEK293-EBNA cell transfected with the plasmid pCEPdE2-FLAG (control) was reacted with the recombinant G1G2, no degradation product reactive with the anti-aggrecanase neo-epitope antibody was detected, whereas such degradation products were detected when the culture supernatant of the HEK293-EBNA cell transfected with the plasmid pCEPdE2-MDTS8Full-FLAG was reacted with the recombinant G1G2.

[0098] The present Example shows that MDTS8 has an aggrecanase activity.

Example 5: Induction of MDTS8 mRNA expression, accompanied by differentiation to cartilage cell

(1) Differentiation induction from mesenchymal stem cells to cartilage cell

[0099] It is known that a human mesenchymal stem cell is differentiated to a cartilage cell when cultivated under spheroid pellet with a stimulation of TGF-β3 or the like (Pittenger M. F., et al., Science, 284, 143-147, 1999).

[0100] Normal human mesenchymal stem cells (manufactured by Bio Whittaker) were cultured in a human mesenchymal stem cells proliferation medium kit (manufactured by Bio Whittaker) to obtain 5×10^5 cells. Then, 2.5×10^5 cells were washed with an incomplete chondrogenesis induction medium [DMEM-high glucose (manufactured by LIFE TECHNOLOGIES), 1 mmol/L-sodium pyruvate (manufactured by LIFE TECHNOLOGIES), 0.35 mmol/L-proline (manufactured by LIFE TECHNOLOGIES), 0.1 μmol/L-dexamethasone (manufactured by Sigma), 0.17 mmol/L-ascorbic acid 2-phosphate (manufactured by Sigma), and ITS+1 Culture Supplement (manufactured by Sigma)], and suspended in 500 μL of a complete chondrogenesis induction medium [an incomplete cartilage differentiation induction medium containing 0.01 μg/mL TGF-β3 (manufactured by Sigma)]. Then, the suspension was transferred into a polypropylene tube and centrifuged at 150 x g for 5 minutes to obtain a cell pellet. The resulting cell pellet was placed in a cell cultivating apparatus as it was, and cultivated. The cultivation was continued for 2 weeks while the medium was changed to a complete cartilage differentiation induction medium every 3 or 4 days, so that the cells were differentiated to cartilage cells.

(2) Confirmation of differentiation to cartilage cells

[0101] A total RNA was prepared from each of undifferentiated human mesenchymal stem cells and cells induced to differentiation, by a commercially available total RNA purifying reagent (ISOGEN; manufactured by Nippon Gene). The resulting total RNA was reacted with DNase (manufactured by Nippon Gene) at 37°C for 15 minutes. The resulting DNase-treated total RNA (0.5 μg) was converted to cDNA by a Superscript first-strand system (for RT-PCR; manufactured by LIFE TECHNOLOGIES).

[0102] The differentiation from the human mesenchymal stem cells to cartilage cells by the differentiation induction treatment as disclosed in Example 5(1) was confirmed by a remarkable increase of an expression of mRNAs of collagen type II and collagen type IX specifically expressed in cartilage cells, with the differentiation induction proceeded. More particularly, a quantitative PCR using a sequence detector (Prism7700 Sequence Detector; manufactured by Applied Biosystems) was used to measure an existing amount and make a comparison. For collagen type II, a combination of the oligonucleotide consisting of the base sequence of SEQ ID NO: 15 and the oligonucleotide consisting of the base sequence of SEQ ID NO: 16 was used as a primer set, and for collagen type IX, a combination of the oligonucleotide consisting of the base sequence of SEQ ID NO: 17 and the oligonucleotide consisting of the base sequence of SEQ ID NO: 18 was used as a primer set. A commercially available PCR agent (SYBR Green PCR core reagent; manufactured by Applied Biosystems) was used to perform the PCR. In the PCR, an initial denaturing reaction was performed at 95°C for 10 minutes. Then, a cycle reaction composed of treatments at 94°C for 15 seconds, at 60°C for 30 seconds and 72°C for 60 seconds was repeated 45 times.

[0103] Further, a PCR was carried out, using human genome DNA as a template and the primer set as above under the same conditions, to obtain a standard curve for calculating an amount of mRNA expressed. A PCR was carried out, using the cDNA as above and human genome DNA as templates and the oligonucleotide consisting of the base sequence of SEQ ID NO: 21 and the oligonucleotide consisting of the base sequence of SEQ ID NO: 22 as the primer set under

the same conditions to calculate an amount of human glyceraldehyde-3-phosphate dehydrogenase (g3pdh) expressed, as an internal standard. The existing amounts of mRNAs of collagen type II and collagen type IX were amended on the basis of the determined values of G3PDH.

(3) Expression of MDTs8 gene in cartilage cells

[0104] The oligonucleotide consisting of the base sequence of SEQ ID NO: 19 and the oligonucleotide consisting of the base sequence of SEQ ID NO: 20 were designed on the basis of the base sequence of SEQ ID NO: 1, as a primer set for measuring an amount of mRNA of the novel aggrecanase gene MDTs8. A PCR was carried out, using the above primer set, cDNA (corresponding to 5 ng of total RNA) prepared in Example 5(2) as a template, and a DNA polymerase (TaKaRa LA Taq™; manufactured by Takara-shuzo). In the PCR, a thermal denaturing reaction was first performed at 94°C for 2 minutes. Then, a cycle composed of treatments at 98°C for 10 seconds, at 60°C for 30 seconds, and 72°C for 1 minute was repeated 40 times.

[0105] A PCR was carried out to monitor the expression of the G3PDH gene, using a combination of the oligonucleotide consisting of the base sequence of SEQ ID NO: 21 and the oligonucleotide consisting of the base sequence of SEQ ID NO: 22 as a primer set, cDNA (corresponding to 5 ng of total RNA) prepared in Example 5(2) as a template, and a DNA polymerase (TaKaRa LA Taq™; manufactured by Takara-shuzo). In the PCR, a thermal denaturing reaction was first performed at 94°C for 2 minutes. Then, a cycle composed of treatments at 98°C for 10 seconds, at 60°C for 30 seconds, and 72°C for 1 minute was repeated 30 times.

[0106] It was revealed that a substantial change of the expression of G3PDH gene, accompanied by the differentiation to cartilage cells, was not observed, whereas mRNA of the novel aggrecanase gene MDTs8 of the present invention was not detected in the human mesenchymal stem cells before differentiation, but was detected as a band of about 0.33 kbp in the cartilage cells. This shows that mRNA of the novel aggrecanase gene MDTs8 of the present invention is expressed in cartilage cells.

Example 6: Chromosome mapping of novel aggrecanase gene MDTs8

[0107] A BLAST search was conducted on the GenBank for the gene sequence of the full-length ORF of the novel aggrecanase gene consisting of the base sequence of SEQ ID NO: 1 of the present invention as an inquiry sequence. It was found that BAC clones AC026498, AC025284, AC010548, and AC009139 contain partial sequences of the MDTs8, and a part of at least 24 exons is present in each of the clones.

[0108] Then, it was confirmed that the four clones as above are located on the chromosome 16q, by the public database (Human Genome Reconstruction Project; http://hgrep.ims.u-tokyo.ac.jp/cgi-bin/HTG_tool/view.cgi?layer=top) established by The Institute of Medical Science, The University of Tokyo and RIKEN and published on the web.

Further, it was also confirmed that the four clones as above contain gene markers WI-22533, stSG3069, SGC32952, and stSG62732 which are located on the chromosome 16q.

[0109] Subsequently, more detailed chromosome mapping was conducted, using the markers WI-22533, stSG3069, SGC32952, and stSG62732 located on the chromosome 16q. More particularly, the above four gene markers were searched for in another public database (The Genome Database; <http://www.gdb.org/>). It was found that each of the four markers were located on 16q22.3-23.1 of the physical map of chromosomes, and thus, the MDTs8 is located on 16q22.3-23.1.

[0110] Therefore, it becomes apparent that the chromosomal position of MDTs8 belongs to a region specified as an OA-susceptibility locus, i.e., a joint disease.

INDUSTRIAL APPLICABILITY

[0111] The polypeptide of the present invention is a novel aggrecanase causative of joint diseases (particularly OA), and thus a substance inhibiting the aggrecanase activity of the polypeptide of the present invention is useful as a substance for treating joint diseases.

[0112] According to the polypeptide of the present invention, a convenient screening system for an agent for treating joint diseases can be provided. Namely, by the detecting method of the present invention using the polypeptide of the present invention, a substance for treating joint diseases can be screened by selecting a substance inhibiting the aggrecanase activity of the polypeptide of the present invention.

[0113] Further, a pharmaceutical composition for treating joint diseases can be produced by preparing a medicament using a substance selected by the screening method, as an active ingredient, and a carrier, a filler, and/or other additives.

[0114] The detecting method of the present method can be used not only for screening a substance for treating joint diseases but also for a quality control test of a pharmaceutical composition for treating joint diseases. Namely, a pharmaceutical composition for treating joint diseases can be produced by detecting whether or not a compound to be tested

inhibits the aggrecanase activity of the polypeptide of the present invention, by the detecting method of the present invention, and then preparing a medicament using the resulting substance inhibiting the aggrecanase activity.

[0115] Further, the polynucleotide, the expression vector, the cell, and the antibody of the present invention are useful for producing the polypeptide of the present invention

FREE TEXT IN SEQUENCE LISTING

[0116] Features of "Artificial Sequence" are described in the numeric identifier <223> in the Sequence Listing. More particularly, each of the base sequences of SEQ ID NOS: 3-6 and 9-12 is an artificially synthesized primer sequence. Each of the base sequences of SEQ ID NOS: 7, 8, 13, and 14 is an artificially synthesized linker sequence. The base sequence of SEQ ID NO: 23 is an artificially synthesized sequence containing a restriction enzyme NotI recognition sequence and a FLAG tag sequence.

[0117] As above, the present invention is explained with reference to particular embodiments, but modifications and improvements obvious to those skilled in the art are included in the scope of the present invention.

SEQUENCE LISTING

[0118]

<110> Yamanouchi Pharmaceutical Co., Ltd.

<120> Novel aggrecanase

<130> Y0130PCT-661

<150> JP 2000-384300

<151> 2000-12-18

<160> 26

<210> 1

<211> 3666

<212> DNA

<213> Homo sapiens

<220>

<221> CDS

<222> (1)..... (3666)

<400> 1

atg gag tgc gcc ctc ctg ctc gcg tgt gcc ttc ccg gct gcg ggt tgg 48
 Met Glu Cys Ala Leu Leu Leu Ala Cys Ala Phe Pro Ala Ala Gly Ser
 1 5 10 15

ggc ccg ccg agg ggc ctg gcg gga ctg ggg cgc gtg gcc aag gcg ctc 96
 Gly Pro Pro Arg Gly Leu Ala Gly Leu Gly Arg Val Ala Lys Ala Leu
 20 25 30

cag ctg tgc tgc ctc tgc tgt gcg tgg gtc gcc gcg gcc tta gcc agt 144
 Gln Leu Cys Cys Leu Cys Cys Ala Ser Val Ala Ala Ala Leu Ala Ser
 35 40 45

gac agc agc agc ggc gcc agc gga tta aat gat gat tac gtc ttt gtc 192
 Asp Ser Ser Ser Gly Ala Ser Gly Leu Asn Asp Asp Tyr Val Phe Val
 50 55 60

acg cca gta gaa gta gac tca gcc ggg tca tat att tca cac gac att 240
 Thr Pro Val Glu Val Asp Ser Ala Gly Ser Tyr Ile Ser His Asp Ile
 65 70 75 80

ttg cac aac ggc agg aaa aag cga tgg gcg cag aat gcc aga agc tcc 288
 Leu His Asn Gly Arg Lys Lys Arg Ser Ala Gln Asn Ala Arg Ser Ser
 85 90 95

5	ctg cac tac cga ttt tca gca ttt gga cag gaa ctg cac tta gaa ctt Leu His Tyr Arg Phe Ser Ala Phe Gly Gln Glu Leu His Leu Glu Leu	336
	100 105 110	
10	aag ccc tcg gcg att ttg agc agt cac ttt att gtc cag gta ctt gga Lys Pro Ser Ala Ile Leu Ser Ser His Phe Ile Val Gln Val Leu Gly	384
	115 120 125	
15	aaa gat ggt gct tca gag act cag aaa ccc gag gtg cag caa tgc ttc Lys Asp Gly Ala Ser Glu Thr Gln Lys Pro Glu Val Gln Gln Cys Phe	432
	130 135 140	
20	tat cag gga ttt atc aga aat gac agc tcc tcc tct gtc gct gtg tct Tyr Gln Gly Phe Ile Arg Asn Asp Ser Ser Ser Val Ala Val Ser	480
	145 150 155 160	
25	acg tgt gct ggc ttg tca ggt tta ata agg aca cga aaa aat gaa ttc Thr Cys Ala Gly Leu Ser Gly Leu Ile Arg Thr Arg Lys Asn Glu Phe	528
	165 170 175	
30	ctc atc tcg cca tta cct cag ctt ctg gcc cag gaa cac aac tac agc Leu Ile Ser Pro Leu Pro Gln Leu Leu Ala Gln Glu His Asn Tyr Ser	576
	180 185 190	
35	tcc cct gcg ggt cac cat cct cac gta ctg tac aaa agg aca gca gag Ser Pro Ala Gly His His Pro His Val Leu Tyr Lys Arg Thr Ala Glu	624
	195 200 205	
40	gag aag atc cag cgg tac cgt ggc tac ccc ggc tct ggc cgg aat tat Glu Lys Ile Gln Arg Tyr Arg Gly Tyr Pro Gly Ser Gly Arg Asn Tyr	672
	210 215 220	
45	cct ggt tac tcc cca agt cac att ccc cat gca tct cag agt cga gag Pro Gly Tyr Ser Pro Ser His Ile Pro His Ala Ser Gln Ser Arg Glu	720
	225 230 235 240	
50	aca gag tat cac cat cga agg ttg caa aag cag cat ttt tgt gga cga Thr Glu Tyr His His Arg Arg Leu Gln Lys Gln His Phe Cys Gly Arg	768
	245 250 255	
55	cgc aag aaa tat gct ccc aag cct ccc aca gag gac acc tat cta agg Arg Lys Lys Tyr Ala Pro Lys Pro Pro Thr Glu Asp Thr Tyr Leu Arg	816
	260 265 270	
	ttt gat gaa tat ggg agc tct ggg cga ccc aga aga tca gct gga aaa	864

	Phe	Asp	Glu	Tyr	Gly	Ser	Ser	Gly	Arg	Pro	Arg	Arg	Ser	Ala	Gly	Lys	
			275					280					285				
5	tca	caa	aag	ggc	ctc	aat	gtg	gaa	acc	ctc	gtg	gtg	gca	gac	aag	aaa	912
	Ser	Gln	Lys	Gly	Leu	Asn	Val	Glu	Thr	Leu	Val	Val	Ala	Asp	Lys	Lys	
			290				295					300					
10	atg	gtg	gaa	aag	cat	ggc	aag	gga	aat	gtc	acc	aca	tac	att	ctc	aca	960
	Met	Val	Glu	Lys	His	Gly	Lys	Gly	Asn	Val	Thr	Thr	Tyr	Ile	Leu	Thr	
	305					310					315				320		
15	gta	atg	aac	atg	gtt	tct	ggc	cta	ttt	aaa	gat	ggg	act	att	gga	agt	1008
	Val	Met	Asn	Met	Val	Ser	Gly	Leu	Phe	Lys	Asp	Gly	Thr	Ile	Gly	Ser	
				325						330					335		
20	gac	ata	aac	gtg	gtt	gtg	gtg	agc	cta	att	ctt	ctg	gaa	caa	gaa	cct	1056
	Asp	Ile	Asn	Val	Val	Val	Val	Ser	Leu	Ile	Leu	Leu	Glu	Gln	Glu	Pro	
				340					345					350			
25	gga	gga	tta	ttg	atc	aac	cat	cat	gca	gac	cag	tct	ctg	aat	agt	ttt	1104
	Gly	Gly	Leu	Leu	Ile	Asn	His	His	Ala	Asp	Gln	Ser	Leu	Asn	Ser	Phe	
			355					360					365				
30	tgt	caa	tgg	cag	tct	gcc	ctc	att	gga	aag	aat	ggc	aag	aga	cat	gat	1152
	Cys	Gln	Trp	Gln	Ser	Ala	Leu	Ile	Gly	Lys	Asn	Gly	Lys	Arg	His	Asp	
		370					375					380					
35	cat	gcc	atc	tta	cta	aca	gga	ttt	gat	att	tgt	tct	tgg	aag	aat	gaa	1200
	His	Ala	Ile	Leu	Leu	Thr	Gly	Phe	Asp	Ile	Cys	Ser	Trp	Lys	Asn	Glu	
	385					390					395					400	
40	cca	tgt	gac	act	cta	ggg	ttt	gcc	ccc	atc	agt	gga	atg	tgc	tct	aag	1248
	Pro	Cys	Asp	Thr	Leu	Gly	Phe	Ala	Pro	Ile	Ser	Gly	Met	Cys	Ser	Lys	
				405					410						415		
45	tac	cga	agt	tgt	acc	atc	aat	gag	gac	aca	gga	ctt	ggc	ctt	gcc	ttc	1296
	Tyr	Arg	Ser	Cys	Thr	Ile	Asn	Glu	Asp	Thr	Gly	Leu	Gly	Leu	Ala	Phe	
				420					425					430			
50	acc	atc	gct	cat	gag	tca	ggg	cac	aac	ttt	ggt	atg	att	cac	gac	gga	1344
	Thr	Ile	Ala	His	Glu	Ser	Gly	His	Asn	Phe	Gly	Met	Ile	His	Asp	Gly	
			435					440					445				
55	gaa	ggg	aat	ccc	tgc	aga	aag	gct	gaa	ggc	aat	atc	atg	tct	ccc	aca	1392
	Glu	Gly	Asn	Pro	Cys	Arg	Lys	Ala	Glu	Gly	Asn	Ile	Met	Ser	Pro	Thr	
		450					455					460					

5	ctg acc gga aac aat gga gtg ttt tca tgg tct tcc tgc agc cgc cag Leu Thr Gly Asn Asn Gly Val Phe Ser Trp Ser Ser Cys Ser Arg Gln 465 470 475 480	1440
10	tat ctc aag aaa ttc ctc agc aca cct cag gcg ggg tgt cta gtg gat Tyr Leu Lys Lys Phe Leu Ser Thr Pro Gln Ala Gly Cys Leu Val Asp 485 490 495	1488
15	gag ccc aag caa gca gga cag tat aaa tat ccg gac aaa cta cca gga Glu Pro Lys Gln Ala Gly Gln Tyr Lys Tyr Pro Asp Lys Leu Pro Gly 500 505 510	1536
20	cag att tat gat gct gac aca cag tgt aaa tgg caa ttt gga gca aaa Gln Ile Tyr Asp Ala Asp Thr Gln Cys Lys Trp Gln Phe Gly Ala Lys 515 520 525	1584
25	gcc aag tta tgc agc ctt ggt ttt gtg aag gat att tgc aaa tca ctt Ala Lys Leu Cys Ser Leu Gly Phe Val Lys Asp Ile Cys Lys Ser Leu 530 535 540	1632
30	tgg tgc cac cga gta ggc cac agg tgt gag acc aag ttt atg ccc gca Trp Cys His Arg Val Gly His Arg Cys Glu Thr Lys Phe Met Pro Ala 545 550 555 560	1680
35	gca gaa ggg acc gtt tgt ggc ttg agt atg tgg tgt cgg caa ggc cag Ala Glu Gly Thr Val Cys Gly Leu Ser Met Trp Cys Arg Gln Gly Gln 565 570 575	1728
40	tgc gta aag ttt ggg gag ctc ggg ccc cgg ccc atc cac ggc cag tgg Cys Val Lys Phe Gly Glu Leu Gly Pro Arg Pro Ile His Gly Gln Trp 580 585 590	1776
45	tcc gcc tgg tgc aag tgg tca gaa tgt tcc cgg aca tgt ggt gga gga Ser Ala Trp Ser Lys Trp Ser Glu Cys Ser Arg Thr Cys Gly Gly Gly 595 600 605	1824
50	gtc aag ttc cag gag aga cac tgc aat aac ccc aag cct cag tat ggt Val Lys Phe Gln Glu Arg His Cys Asn Asn Pro Lys Pro Gln Tyr Gly 610 615 620	1872
55	ggc tta ttc tgt cca ggt tct agc cgt att tat cag ctg tgc aat att Gly Leu Phe Cys Pro Gly Ser Ser Arg Ile Tyr Gln Leu Cys Asn Ile 625 630 635 640	1920
	aac cct tgc aat gaa aat agc ttg gat ttt cgg gct caa cag tgt gca	1968

	Asn	Pro	Cys	Asn	Glu	Asn	Ser	Leu	Asp	Phe	Arg	Ala	Gln	Gln	Cys	Ala	
					645					650					655		
5																	
	gaa	tat	aac	agc	aaa	cct	ttc	cgt	gga	tgg	ttc	tac	cag	tgg	aaa	ccc	2016
	Glu	Tyr	Asn	Ser	Lys	Pro	Phe	Arg	Gly	Trp	Phe	Tyr	Gln	Trp	Lys	Pro	
				660					665					670			
10																	
	tat	aca	aaa	gtg	gaa	gag	gaa	gat	cga	tgc	aaa	ctg	tac	tgc	aag	gct	2064
	Tyr	Thr	Lys	Val	Glu	Glu	Glu	Asp	Arg	Cys	Lys	Leu	Tyr	Cys	Lys	Ala	
			675					680					685				
15																	
	gag	aac	ttt	gaa	ttt	ttt	ttt	gca	atg	tcc	ggc	aaa	gtg	aaa	gat	gga	2112
	Glu	Asn	Phe	Glu	Phe	Phe	Phe	Ala	Met	Ser	Gly	Lys	Val	Lys	Asp	Gly	
		690						695					700				
20																	
	act	ccc	tgc	tcc	cca	aac	aaa	aat	gat	gtt	tgt	att	gac	ggg	gtt	tgt	2160
	Thr	Pro	Cys	Ser	Pro	Asn	Lys	Asn	Asp	Val	Cys	Ile	Asp	Gly	Val	Cys	
	705					710					715					720	
25																	
	gaa	cta	gtg	gga	tgt	gat	cat	gaa	cta	ggc	tct	aaa	gca	gtt	tca	gat	2208
	Glu	Leu	Val	Gly	Cys	Asp	His	Glu	Leu	Gly	Ser	Lys	Ala	Val	Ser	Asp	
				725						730					735		
30																	
	gct	tgt	ggc	gtt	tgc	aaa	ggc	gat	aat	tca	act	tgc	aag	ttt	tat	aaa	2256
	Ala	Cys	Gly	Val	Cys	Lys	Gly	Asp	Asn	Ser	Thr	Cys	Lys	Phe	Tyr	Lys	
			740					745						750			
35																	
	ggc	ctg	tac	ctc	aac	cag	cat	aaa	gca	aat	gaa	tat	tat	cgc	gtg	gtc	2304
	Gly	Leu	Tyr	Leu	Asn	Gln	His	Lys	Ala	Asn	Glu	Tyr	Tyr	Pro	Val	Val	
		755						760					765				
40																	
	atc	att	cca	gct	ggc	gcc	cga	agc	atc	gaa	atc	cag	gag	ctg	cag	gtt	2352
	Ile	Ile	Pro	Ala	Gly	Ala	Arg	Ser	Ile	Glu	Ile	Gln	Glu	Leu	Gln	Val	
	770						775					780					
45																	
	tcc	tcc	agt	tac	ctc	gca	gtt	cga	agc	ctc	agt	caa	aag	tat	tac	ctc	2400
	Ser	Ser	Ser	Tyr	Leu	Ala	Val	Arg	Ser	Leu	Ser	Gln	Lys	Tyr	Tyr	Leu	
	785					790					795					800	
50																	
	acc	ggg	ggc	tgg	agc	atc	gac	tgg	cct	ggg	gag	ttc	ccc	ttc	gct	ggg	2448
	Thr	Gly	Gly	Trp	Ser	Ile	Asp	Trp	Pro	Gly	Glu	Phe	Pro	Phe	Ala	Gly	
				805						810					815		
55																	
	acc	acg	ttt	gaa	tac	cag	cgc	tct	ttc	aac	cgc	ccg	gaa	cgt	ctg	tac	2496
	Thr	Thr	Phe	Glu	Tyr	Gln	Arg	Ser	Phe	Asn	Arg	Pro	Glu	Arg	Leu	Tyr	
			820						825						830		

5	gcg cca ggg ccc aca aat gag acg ctg gtc ttt gaa att ctg atg caa Ala Pro Gly Pro Thr Asn Glu Thr Leu Val Phe Glu Ile Leu Met Gln 835 840 845	2544
10	ggc aaa aat cca ggg ata gct tgg aag tat gca ctt ccc aag gtc atg Gly Lys Asn Pro Gly Ile Ala Trp Lys Tyr Ala Leu Pro Lys Val Met 850 855 860	2592
15	aat gga act cca cca gcc aca aaa aga cct gcc tat acc tgg agt atc Asn Gly Thr Pro Pro Ala Thr Lys Arg Pro Ala Tyr Thr Trp Ser Ile 865 870 875 880	2640
20	gtg cag tca gag tgc tcc gtc tcc tgt ggt gga ggt tac ata aat gta Val Gln Ser Glu Cys Ser Val Ser Cys Gly Gly Gly Tyr Ile Asn Val 885 890 895	2688
25	aag gcc att tgc ttg cga gat caa aat act caa gtc aat tcc tca ttc Lys Ala Ile Cys Leu Arg Asp Gln Asn Thr Gln Val Asn Ser Ser Phe 900 905 910	2736
30	tgc agt gca aaa acc aag cca gta act gag ccc aaa atc tgc aac gct Cys Ser Ala Lys Thr Lys Pro Val Thr Glu Pro Lys Ile Cys Asn Ala 915 920 925	2784
35	ttc tcc tgc ccg gct tac tgg atg cca ggt gaa tgg agt aca tgc agc Phe Ser Cys Pro Ala Tyr Trp Met Pro Gly Glu Trp Ser Thr Cys Ser 930 935 940	2832
40	aag gcc tgt gct gga ggc cag cag agc cga aag atc cag tgt gtg caa Lys Ala Cys Ala Gly Gly Gln Gln Ser Arg Lys Ile Gln Cys Val Gln 945 950 955 960	2880
45	aag aag ccc ttc caa aag gag gaa gca gtg ttg cat tct ctc tgt cca Lys Lys Pro Phe Gln Lys Glu Glu Ala Val Leu His Ser Leu Cys Pro 965 970 975	2928
50	gtg agc aca ccc act cag gtc caa gcc tgc aac agc cat gcc tgc cct Val Ser Thr Pro Thr Gln Val Gln Ala Cys Asn Ser His Ala Cys Pro 980 985 990	2976
55	cca caa tgg agc ctt gga ccc tgg tct cag tgt tcc aag acc tgt gga Pro Gln Trp Ser Leu Gly Pro Trp Ser Gln Cys Ser Lys Thr Cys Gly 995 1000 1005	3024
	cga ggg gtg agg aag cgt gaa ctc ctc tgc aag ggc tct gcc gca gaa	3072

	Arg Gly Val Arg Lys Arg Glu Leu Leu Cys Lys Gly Ser Ala Ala Glu	
	1010 1015 1020	
5	acc ctc ccc gag agc cag tgt acc agt ctc ccc aga cct gag ctg cag Thr Leu Pro Glu Ser Gln Cys Thr Ser Leu Pro Arg Pro Glu Leu Gln	3120
	1025 1030 1035 1040	
10	gag ggc tgt gtg ctt gga cga tgc ccc aag aac agc cgg cta cag tgg Glu Gly Cys Val Leu Gly Arg Cys Pro Lys Asn Ser Arg Leu Gln Trp	3168
	1045 1050 1055	
15	gtc gct tct tcg tgg agc gag tgt tct gca acc tgt ggt ttg ggt gtg Val Ala Ser Ser Trp Ser Glu Cys Ser Ala Thr Cys Gly Leu Gly Val	3216
	1060 1065 1070	
20	agg aag agg gag atg aag tgc agc gag aag ggc ttc cag gga aag ctg Arg Lys Arg Glu Met Lys Cys Ser Glu Lys Gly Phe Gln Gly Lys Leu	3264
	1075 1080 1085	
25	ata act ttc cca gag cga aga tgc cgt aat att aag aaa cca aat ctg Ile Thr Phe Pro Glu Arg Arg Cys Arg Asn Ile Lys Lys Pro Asn Leu	3312
	1090 1095 1100	
30	gac ttg gaa gag acc tgc aac cga cgg gct tgc cca gcc cat cca gtg Asp Leu Glu Glu Thr Cys Asn Arg Arg Ala Cys Pro Ala His Pro Val	3360
	1105 1110 1115 1120	
35	tac aac atg gta gct gga tgg tat tca ttg cgg tgg cag cag tgc aca Tyr Asn Met Val Ala Gly Trp Tyr Ser Leu Pro Trp Gln Gln Cys Thr	3408
	1125 1130 1135	
40	gtc acc tgt ggg gga ggg gtc cag acc cgg tca gtc cac tgt gtt cag Val Thr Cys Gly Gly Gly Val Gln Thr Arg Ser Val His Cys Val Gln	3456
	1140 1145 1150	
45	caa ggc cgg cct tcc tca agt tgt ctg ctc cat cag aaa cct ccg gtg Gln Gly Arg Pro Ser Ser Ser Cys Leu Leu His Gln Lys Pro Pro Val	3504
	1155 1160 1165	
50	cta cga gcc tgt aat aca aac ttc tgt cca gct cct gaa aag aga gag Leu Arg Ala Cys Asn Thr Asn Phe Cys Pro Ala Pro Glu Lys Arg Glu	3552
	1170 1175 1180	
55	gat cca tcc tgc gta gat ttc ttc aac tgg tgt cac cta gtt cct cag Asp Pro Ser Cys Val Asp Phe Phe Asn Trp Cys His Leu Val Pro Gln	3600
	1185 1190 1195 1200	

cat ggt gtc tgc aac cac aag ttt tac gga aaa caa tgc tgc aag tca 3648
 His Gly Val Cys Asn His Lys Phe Tyr Gly Lys Gln Cys Cys Lys Ser
 1205 1210 1215

tgc aca agg aag atc tga 3666
 Cys Thr Arg Lys Ile
 1220

<210> 2
 <211> 1221
 <212> PRT
 <213> Homo sapiens

<400> 2

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

	Thr	Glu	Tyr	His	His	Arg	Arg	Leu	Gln	Lys	Gln	His	Phe	Cys	Gly	Arg	
				245						250					255		
5	Arg	Lys	Lys	Tyr	Ala	Pro	Lys	Pro	Pro	Thr	Glu	Asp	Thr	Tyr	Leu	Arg	
				260					265						270		
	Phe	Asp	Glu	Tyr	Gly	Ser	Ser	Gly	Arg	Pro	Arg	Arg	Ser	Ala	Gly	Lys	
			275					280					285				
10	Ser	Gln	Lys	Gly	Leu	Asn	Val	Glu	Thr	Leu	Val	Val	Ala	Asp	Lys	Lys	
			290				295					300					
	Met	Val	Glu	Lys	His	Gly	Lys	Gly	Asn	Val	Thr	Thr	Tyr	Ile	Leu	Thr	
	305					310					315					320	
15	Val	Met	Asn	Met	Val	Ser	Gly	Leu	Phe	Lys	Asp	Gly	Thr	Ile	Gly	Ser	
				325					330						335		
	Asp	Ile	Asn	Val	Val	Val	Val	Ser	Leu	Ile	Leu	Leu	Glu	Gln	Glu	Pro	
				340					345					350			
20	Gly	Gly	Leu	Leu	Ile	Asn	His	His	Ala	Asp	Gln	Ser	Leu	Asn	Ser	Phe	
			355					360					365				
	Cys	Gln	Trp	Gln	Ser	Ala	Leu	Ile	Gly	Lys	Asn	Gly	Lys	Arg	His	Asp	
			370				375					380					
25	His	Ala	Ile	Leu	Leu	Thr	Gly	Phe	Asp	Ile	Cys	Ser	Trp	Lys	Asn	Glu	
	385					390					395					400	
	Pro	Cys	Asp	Thr	Leu	Gly	Phe	Ala	Pro	Ile	Ser	Gly	Met	Cys	Ser	Lys	
				405						410					415		
30	Tyr	Arg	Ser	Cys	Thr	Ile	Asn	Glu	Asp	Thr	Gly	Leu	Gly	Leu	Ala	Phe	
			420						425					430			
	Thr	Ile	Ala	His	Glu	Ser	Gly	His	Asn	Phe	Gly	Met	Ile	His	Asp	Gly	
			435					440					445				
35	Glu	Gly	Asn	Pro	Cys	Arg	Lys	Ala	Glu	Gly	Asn	Ile	Met	Ser	Pro	Thr	
			450				455					460					
	Leu	Thr	Gly	Asn	Asn	Gly	Val	Phe	Ser	Trp	Ser	Ser	Cys	Ser	Arg	Gln	
	465					470					475					480	
	Tyr	Leu	Lys	Lys	Phe	Leu	Ser	Thr	Pro	Gln	Ala	Gly	Cys	Leu	Val	Asp	
				485						490					495		
40	Glu	Pro	Lys	Gln	Ala	Gly	Gln	Tyr	Lys	Tyr	Pro	Asp	Lys	Leu	Pro	Gly	
				500					505					510			
	Gln	Ile	Tyr	Asp	Ala	Asp	Thr	Gln	Cys	Lys	Trp	Gln	Phe	Gly	Ala	Lys	
			515					520					525				
45	Ala	Lys	Leu	Cys	Ser	Leu	Gly	Phe	Val	Lys	Asp	Ile	Cys	Lys	Ser	Leu	
			530				535					540					
	Trp	Cys	His	Arg	Val	Gly	His	Arg	Cys	Glu	Thr	Lys	Phe	Met	Pro	Ala	
	545					550					555					560	
50	Ala	Glu	Gly	Thr	Val	Cys	Gly	Leu	Ser	Met	Trp	Cys	Arg	Gln	Gly	Gln	
				565						570					575		
	Cys	Val	Lys	Phe	Gly	Glu	Leu	Gly	Pro	Arg	Pro	Ile	His	Gly	Gln	Trp	
			580					585					590				
55	Ser	Ala	Trp	Ser	Lys	Trp	Ser	Glu	Cys	Ser	Arg	Thr	Cys	Gly	Gly	Gly	
			595					600					605				

	Val	Lys	Phe	Gln	Glu	Arg	His	Cys	Asn	Asn	Pro	Lys	Pro	Gln	Tyr	Gly
	610						615					620				
5	Gly	Leu	Phe	Cys	Pro	Gly	Ser	Ser	Arg	Ile	Tyr	Gln	Leu	Cys	Asn	Ile
	625					630					635					640
	Asn	Pro	Cys	Asn	Glu	Asn	Ser	Leu	Asp	Phe	Arg	Ala	Gln	Gln	Cys	Ala
				645						650					655	
10	Glu	Tyr	Asn	Ser	Lys	Pro	Phe	Arg	Gly	Trp	Phe	Tyr	Gln	Trp	Lys	Pro
			660						665					670		
	Tyr	Thr	Lys	Val	Glu	Glu	Glu	Asp	Arg	Cys	Lys	Leu	Tyr	Cys	Lys	Ala
			675					680					685			
15	Glu	Asn	Phe	Glu	Phe	Phe	Phe	Ala	Met	Ser	Gly	Lys	Val	Lys	Asp	Gly
	690						695					700				
	Thr	Pro	Cys	Ser	Pro	Asn	Lys	Asn	Asp	Val	Cys	Ile	Asp	Gly	Val	Cys
	705					710					715					720
20	Glu	Leu	Val	Gly	Cys	Asp	His	Glu	Leu	Gly	Ser	Lys	Ala	Val	Ser	Asp
				725						730					735	
	Ala	Cys	Gly	Val	Cys	Lys	Gly	Asp	Asn	Ser	Thr	Cys	Lys	Phe	Tyr	Lys
				740					745					750		
25	Gly	Leu	Tyr	Leu	Asn	Gln	His	Lys	Ala	Asn	Glu	Tyr	Tyr	Pro	Val	Val
			755					760					765			
	Ile	Ile	Pro	Ala	Gly	Ala	Arg	Ser	Ile	Glu	Ile	Gln	Glu	Leu	Gln	Val
	770						775					780				
30	Ser	Ser	Ser	Tyr	Leu	Ala	Val	Arg	Ser	Leu	Ser	Gln	Lys	Tyr	Tyr	Leu
	785					790					795					800
	Thr	Gly	Gly	Trp	Ser	Ile	Asp	Trp	Pro	Gly	Glu	Phe	Pro	Phe	Ala	Gly
				805						810					815	
35	Thr	Thr	Phe	Glu	Tyr	Gln	Arg	Ser	Phe	Asn	Arg	Pro	Glu	Arg	Leu	Tyr
			820						825					830		
	Ala	Pro	Gly	Pro	Thr	Asn	Glu	Thr	Leu	Val	Phe	Glu	Ile	Leu	Met	Gln
			835					840					845			
40	Gly	Lys	Asn	Pro	Gly	Ile	Ala	Trp	Lys	Tyr	Ala	Leu	Pro	Lys	Val	Met
	850					855					860					
	Asn	Gly	Thr	Pro	Pro	Ala	Thr	Lys	Arg	Pro	Ala	Tyr	Thr	Trp	Ser	Ile
	865					870					875					880
45	Val	Gln	Ser	Glu	Cys	Ser	Val	Ser	Cys	Gly	Gly	Gly	Tyr	Ile	Asn	Val
				885						890					895	
	Lys	Ala	Ile	Cys	Leu	Arg	Asp	Gln	Asn	Thr	Gln	Val	Asn	Ser	Ser	Phe
				900					905					910		
50	Cys	Ser	Ala	Lys	Thr	Lys	Pro	Val	Thr	Glu	Pro	Lys	Ile	Cys	Asn	Ala
			915					920					925			
	Phe	Ser	Cys	Pro	Ala	Tyr	Trp	Met	Pro	Gly	Glu	Trp	Ser	Thr	Cys	Ser
	930						935					940				
55	Lys	Ala	Cys	Ala	Gly	Gly	Gln	Gln	Ser	Arg	Lys	Ile	Gln	Cys	Val	Gln
	945					950					955					960
	Lys	Lys	Pro	Phe	Gln	Lys	Glu	Glu	Ala	Val	Leu	His	Ser	Leu	Cys	Pro
				965						970					975	

Val Ser Thr Pro Thr Gln Val Gln Ala Cys Asn Ser His Ala Cys Pro
 980 985 990
 5 Pro Gln Trp Ser Leu Gly Pro Trp Ser Gln Cys Ser Lys Thr Cys Gly
 995 1000 1005
 Arg Gly Val Arg Lys Arg Glu Leu Leu Cys Lys Gly Ser Ala Ala Glu
 1010 1015 1020
 10 Thr Leu Pro Glu Ser Gln Cys Thr Ser Leu Pro Arg Pro Glu Leu Gln
 1025 1030 1035 1040
 Glu Gly Cys Val Leu Gly Arg Cys Pro Lys Asn Ser Arg Leu Gln Trp
 1045 1050 1055
 15 Val Ala Ser Ser Trp Ser Glu Cys Ser Ala Thr Cys Gly Leu Gly Val
 1060 1065 1070
 Arg Lys Arg Glu Met Lys Cys Ser Glu Lys Gly Phe Gln Gly Lys Leu
 1075 1080 1085
 20 Ile Thr Phe Pro Glu Arg Arg Cys Arg Asn Ile Lys Lys Pro Asn Leu
 1090 1095 1100
 Asp Leu Glu Glu Thr Cys Asn Arg Arg Ala Cys Pro Ala His Pro Val
 1105 1110 1115 1120
 25 Tyr Asn Met Val Ala Gly Trp Tyr Ser Leu Pro Trp Gln Gln Cys Thr
 1125 1130 1135
 Val Thr Cys Gly Gly Gly Val Gln Thr Arg Ser Val His Cys Val Gln
 1140 1145 1150
 30 Gln Gly Arg Pro Ser Ser Ser Cys Leu Leu His Gln Lys Pro Pro Val
 1155 1160 1165
 Leu Arg Ala Cys Asn Thr Asn Phe Cys Pro Ala Pro Glu Lys Arg Glu
 1170 1175 1180
 35 Asp Pro Ser Cys Val Asp Phe Phe Asn Trp Cys His Leu Val Pro Gln
 1185 1190 1195 1200
 His Gly Val Cys Asn His Lys Phe Tyr Gly Lys Gln Cys Cys Lys Ser
 1205 1210 1215
 40 Cys Thr Arg Lys Ile
 1220

<210> 3

<211> 40

45 <212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: an artificially synthesized primer sequence

50

<400> 3

gctctagacc atggagtgcg cccctctgct cgcgtgtgcc 40

<210> 4

55 <211> 41

<212> DNA

<213> Artificial Sequence

EP 1 344 821 B9

<220>

<223> Description of Artificial Sequence: an artificially synthesized primer sequence

<400> 4

5 agagcggccg cgccttata aaactgcaa gttgaattat c 41

<210> 5

<211> 28

<212> DNA

10 <213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: an artificially synthesized primer sequence

<400> 5

15 acgggggttg tgaactagt ggatgtga 28

<210> 6

<211> 41

20 <212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: an artificially synthesized primer sequence

<400> 6

25 agagcggccg cgccttctc tgtcatgac ttgcagcatt g 41

<210> 7

30 <211> 50

<212> DNA

<213> Artificial Sequence

<220>

35 <223> Description of Artificial Sequence: an artificially synthesized linker sequence

<400> 7

ctagcgcgcg cgcaggatcc gactacaagg acgacgatga caaatgataa 50

<210> 8

40 <211> 50

<212> DNA

<213> Artificial Sequence

<220>

45 <223> Description of Artificial Sequence: an artificially synthesized linker sequence

<400> 8

50 gatcttatca ttgtcatcg tcgtccttgt agtcggatcc tgcggccgcg 50

<210> 9

<211> 34

<212> DNA

55 <213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: an artificially synthesized primer sequence

EP 1 344 821 B9

<400> 9
 ggactagtct agaagctggg taccagctgc tagc 34

 <210> 10
 <211> 29
 <212> DNA
 <213> Artificial Sequence

 <220>
 <223> Description of Artificial Sequence: an artificially synthesized primer sequence

 <400> 10
 ggactagtgt cgaccgggtca tggctgcgc 29

 <210> 11
 <211> 37
 <212> DNA
 <213> Artificial Sequence

 <220>
 <223> Description of Artificial Sequence: an artificially synthesized primer sequence

 <400> 11 37
 taggatcctt gtagaaactt cagaccatga caactcg 37

 <210> 12
 <211> 59
 <212> DNA
 <213> Artificial Sequence

 <220>
 <223> Description of Artificial Sequence: an artificially synthesized primer sequence

 <400> 12
 atggatcctc aatggtgatg gtgatgatga ccgaagcaga aggcattggtg ccgggacag 59

 <210> 13
 <211> 97
 <212> DNA
 <213> Artificial Sequence

 <220>
 <223> Description of Artificial Sequence: an artificially synthesized linker sequence

 <400> 13

agcttggccac catgaagaag atcatcgccc tgagctacat cttctgcctg gtattcgccg 60
actacaagga cgatgatgac aaggggatcc actagtc 97

 <210> 14
 <211> 97
 <212> DNA
 <213> Artificial Sequence

 <220>
 <223> Description of Artificial Sequence: an artificially synthesized linker sequence

<400> 14

5 **tcgagactag tggatcccct tgtcatcatc gtcctttag tagcggaata ccaggcagaa 60**
gatgtagctc agggcgatga tcgtcttcat ggtggca 97

10 <210> 15
 <211> 20
 <212> DNA
 <213> Homo sapiens

15 <400> 15 20
 tttcccaggt caagatggc 20

20 <210> 16*
 <211> 20
 <212> DNA
 <213> Homo sapiens

<400> 16 20
 cttcagcacc tgtctacca 20

25 <210> 17
 <211> 20
 <212> DNA
 <213> Homo sapiens

30 <400> 17 20
 gtgttgctgg tgaaaagggt 20

35 <210> 18
 <211> 20
 <212> DNA
 <213> Homo sapiens

40 <400> 18 20
 gggatccac tggctctaat 20

<210> 19

45 <211> 30
 <212> DNA
 <213> Homo sapiens
 <400> 19

cagtggaatg tgctctaagt accgaagtg 30

50 <210> 20
 <211> 30
 <212> DNA
 <213> Homo sapiens

55 <400> 20
 acactgtgtg tcagcatcat aaatctgtcc 30

<210> 21

EP 1 344 821 B9

<211> 22
 <212> DNA
 <213> Homo sapiens

5 <400> 21
 caacgaattt ggctacagca ac 22

<210> 22
 <211> 22
 10 <212> DNA
 <213> Homo sapiens

<400> 22
 15 ctacatggca actgtgagga gg 22

<210> 23
 <211> 11
 <212> PRT
 <213> Artificial Sequence

20 <220>
 <223> Description of Artificial Sequence: an artificially synthesized sequence containing a restriction enzyme Not
 I recognition sequence and a FLAG tag sequence

25 <400> 23

30 Ala Ala Ala Asp Tyr Lys Asp Asp Asp Asp Lys
 1 5 10

<210> 24
 <211> 5
 <212> PRT
 35 <213> Homo sapiens

<220>
 <221> misc_feature
 <222> (3)..(4)
 40 <223> Xaa means an arbitrary amino acid.

<400> 24

45 His Glu Xaa Xaa His
 1 5

<210> 25
 <211> 5
 50 <212> PRT
 <213> Homo sapiens

<400> 25

55 Asn Ile Thr Gly Glu
 1 5

<210> 26
 <211> 5
 <212> PRT
 <213> Homo sapiens

<400> 26

Ala Arg Gly Ser Val
 1 5

Claims

1. A polypeptide exhibiting an aggrecanase activity and comprising (1) the amino acid sequence of SEQ ID No: 2 or (2) an amino acid sequence in which 1 to 10 amino acids are deleted, substituted, and/or inserted in the amino acid sequence of SEQ ID No: 2.
2. The polypeptide according to claim 1, comprising the amino acid sequence of SEQ ID No: 2, and exhibiting an aggrecanase activity.
3. A polypeptide comprising an amino acid sequence having a 90% or more homology with the amino acid sequence of SEQ ID No: 2, and exhibiting an aggrecanase activity.
4. A polypeptide consisting of the amino acid sequence of SEQ ID No: 2.
5. A polynucleotide encoding the polypeptide according to any one of claims 1 to 4.
6. An expression vector comprising the polynucleotide according to claim 5.
7. A cell transfected with the expression vector according to claim 6.
8. A method for detecting whether or not a compound to be tested inhibits an aggrecanase activity of a polypeptide according to any one of claims 1 to 4, comprising the steps of:
 - bringing into contact (1) said polypeptide, (2) a substrate polypeptide capable of digesting with aggrecanase, and (3) the compound to be tested and
 - analysing the digestion of the substrate polypeptide.
9. A method for screening for a substance which inhibits an aggrecanase activity of a polypeptide according to any one of claims 1 to 4, comprising the steps of:
 - detecting by the method according to claim 8 and
 - selecting a substance inhibiting an aggrecanase activity.
10. A method for screening for a substance according to claim 9, wherein the substance is for treating joint diseases.
11. An antibody or a fragment thereof, which binds to the polypeptide according to any one of claims 1 to 4.

Patentansprüche

1. Polypeptid, das Aggrecanase-Aktivität aufweist und (1) die Aminosäuresequenz in SEQ ID NO: 2 oder (2) eine Aminosäuresequenz, in der 1 bis 10 Aminosäuren deletiert, substituiert und/oder in der Aminosäuresequenz in SEQ ID NO: 2 inseriert sind, umfaßt.
2. Polypeptid gemäß Anspruch 1, umfassend die Aminosäuresequenz nach SEQ ID NO: 2 und Aggrecanase-Aktivität

aufweisend.

3. Polypeptid, umfassend eine Aminosäuresequenz mit einer 90 %igen oder größer Homologie mit der Aminosäuresequenz in SEQ ID NO: 2, das Aggrecanase-Aktivität aufweist.

4. Polypeptid, bestehend aus der Aminosäuresequenz in SEQ ID NO: 2.

5. Polynukleotid, codierend als Polypeptid gemäß irgendeinem der Ansprüche 1 bis 4.

6. Expressionsvektor, umfassend das Polynukleotid gemäß Anspruch 5.

7. Zelle, transfiziert mit dem Expressionsvektor gemäß Anspruch 6.

8. Verfahren zum Nachweisen, ob eine Verbindung, die getestet werden soll, die Aggrecanase-Aktivität eines Polypeptid gemäß irgendeinem der Ansprüche 1 bis 4 inhibiert, umfassend die Schritte:

Inkontaktbringen (1) des Polypeptids, (2) eines Substratpolypeptids, das in der Lage ist, durch Aggrecanase verdaut zu werden, und (3) der zu testenden Verbindung und Analysieren des Verdaus des Substratpolypeptids.

9. Verfahren zum Durchmustern nach einer Substanz, die die Aggrecanase-Aktivität eines Polypeptids gemäß irgendeinem der Ansprüche 1 bis 4 inhibiert, umfassend die Schritte:

Nachweisen durch das Verfahren gemäß Anspruch 8 und
Auswählen einer Substanz, die die Aggrecanase-Aktivität inhibiert.

10. Verfahren zum Durchmustern nach einer Substanz gemäß Anspruch 9, wobei die Substanz zur Behandlung von Gelenkerkrankungen dient.

11. Antikörper oder Fragment davon, der an das Polypeptid gemäß irgendeinem der Ansprüche 1 bis 4 bindet.

Revendications

1. Polypeptide présentant une activité aggrécanase et comprenant (1) la séquence d'acides aminés de SEQ ID No. 2 ou (2) une séquence d'acides aminés dans laquelle 1 à 10 acides aminés sont délétés, substitués et/ou insérés dans la séquence d'acides aminés de SEQ ID No. 2.

2. Polypeptide selon la revendication 1, comprenant la séquence d'acides aminés de SEQ ID No. 2, et présentant une activité aggrécanase.

3. Polypeptide comprenant une séquence d'acides aminés ayant une homologie de 90 % ou plus avec la séquence d'acides aminés de SEQ ID No. 2, et présentant une activité aggrécanase.

4. Polypeptide constitué de la séquence d'acides aminés de SEQ ID No. 2.

5. Polynucléotide codant pour le polypeptide selon l'une quelconque des revendications 1 à 4.

6. Vecteur d'expression comprenant le polynucléotide selon la revendication 5.

7. Cellule transfectée avec le vecteur d'expression selon la revendication 6.

8. Procédé pour détecter si oui ou non un composé à tester inhibe une activité aggrécanase d'un polypeptide selon l'une quelconque des revendications 1 à 4, comprenant les étapes de :

mettre en contact (1) ledit polypeptide, (2) un polypeptide substrat capable d'être digéré avec l'aggrécanase, et (3) le composé à tester et analyser la digestion du polypeptide substrat.

- 9.** Procédé pour le criblage d'une substance qui inhibe une activité aggrécanase d'un polypeptide selon l'une quelconque des revendications 1 à 4, comprenant les étapes de :

détection par le procédé selon la revendication 8 et
sélection d'une substance inhibant une activité aggrécanase.

- 10.** Procédé pour le criblage d'une substance selon la revendication 9, dans lequel la substance est pour traiter des maladies des articulations.

- 11.** Anticorps ou fragment de celui-ci, qui se lie au polypeptide selon l'une quelconque des revendications 1 à 4.

REFERENCES CITED IN THE DESCRIPTION

This list of references cited by the applicant is for the reader's convenience only. It does not form part of the European patent document. Even though great care has been taken in compiling the references, errors or omissions cannot be excluded and the EPO disclaims all liability in this regard.

Patent documents cited in the description

- WO 03027282 A [0008]
- WO 9528963 A [0064]
- JP 2000384300 A [0118]

Non-patent literature cited in the description

- **ELDERS M. J.** *J. Rheumatol.*, 2000, vol. 27 (60), 6-8 [0002]
- **DIEPPE P.** *Scand. J. Rheumatol.*, 2000, vol. 29, 279-281 [0002]
- **GREENWALD R. A.** *Ann. New York Acad. Sci.*, 1999, vol. 878, 413-419 [0004]
- **SANDY J.D. et al.** *J. Clin. Invest.*, 1992, vol. 89, 1512-1516 [0005]
- **LOHMANDER L.S. et al.** *Arthritis Rheum.*, 1993, vol. 36, 1214-1222 [0005]
- **DINGLE L.T. et al.** *Ann. Rheum. Dis.*, 1975, vol. 34, 303-311 [0005]
- **CAWSTON T.E. et al.** *Biochem. Biophys. Res. Comm.*, 1995, vol. 215, 377-385 [0005]
- **KOZACI L.D. et al.** *Arthritis Rheum.*, 1997, vol. 40, 164-174 [0005]
- **VAN MEURS J.B. et al.** *Arthritis Rheum.*, 1999, vol. 42, 1128-1139 [0005]
- **TORTORELLA M. D. et al.** *Science*, 1999, vol. 284, 1664-1666 [0006]
- **ABBASZADE I. et al.** *J. Biol. Chem.*, 1999, vol. 274, 23443-23450 [0006]
- **FLANNERY C. R. et al.** *Biochem. Biophys. Res. Commun.*, 1999, vol. 260, 318-322 [0006]
- **CHAPMAN K. et al.** *Am. J. Hum. Genet.*, 1999, vol. 65, 167-174 [0007]
- **LOUGHLIN J. et al.** *Am. J. Hum. Genet.*, 1999, vol. 65, 1795-1798 [0007]
- **HUGHES C. E. et al.** *Biochem. J.*, 1995, vol. 305, 799-804 [0016]
- **SAMBROOK, J. et al.** *Molecular Cloning-A Laboratory Manual*. Cold Spring Harbor Laboratory, 1989 [0016] [0038] [0050]
- **SAIKI, R. K. et al.** *Science*, 1988, vol. 239, 487-491 [0016]
- **MARK, D. F. et al.** *Proc. Natl. Acad. Sci. USA*, 1984, vol. 81, 5662-5666 [0016] [0040]
- **ALTSCHUL, S. F. et al.** *J. Mol. Biol.*, 1990, vol. 215, 403-410 [0018]
- **TATIANA A. TATUSOVA ; THOMAS L. MADDEN.** *FEMS Microbiol. Lett.*, 1999, vol. 174, 247-250 [0018]
- **EFSTRATIADIS, A. et al.** *Cell*, 1976, vol. 7, 279-288 [0027]
- **LAND, H. et al.** *Nucleic Acids Res.*, 1981, vol. 9, 2251-2266 [0027]
- **YOO, O. J. et al.** *Proc. Natl. Acad. Sci. USA*, 1983, vol. 79, 1049-1053 [0027]
- **OKAYAMA, H. ; BERG, P.** *Mol. Cell. Biol.*, 1982, vol. 2, 161-170 [0027]
- **HANAHAN, D. J.** *Mol. Biol.*, 1983, vol. 166, 557-580 [0028]
- **HUNKAPILLER, M. et al.** *Nature*, 1984, vol. 10, 105-111 [0040]
- **CRANTHAM, R. et al.** *Nucleic Acids Res.*, 1981, vol. 9, r43-r74 [0040]
- **MAXAM, A. M. ; GILBERT, W.** *Methods in Enzymology*, 1980, vol. 65, 499-559 [0041]
- **MESSING, J. ; VIEIRA, J.** *Gene*, 1982, vol. 19, 269-276 [0041]
- **GLUZMAN, Y.** *Cell*, 1981, vol. 23, 175-182 [0045]
- **URLAUB, G. ; CHASIN, L. A.** *Proc. Natl. Acad. Sci. USA*, 1980, vol. 77, 4216-4220 [0045]
- **SUBRAMANI, S. et al.** *Mol. Cell. Biol.*, 1981, vol. 1, 854-864 [0046]
- **MIZUSHIMA, S. ; NAGATA, S.** *Nucleic Acids Res.*, 1990, vol. 18, 5322 [0046] [0048]
- **MARUYAMA, K. ; TAKEBE, Y.** *Med. Immunol.*, 1990, vol. 20, 27-32 [0048]
- **SEED, B.** *Nature*, 1987, vol. 329, 840-842 [0048]
- **LUTHMAN, H. ; MAGNUSSON, G.** *Nucleic Acids Res.*, 1983, vol. 11, 1295-1308 [0049]
- **GRAHAM, F. L. ; VANDERED, A. J.** *Virology*, 1973, vol. 52, 456-457 [0049]
- **NEUMANN, E. et al.** *EMBO J.*, 1982, vol. 1, 841-845 [0049]
- **SOUTHERN, P. J. ; BERG, P. J.** *Mol. Appl. Genet.*, 1982, vol. 1, 327-341 [0050]
- Shin Seikagaku Jikken Koza 18. Saibou Baiyou Gi-jyutsu. Tokyo Kagaku Dojin, 1990 [0051]
- **OKADA, M. ; MIYAZAKI K.** *Kaitei, Tanpakushitsu Jikken Noto, Jyo.Ge. Yodo-sha*, 1999 [0052]
- **TERRETT, N. K. et al.** *Tetrahedron*, 1995, vol. 51, 8135-8137 [0055]

EP 1 344 821 B9 (W1B1)

- **FELICI, F. et al.** *J. Mol. Biol.*, 1991, vol. 222, 301-310 [0055]
- **RAZ, E. et al.** *Proc. Natl. Acad. Sci. USA*, 1994, vol. 91, 9519-9523 [0070]
- **DONNELLY, J. J. et al.** *J. Infect. Dis.*, 1996, vol. 173, 314-320 [0070]
- **KOHLER, G. ; MILSTEIN, C.** *Nature*, 1975, vol. 256, 495-497 [0072]
- **CLACKSON, T. et al.** *Nature*, 1991, vol. 352, 624-628 [0078]
- **ZEBEDEE, S. et al.** *Proc. Natl. Acad. Sci. USA*, 1992, vol. 89, 3175-3179 [0078]
- **LONBERG, N. et al.** *Nature*, 1994, vol. 368, 856-859 [0078]
- **SAMBROOK, J. et al.** *Molecular Cloning - A Laboratory Manual*. Cold Spring Harbor Laboratory, 1989 [0079]
- **DOEGE K. et al.** *Biochem. Soc. Trans.*, 1990, vol. 18, 200-202 [0092]
- **GUAN X-M. et al.** *J. Biol. Chem.*, 1992, vol. 267, 21995-21998 [0093]
- **HUGHES C.E. et al.** *Biochemical J.*, 1995, vol. 305, 799-804 [0096]
- **PITTENGER M. F. et al.** *Science*, 1999, vol. 284, 143-147 [0099]