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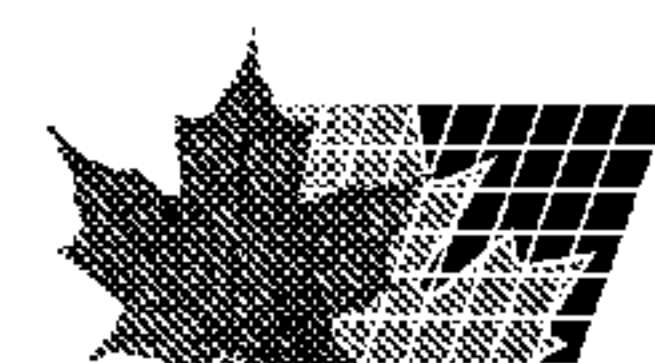
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(57) **Abrégé/Abstract:**

Novel aggrecanase proteins and the nucleotide sequences encoding them as well as processes for producing them are disclosed. Methods of identifying and developing inhibitors of the aggrecanase enzymes and antibodies to the enzymes for treatment of conditions characterized by the degradation of aggrecan are also disclosed.



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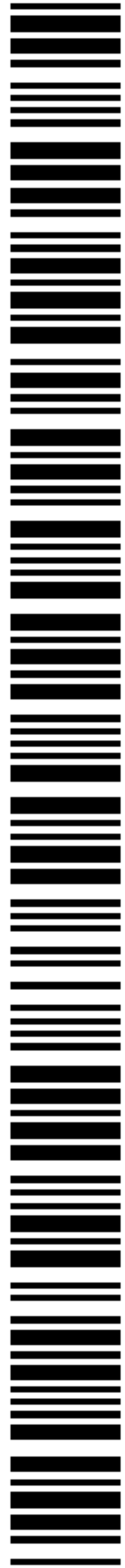
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(54) Title: AGGRECANASE MOLECULES

(57) Abstract: Novel aggrecanase proteins and the nucleotide sequences encoding them as well as processes for producing them are disclosed. Methods of identifying and developing inhibitors of the aggrecanase enzymes and antibodies to the enzymes for treatment of conditions characterized by the degradation of aggrecan are also disclosed.



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AGGRECANASE MOLECULES

RELATED APPLICATION

[001] This application relies on the benefit of priority of U.S. provisional patent application No. 60/353,680, filed on January 31, 2002, the entire disclosure of which is incorporated by reference.

FIELD OF THE INVENTION

[002] The present invention relates to the discovery of nucleotide sequences encoding novel aggrecanase molecules, aggrecanase proteins and fragments thereof, and processes for producing them. The invention further relates to identification and development of inhibitors of and antibodies to the aggrecanase enzymes. These inhibitors and antibodies may be useful for the treatment of various aggrecanase-associated conditions including osteoarthritis.

BACKGROUND OF THE INVENTION

[003] Aggrecan is a major extracellular component of articular cartilage. It is a proteoglycan responsible for providing cartilage with its mechanical properties of compressibility and elasticity. The loss of aggrecan has been implicated in the degradation of articular cartilage in arthritic diseases. Osteoarthritis is a debilitating disease which affects at least 30 million Americans (MacLean *et al.*, *J Rheumatol* 25:2213-8 (1998)). Osteoarthritis can severely reduce quality of life due to degradation of articular cartilage and the resulting chronic pain. An early and important characteristic of the osteoarthritic process is loss of aggrecan from the extracellular matrix (Brandt and Mankin, *Pathogenesis of Osteoarthritis*, in Textbook of Rheumatology, WB Saunders Company, Philadelphia, PA, at 1355-1373 (1993)). The large, sugar-containing portion of aggrecan is thereby lost from the extra-cellular matrix, resulting in deficiencies in the biomechanical characteristics of the cartilage.

[004] A proteolytic activity termed "aggrecanase" is believed to be responsible for the cleavage of aggrecan thereby having a role in cartilage degradation associated with osteoarthritis and inflammatory joint disease. Research has been conducted to identify the enzymes responsible for the degradation of aggrecan in human osteoarthritic cartilage. At least two enzymatic cleavage sites have been identified within the interglobular domain of aggrecan. One enzymatic cleavage site within the interglobular domain of aggrecan (Asn³⁴¹-Phe³⁴²) has been observed to be cleaved by several known metalloproteases. Flannery *et al.*, *J Biol Chem* 267:1008-14 (1992); Fosang *et al.*, *Biochemical J.* 304:347-351 (1994). Cleavage at a second aggrecan cleavage site within aggrecan (Glu³⁷³-Ala³⁷⁴) due to IL-1 induced cartilage aggrecan cleavage results in the generation of an aggrecan fragment found in human synovial fluid (Sandy *et al.*, *J Clin Invest* 69:1512-1516 (1992); Lohmander *et al.*, *Arthritis Rheum* 36: 1214-1222 (1993); Sandy *et al.*, *J Biol Chem* 266: 8683-8685 (1991)). Aggrecan cleavage at (Glu³⁷³-Ala³⁷⁴) has been attributed to aggrecanase activity (Sandy *et al.*, *J Clin Invest* 69:1512-1516 (1992). This Glu³⁷³-Ala³⁷⁴ cleavage site will be referred to as the aggrecanase cleavage site.

[005] Recently, identification of two enzymes, aggrecanase-1 (ADAMTS-4) and aggrecanase-2 (ADAMTS-11) within the "Disintegrin-like and Metalloprotease with Thrombospondin type 1 motif" (ADAMTS) family have been identified which are synthesized by IL-1 stimulated cartilage and cleave aggrecan at the Glu³⁷³-Ala³⁷⁴ site (Tortorella *et al.*, *Science* 284:1664-6 (1999); Abbaszade *et al.*, *J Biol Chem* 274: 23443-23450 (1999)). It is possible that these enzymes could be synthesized by osteoarthritic human articular cartilage. It is also contemplated that there are other, related enzymes in the ADAMTS family which are capable of cleaving aggrecan at the Glu³⁷³-Ala³⁷⁴ bond and could contribute to aggrecan cleavage in osteoarthritis. Therefore, there is a need to identify various aggrecanase enzymes and determine ways to block their enzymatic activity.

SUMMARY OF THE INVENTION

[006] The present invention is directed to the identification of novel aggrecanase protein molecules capable of cleaving aggrecan, nucleotide sequences which encode the aggrecanase enzymes, and processes for the production of aggrecanases. These enzymes are contemplated to be characterized as having proteolytic aggrecanase activity. The invention further includes compositions comprising these enzymes.

[007] The invention also includes antibodies to these enzymes, in one embodiment, for example, antibodies that block aggrecanase activity. In addition, the invention includes methods for identifying and developing inhibitors of aggrecanase which block the enzyme's proteolytic activity. These inhibitors and antibodies may be used in various assays and therapies for treatment of conditions characterized by the degradation of articular cartilage. This invention provides nucleotide molecules that encode novel aggrecanase proteins. Accordingly, in one embodiment, the invention features an isolated DNA molecule comprising a DNA sequence chosen from: nucleotide #1 to nucleotide #3663 of SEQ ID NO: 1 (Figure 1A and 1B); fragments of SEQ ID NO: 1 which encode polypeptides or proteins that exhibit aggrecanase activity; variants of SEQ ID NO: 1 that encode proteins or polypeptides that exhibit aggrecanase activity, and fragments thereof; sequences which hybridize under stringent conditions with SEQ ID NO: 1; naturally occurring human allelic sequences; and equivalent degenerative codon sequences

[008] In another aspect, the invention comprises an isolated aggrecanase protein comprising an amino acid sequence chosen from: amino acid #1 (methionine) to amino acid #1221 (isoleucine) of SEQ ID NO: 2 (Figure 2); fragments of SEQ ID NO: 2 which exhibit aggrecanase activity, and variants and fragments of aggrecanase proteins that exhibit proteolytic activity, including deletion and substitution mutants. In yet another aspect, the invention provides methods for producing an isolated aggrecanase protein. One such method includes (1) transforming a host cell with a DNA sequence,

such as the DNA sequence depicted in SEQ ID NO: 1 ; (2) culturing the host cell; and (3) purifying the aggrecanase enzyme set forth in SEQ ID NO: 2 that is encoded by the DNA sequence, from the cell culture medium.

[009] The invention also provides antibodies that bind to isolated aggrecanase proteins of the invention. In one embodiment, such an antibody reduces, inhibits or antagonizes aggrecanase activity. The invention further provides methods for developing and identifying inhibitors of aggrecanase activity comprising the use of aggrecanase protein chosen from SEQ ID NO: 2 or a fragment or a variant thereof. In one embodiment, inhibitors of aggrecanase activity prevent cleavage of aggrecan.

[010] Additionally, the invention provides pharmaceutical compositions for inhibiting the proteolytic activity of aggrecanase, wherein the compositions comprise at least one antibody according to the invention and at least one pharmaceutical carrier. The invention also provides methods for inhibiting aggrecanase activity in a mammal comprising administering to the mammal an effective amount of a pharmaceutical composition according to the invention to inhibit aggrecanase activity.

[011] Additional aspects of the disclosure will be set forth in part in the description, and in part be obvious from the description, or may be learned from practicing the invention. The invention is set forth and particularly pointed out in the claims, and the disclosure should not be construed as limiting the scope of the claims. The following detailed description includes exemplary representations of various embodiments of the invention, which are not restrictive of the invention as claimed. The accompanying figures constitute a part of this specification and, together with the description, serve to illustrate embodiments and not limit the invention.

BRIEF DESCRIPTION OF THE FIGURES AND SEQUENCES

[012] Figures 1A and 1B show the full-length nucleotide sequence for ADAMTS-18 (EST18). (SEQ ID NO: 1)

[013] Figure 2 shows the full-length amino acid sequence for ADAMTS-18, based on the nucleotide sequence of SEQ ID NO: 1. (SEQ ID NO: 2)

[014] Figures 3A and 3B show a nucleotide sequence of ADAMTS-18 (EST18). (SEQ ID NO: 3).

[015] Figure 4 shows the predicted amino acid sequence of ADAMTS-18 based on the nucleotide sequence of SEQ ID NO: 3 (SEQ ID NO: 4).

[016] Figures 5A and 5B show a virtual nucleotide sequence for ADAMTS-18, which was identified by Celera database-mining techniques. (SEQ ID NO: 5).

[017] Figure 6A shows a schematic representation of the PCR primers used for amplification of fragments of a EST18 nucleotide sequence. Figure 6B shows a schematic representation of the overlapping nucleotide sequence fragments of EST18 including sites for restriction enzymes.

[018] Figure 7 shows a nucleotide sequence encoding for a truncated form of ADAMTS-18 linked to a Streptavidin-tag. (SEQ ID NO: 7).

[019] Figure 8 shows an amino acid sequence for a truncated form of ADAMTS-18 including a Streptavidin-tag, based on SEQ ID NO: 7. (SEQ ID NO: 8)

[020] Figure 9 shows a schematic representation of the hydrophobic plot generated for the protein of SEQ ID NO: 2 using the GCG plotstructure program.

[021] Figure 10 shows a schematic representation of an assay for detecting aggrecanase activity.

DETAILED DESCRIPTION OF THE INVENTION

I. Definitions

[022] In order that the present invention may be more readily understood, certain terms are first defined. Additional definitions are set forth throughout the detailed description.

[023] The term “**aggrecanase**” refers to a family of polypeptides that are capable of cleaving the aggrecan protein. Generally, these are proteins that cleave aggrecan at the Glu³⁷³-Ala³⁷⁴ aggrecanase cleavage site. Aggrecanases of the present invention encompass but are not limited to the amino acid sequence of SEQ ID NO: 2. The term “aggrecanase” includes naturally occurring variants of the amino acid sequence set forth in SEQ ID NO: 2, as well as fragments of SEQ ID NO: 2 that are active in one or more of the assays provided. For example, included in this definition are amino acid sequences substantially similar or substantially identical to the amino acid of SEQ ID NO: 2 or a fragment thereof; or an amino acid sequence at least about 50%, about 55%, about 60%, about 65%, about 70%, about 75%, about 80%, about 85%, about 90%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98% or about 99% identical to the amino acid sequence of SEQ ID NO: 2, or a fragment thereof. The term “aggrecanase” further includes the proteins encoded by the nucleic acid sequence of SEQ ID NO: 1 disclosed, fragments and variants thereof. In one embodiment, the nucleic acids of the present invention will possess a sequence which is either derived from, or is a variant of a natural aggrecanase encoding gene, or a fragment thereof.

[024] The term “**aggrecanase activity**” refers to at least one cellular process interrupted or initiated by an aggrecanase enzyme binding to aggrecan. Generally, activity refers to proteolytic cleavage of aggrecan by aggrecanase. Aggrecanase activities include, but are not limited to, binding of aggrecanase to aggrecan and cleavage of aggrecan by aggrecanase. Activity

can also include a biological response resulting from the binding to or cleavage of aggrecan by aggrecanases of the invention.

[025] The term “**antibody**” refers to an immunoglobulin or a fragment thereof, and encompasses any polypeptide comprising an antigen-binding site. The term includes but is not limited to polyclonal, monoclonal, monospecific, polyspecific, non-specific, humanized, human, single-chain, chimeric, synthetic, recombinant, hybrid, mutated, grafted, and *in vitro* generated antibodies. It also includes, unless otherwise stated, antibody fragments such as Fab, F(ab')₂, Fv, scFv, Fd, dAb, and other antibody fragments which retain the antigen binding function.

[026] The term “**effective amount**” refers to a dosage or amount of a composition at least one aggrecanase inhibitor or antibody of the invention that is sufficient to treat a patient.

[027] The term “**inhibit**” or “**inhibition**” of aggrecanase or aggrecanase activity refers to a reduction, inhibition or otherwise diminution of at least one activity of aggrecanase due to binding of an inhibitor to the aggrecanase or aggrecan. The reduction, inhibition or diminution of binding can be measured by one of many assays provided. Inhibition of aggrecanase activity does not necessarily indicate a complete negation of aggrecanase activity. A reduction in activity can be, for example, at least about 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or more. In one embodiment, inhibition is measured by a reduction in the detection of cleavage products of aggrecan.

[028] The term “**isolated**” describes a nucleic acid molecule or polypeptide molecule that is substantially free of its natural environment. For instance, an isolated protein is substantially free of cellular material or other contaminating proteins from the cell or tissue source from which it is derived. The term “isolated” also refers to an aggrecanase protein according to the invention which is free from association with other proteases and retains aggrecanase proteolytic activity. In addition, the term “isolated” refers to

nucleic acid molecules that encode aggrecanases of the invention and are free from other cellular material and contaminants.

[029] The term “**neoepitope antibody**” refers to an antibody that specifically recognizes a new N- or C-terminal amino acid sequence generated by proteolytic cleavage but which does not bind to such an epitope on the intact (uncleaved) substrate.

[030] The term “**operative association**” with an expression control sequence generally refers to the presence of a specific nucleotide sequence or sequences that control or affect transcription rate or efficiency of a nucleotide molecule linked to the sequence. For example, a promoter sequence that is located proximally to the 5' end of an aggrecanase coding nucleotide sequence may be in operative association with the aggrecanase encoding nucleotide sequence. Expression control sequences include, but are not limited to, for example, promoters, enhancers, and other expression control sequences, or any combination of such elements, either 5' or 3' to an aggrecanase encoding nucleotide sequence in order to control its expression. Not all of these elements are required, however. A skilled artisan can select the appropriate expression control sequences, for example, depending on desired expression levels for the aggrecanases of the invention.

[031] The term “**specific binding**” of an antibody means that the antibody binds to at least one novel aggrecanase molecule of the present invention and the antibody will not show any significant binding to molecules other than at least one novel aggrecanase molecule. The term is also applicable where, e.g., an antigen binding domain of an antibody is specific for a particular epitope, which is represented on a number of antigens, and the specific binding member (the antibody) carrying the antigen binding domain will be able to bind to the various antigens carrying the epitope. Therefore, it is contemplated that an antibody of the invention will bind to an epitope on multiple novel aggrecanase proteins. Typically, the binding is considered specific when the affinity constant K_a is higher than 10^8 M^{-1} . An

antibody is said to "specifically bind" to an antigen if, under appropriately selected conditions, such binding is not substantially inhibited, while at the same time non-specific binding is inhibited. The conditions are usually defined in terms of concentration of antibodies, ionic strength of the solution, temperature, time allowed for binding, concentration of additional molecules associated with the binding reaction (e.g., serum albumin, milk casein), etc. Such conditions are well known in the art, and a skilled artisan using routine techniques can select appropriate conditions.

[032] The term "**highly stringent**" or "**high stringency**" describes conditions for hybridization and washing used for determining nucleic acid-nucleic acid interactions. Nucleic acid hybridization will be affected by such conditions as salt concentration, temperature, or organic solvents, in addition to the base composition, length of the complementary strands, and the number of nucleotide base mismatches between the hybridizing nucleic acids, as will be readily appreciated by those skilled in the art. The stringency conditions are dependent on the length of the nucleic acid and the base composition of the nucleic acid and can be determined by techniques well known in the art. Generally, stringency can be altered or controlled by, for example, manipulating temperature and salt concentration during hybridization and washing. For example, a combination of high temperature and low salt concentration increases stringency. Such conditions are known to those skilled in the art and can be found in, for example, "Current Protocols in Molecular Biology," John Wiley & Sons, N.Y. (1989), 6.3.1-6.3.6. Both aqueous and nonaqueous conditions as described in the art can be used. One example of highly stringent hybridization conditions is hybridization in 6X sodium chloride/sodium citrate (SSC) at about 45°C, followed by at least one wash in 0.2X SSC, 0.1% SDS at 50°C. A second example of highly stringent hybridization conditions is hybridization in 6X SSC at about 45°C, followed by at least one wash in 0.2X SSC, 0.1% SDS at 55°C. Another example of highly stringent hybridization conditions is hybridization in 6X SSC at about

45°C, followed by at least one wash in 0.2X SSC, 0.1% SDS at 60°C. A further example of highly stringent hybridization conditions is hybridization in 6X SSC at about 45°C, followed by at least one wash in 0.2X SSC, 0.1% SDS at 65°C. Highly stringent conditions include hybridization in 0.5M sodium phosphate, 7% SDS at 65°C, followed by at least one wash at 0.2X SSC, 1% SDS at 65°C.

[033] The phrase "**moderately stringent**" or "**moderate stringency**" hybridization refers to conditions that permit a nucleic acid to bind a complementary nucleic acid that has at least about 60%, at least about 75%, or at least about 85%, identity to the nucleic acid; with greater than about 90% identity to the nucleic acid especially preferred. Moderately stringent conditions comprise but are not limited to, for example, hybridization in 50% formamide, 5X Denhart's solution, 5X SSPE, 0.2% SDS at 42°C., followed by washing in 0.2 X SSPE, 0.2% SDS, at 65°C. (see, e.g., Sambrook *et al.*, *Molecular Cloning A Laboratory Manual*, Cold Spring Harbor Laboratory Press, 1989).

[034] The phrase "**substantially identical**" or "**substantially similar**" means that the relevant amino acid or nucleotide sequence will be identical to or have insubstantial differences (through conserved amino acid substitutions) in comparison to the sequences which are disclosed. Nucleotide and polypeptides of the invention include, for example, those that are at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99% identical in sequence to nucleic acid molecules and polypeptides disclosed.

[035] For polypeptides, at least 20, 30, 50, 100, or more amino acids will be compared between the original polypeptide and the variant polypeptide that is substantially identical to the original. For nucleic acids, at least 50,

100, 150, 300 or more nucleotides will be compared between the original nucleic acid and the variant nucleic acid that is substantially identical to the original. Thus, a variant could be substantially identical in a region or regions, but divergent in others, while still meeting the definition of "substantially identical." Percent identity between two sequences is determined by standard alignment algorithms such as, for example, Basic Local Alignment Tool (BLAST) described in Altschul et al., J. Mol. Biol., 215:403-410 (1990), the algorithm of Needleman et al., J. Mol. Biol., 48:444-453 (1970), or the algorithm of Meyers et al., Comput. Appl. Biosci., 4:11-17 (1988).

[036] The term "**treating**" or "**treatment**" refers to both therapeutic treatment and prophylactic or preventative measures. Those in need of treatment may include individuals already having a particular medical disorder as well as those who may ultimately acquire the disorder (*i.e.*, those needing preventative measures). Treatment may regulate aggrecanase activity or the level of aggrecanase to prevent or ameliorate clinical symptoms of at least one diseases. The inhibitors and/or antibodies may function by, for example, preventing the interaction or binding of aggrecanase to aggrecan, or by reducing or inhibiting aggrecanase activity.

[037] The term "**variant**" refers to nucleotide and amino acid sequences that are substantially identical or similar to the nucleotide and amino acid sequences provided, respectively. Variants can be naturally occurring, for example, naturally occurring human and non-human nucleotide sequences that encode aggrecanase or aggrecanase-like proteins, or be generated artificially. Examples of variants are aggrecanases resulting from alternative splicing of the aggrecanase mRNA, including both 3' and 5' spliced variants of the aggrecanases of the invention, point mutations and other mutations, or proteolytic cleavage of the aggrecanase protein. Variants of aggrecanases of the invention include nucleic acid molecules or fragments thereof and amino acid sequences and fragments thereof, that are substantially identical or similar to other nucleic acids (or their complementary

strands when they are optimally aligned (with appropriate insertions or deletions) or amino acid sequences respectively. In one embodiment, there is at least about 50% identity, at least about 55% identity, at least about 60% identity, at least about 65% identity, at least about 70% identity, at least about 75% identity, at least about 80% identity, at least about 85% identity, at least at least about 90%, at least about 92% identity, at least about 93% identity, at least about 94% identity, at least about 95% identity, at least about 96% identity, at least about 97% identity, at least about 98% identity, or at least about 99% identity between a nucleic acid molecule or protein of the invention and another nucleic acid molecule or protein respectively, when optimally aligned. Additionally, variants include proteins or polypeptides that exhibit aggrecanase activity, as defined.

[038] To assist in the identification of the sequences listed in the specification and figures, the following table (Table 1) is provided, which lists the SEQ ID NOs, the figure location, and a brief description of each sequence.

Table 1

a.a. = amino acid

SEQUENCES	FIGURES	DESCRIPTION
SEQ ID NO: 1	Figures 1A and 1B	full-length nucleotide sequence of ADAMTS-18 (EST-18)
SEQ ID NO: 2	Figure 2	full-length a.a. sequence of ADAMTS-18 encoded by SEQ ID NO: 1
SEQ ID NO: 3	Figure 3A and 3B	a nucleotide sequence of ADAMTS-18 (EST18)
SEQ ID NO: 4	Figure 4	predicted a.a. sequence of ADAMTS-18 based on SEQ ID NO: 3
SEQ ID NO: 5	Figure 5A and 5B	virtual nucleotide sequence for ADAMTS-18
SEQ ID NO: 6		zinc binding signature

		region of aggrecanase-1
SEQ ID NO: 7	Figure 7	truncated EST18 nucleotide sequence including a Streptavidin tag
SEQ ID NO: 8	Figure 8	truncated a.a. sequence of EST18 protein including a Streptavidin tag encoded by SEQ ID NO: 7
SEQ ID NO: 9	Figure 6A	primer
SEQ ID NO: 10	Figure 6A	primer
SEQ ID NO: 11	Figure 6A	primer
SEQ ID NO: 12	Figure 6A	primer
SEQ ID NO: 13		peptide sequence
SEQ ID NO: 14		peptide sequence
SEQ ID NO: 15		CD-36 binding motif
SEQ ID NO: 16		primer
SEQ ID NO: 17		primer
SEQ ID NO: 18		primer
SEQ ID NO: 19		primer
SEQ ID NO: 20		primer
SEQ ID NO: 21		oligonucleotide
SEQ ID NO: 22		oligonucleotide
SEQ ID NO: 23		oligonucleotide
SEQ ID NO: 24		oligonucleotide
SEQ ID NO: 25		oligonucleotide
SEQ ID NO: 26		oligonucleotide
SEQ ID NO: 27		primer
SEQ ID NO: 28		primer
SEQ ID NO: 29		epitope tag
SEQ ID NO: 30		nucleotide insert
SEQ ID NO: 31		nucleotide sequence containing an XhoI site
SEQ ID NO: 32		a 68 base pair adapter nucleotide sequence
SEQ ID NO: 33		neoepitope sequence

II. Novel Aggrecanase Molecules

[039] In one embodiment, a nucleotide sequence of an aggrecanase molecule according to the present invention is set forth in SEQ ID NO: 1, including nucleotide #1 to nucleotide #3663 of SEQ ID NO: 1 (Figures 1A and 1B). The invention further includes equivalent degenerative codon sequences of the sequence set forth in SEQ ID NO: 1, as well as fragments and variants

thereof which encode proteins that exhibit aggrecanase activity. The nucleic acid sequences of the invention include both naturally occurring sequences and variants thereof and those that are artificially generated. Full length nucleotide sequences encoding the aggrecanase molecules of the present invention may be obtained in one embodiment, for example, by using the nucleotide sequence set forth in SEQ ID NO: 3 to design probes for screening for the full-length aggrecanase nucleotide sequence using standard techniques.

[040] The amino acid sequence of the isolated aggrecanase-like molecule is set forth in SEQ ID NO: 2, including amino acid #1 (methionine) to amino acid #1221 (isoleucine) of SEQ ID NO: 2 (Figure 2).

[041] The invention further includes fragments of the amino acid sequence which encode molecules exhibiting aggrecanase activity.

[042] The invention includes methods for obtaining full length aggrecanase molecules, the nucleotide sequences that encode aggrecanase molecules obtained by the methods and proteins encoded by the nucleotide sequences. Methods for isolation of the full length sequence include, for example, utilizing the aggrecanase nucleotide sequence set forth in SEQ ID NO: 3 (Figures 3A and 3B) for designing probes for screening, or otherwise screen for full-length nucleotide sequence using standard procedures known to those skilled in the art.

[043] The human aggrecanase protein or a fragment thereof may be produced by culturing a cell transformed with a DNA sequence chosen from SEQ ID NO: 1 and recovering and purifying from the culture medium a protein characterized by an amino acid sequence set forth in SEQ ID NO: 2, which is substantially free from other proteinaceous materials with which it is co-produced. For production in mammalian cells, the DNA sequence further comprises a DNA sequence encoding a suitable propeptide 5' to and linked in frame to the nucleotide sequence encoding an aggrecanase enzyme.

[044] Human aggrecanase proteins produced by methods of the invention are characterized by having the ability to cleave aggrecan and having an amino acid sequence chosen from SEQ ID NO: 2, variants of the amino acid sequence of SEQ ID NO: 2, including naturally occurring mutant proteins spliced products, and other variants, in which the proteins retain the ability to cleave aggrecan which is characteristic of aggrecanase proteins. These proteins may include a protein which is at least about 30% identical, about 35% identical, about 40% identical, about 45% identical, about 50% identical, about 55% identical, about 60% identical, about 65% identical, about 70% identical, about 75% identical, about 80% identical, about 85% identical, about 90% identical, about 92% identical, about 94% identical, about 95% identical, about 96% identical, about 97% identical, about 98% identical or about 99% identical, to the amino acid sequence shown in SEQ ID NO: 2. Finally, proteins including variations of the sequence depicted in SEQ ID NO: 2, including amino acid changes induced by mutagenesis, chemical alteration, or by alteration of DNA sequence used to produce the protein, whereby the peptide sequence still has aggrecanase activity, are also included in the present invention. The present invention also includes fragments of the amino acid sequence of SEQ ID NO: 2 which retain the activity of aggrecanase protein, and variants of the fragments as well.

III. Identification of Aggrecanase Proteins and DNA Molecules Encoding Them, and Variants Thereof.

[045] It is expected that there are additional human sequences that encode for aggrecanases or related proteins with aggrecanase activity and that other species also have DNA sequences encoding proteins that are variants of human aggrecanase enzymes. The invention, therefore, includes methods for obtaining DNA sequences encoding aggrecanase proteins and variants thereof, DNA sequences obtained by those methods, and proteins or polypeptides encoded by the DNA sequences. One such method entails utilizing a nucleotide sequence of the invention or portions thereof to design probes for screening libraries for the corresponding nucleotide sequence from

other species or coding sequences or fragments thereof using standard techniques. Thus, the present invention may include DNA sequences from other species, which encode aggrecanase or aggrecanase-like polypeptides or proteins, which can be obtained using the human aggrecanase nucleotide sequence. The present invention may also include functional fragments of the aggrecanase protein, and DNA sequences encoding such functional fragments, as well as functional fragments of related proteins with aggrecanase or aggrecanase-like activity. The ability of such a fragment to function like an aggrecanase is determinable by using the polypeptide or protein in one of many biological assays described for detecting activity of the aggrecanase protein.

[046] For example, SEQ ID NO: 1, set forth in Figures 1A and 1B, was used as a query against GenBank and GenSeq to find similar nucleotide sequences from humans. Several sequences were identified as being similar either to the full-length or partial nucleic acid sequence of SEQ ID NO: 1. The published sequences were identified by the following accession numbers: AJ311903; Ax319854 (sequence 18 from WO 01/183782); AC025284; AC010548; AC009139; AQ407949; AQ309991; AQ543125; AQ052241; Abn89277 (disclosed in WO 02/250258); G65591; G53009; BD040395; Abn89277; Aas97176; Aad16756; Aad16759; Abq79948; Aas65280; Aad16771; Aad16774; Aas75293; Aas65278; Aac16650; Aah36077; Aba11592; Aba15654; Aba15653; and Aba15655.

[047] In addition, SEQ ID NO: 1 was used to search a database BLASTX which includes translations of the genes in the Genbank database and the protein components of the GeneSeq database. The search revealed several human protein sequences which include sequences identified by the following accession numbers: GENESEQP:ABB81460 (disclosed in WO 02/250,258); Genbank:CAC83612; GENESEQP:AAU72893; GENESEQP:AAE09696; GENESEQP:AAE09699; GENESEQP:ABB82162;

GENESEQP:AAE09711; GENESEQP:ABG11106; GENESEQP:AAB08954; and GENESEQP:AAB08913.

[048] It is expected that similar sequences exist in non-human species that are likely to encode aggrecanases or aggrecanase-like proteins. Various non-human variants of the aggrecanase protein were identified by searching the BLASTX database using the nucleotide sequence set forth in SEQ ID NO: 1. These include, for example, BAC35556_1 (mouse); AAH34739_1 (mouse); BAC29190_1 (mouse); AAO17380_1 (mouse); BAC33391_1 (mouse); AAG29823_1 (rat); AAD34012_1 (rat); BAA11088_1 (mouse); BAA24501_1 (mouse); AAH40382_1 (mouse); CAA65253_1 (*Bos.taurus*); CAA93287_1 (*C. elegans*); AAF46065_2 (*D. melanogaster*); AAN17331_1 (*Equus caballus*); AAM50192_1 (*D. melanogaster*); AAF55199_2 (*D. melanogaster*); AAF25805_1 (mouse); AAG37995_1 (*D. melanogaster*); AAG41980_1 (mouse); AAD56356_1 (mouse); AAF56794_3 (*D. melanogaster*); AAF56795_3; GENESEQP:ABB71150 (*D. melanogaster*); GENESEQP:AAB72280 (mouse); GENESEQP:ABB62044 (*D. melanogaster*); GENESEQP:AAB72284 (mouse); GENESEQP:AAB21265 (mouse); GENESEQP:AAY53899 (mouse); GENESEQP:AAY53900 (bovine); GENESEQP:ABB60410 (*D. melanogaster*); GENESEQP:AAB50004 (bovine); GENESEQP:AAY53898 (*C. elegans*); GENESEQP:AAW47030 (bovine); GENESEQP:AAB72287(mouse); NR:25053113 (mouse); NR:20888361 (mouse); NR:23634336 (mouse); NR27721019 (rat); NR27688211 (rat); NR:27712734; NR: 20898418 (mouse); NR:27681743 (mouse); NR:21288693 (*Anopheles gambiae*); NR:27705982 (rat); NR:27693936 (rat); NR:27664306 (rat); NR:20861058 (mouse); NR:27681747 (rat); NR:27719839 (rat); NR:25056874 (mouse); and NR:25052431 (mouse).

[049] Several ESTs similar to the nucleotide sequence of SEQ ID NO: 1 are also published in Genbank, including the following accession numbers: AW295437; BF224279; BE674425; BF512077; AA057097; AA057097;

AA057408; AV730422; BM696215; BM664487; BG396090; BE253544; AA442575; and AA436819.

[050] It is contemplated, based on the results of the BLAST searches described that the EST18 mRNA is expressed at least in carcinoid tissue, retinoblastoma, retina, testis, hypothalamus, kidney and the brain. Additionally, the gene for EST18 is speculated to be located on chromosome 16 in humans.

[051] The full-length EST18 sequence, set forth in SEQ NO: 1, was further used to search a genomic sequence database provided by Celera for spliced variants of the EST18 mRNA, including, for example, both 5' and 3' spliced variants. Some of the putative spliced variants are identified by accession numbers: Geneseq:aac16650; Geneseq:aah36077; Geneseq:aas65278; Geneseq:aas65279; Geneseq:aas65280; Geneseq:aas97176; Genbank:AJ311903; and Genbank:AX319854. Sequence alignments of these sequences with the EST18 nucleotide sequence suggests that majority of the spliced variants described herein have differences at the 3' ends.

[052] The Celera single nucleotide polymorphism database was searched with the sequence set forth in SEQ ID NO: 1. The table below summarizes the results of such a search, which lists the genetic variations found within the EST18 sequence, for example, across different races and ethnicities in humans.

Table 2

SNP name	Source	Allele	Protein Variation	Location
hCV3284477	Celera	T/C		Intron
hCV3284476	Celera	G/A	Cys(TGC)1057Cys(TGT)	Silent Mutation
hCV11516846	Celera	A/-		Intron
hCV3284474	Celera	A/T		Intron
hCV3284473	Celera	A/G		Intron
hCV3284472	Celera	T/G		Intron
hCV9478412	dbSNP	A/C		Intron

SNP name	Source	Allele	Protein Variation	Location
hCV3284471	Celera	C/G		Intron
hCV3284470	Celera	T/A		Intron
hCV3284469	Celera	T/C		Intron
hCV3284468	Celera	C/T		Intron
hCV3284467	Celera	A/G		Intron
hCV3284466	Celera	T/C	Val(GTA)986Val(GTG)	Silent Mutation
hCV3284465	Celera	C/A	Ala(GCC)955Ser(TCC)	Mis-sense Mutation
hCV3284464	Celera	A/G		Intron
hCV3284463	Celera	G/C		Intron
hCV3284462	Celera	T/C		Intron
hCV11516852	Celera	-/T		Intron
hCV3284461	Celera	T/C		Intron
hCV3284460	Celera	C/T		Intron
hCV16210086	dbSNP	G/A		Intron
hCV11937057	dbSNP	C/T		Intron
hCV11937062	dbSNP	C/T		Intron
hCV9602010	dbSNP	A/G		Intron
hCV9602009	dbSNP	A/G		Intron
hCV9602008	dbSNP	T/C		Intron
hCV9602001	dbSNP	T/G T/G T/G		Intron
hCV11937070	dbSNP	T/C		Intron
hCV2852198	Celera	C/A		Intron
hCV2852197	Celera	A/G		Intron
hCV2828126	Celera	C/A		Intron
hCV2828125	Celera	T/C		Intron
hCV2828124	Celera	G/C		Intron
hCV2828123	Celera	T/C		Intron
hCV7606027	dbSNP	T/C		Intron
hCV7606023	dbSNP	G/A		Intron
hCV7606022	dbSNP	T/C		Intron
hCV2828122	Celera	T/-		Intron
hCV2828121	Celera	C/T		Intron
hCV11935339	dbSNP	G/A		Intron
hCV16018212	dbSNP	T/G		Intron
hCV2828119	dbSNP Celera	G/A A/G G/A		Intron
hCV2828118	dbSNP Celera	A/T T/A T/A T/A		Intron
hCV2381371	dbSNP	A/G G/A G/A G/A		Intron
hCV2828117	dbSNP	G/A G/A G/A		Intron

SNP name	Source	Allele	Protein Variation	Location
hCV2381370	dbSNP	A/G A/G G/A		Intron
hCV11669939	Celera	T/-		Intron
hCV2381369	dbSNP	G/A A/G A/G		Intron
hCV2828115	Celera	T/G		Intron
hCV7606016	dbSNP	G/A		Intron
hCV7606010	dbSNP Celera	C/T C/T		Intron
hCV11669940	dbSNP Celera	G/A A/G		Intron
hCV9478393	dbSNP	C/T		Intron
hCV2828114	Celera	C/G		Intron
hCV11439282	dbSNP	C/T		Intron
hCV2828113	dbSNP Celera	C/G G/C		Intron
hCV2828112	Celera	G/A		Intron
hCV11439283	dbSNP	C/G		Intron
hCV7606009	dbSNP	T/C		Intron
hCV16139205	dbSNP	C/T		Intron
hCV11669941	Celera	A/-		Intron
hCV11669944	Celera	A/-		Intron
hCV11439286	dbSNP	A/G		Intron
hCV16271258	dbSNP	A/G		Intron
hCV16271259	dbSNP	C/T		Intron
hCV2828109	dbSNP Celera	T/C C/T		Intron
hCV2828108	dbSNP Celera	C/T C/T		Intron
hCV9478420	dbSNP	A/C A/C A/C A/C		Intron
hCV2828107	dbSNP Celera	T/C T/C		Intron
hCV2828106	dbSNP Celera	C/T C/T		Intron
hCV2828105	dbSNP Celera	C/T T/C		Intron
hCV2828104	Celera	G/A		Intron
hCV16271260	dbSNP	A/G		Intron
hCV3284520	Celera	C/A		Intron
hCV3284521	dbSNP Celera	G/A A/G G/A		Intron
hCV11669953	Celera	T/G		Intron
hCV11669954	Celera	T/A		Intron
hCV11669955	Celera	C/A		Intron
hCV16271264	dbSNP	C/T		Intron
hCV11439287	dbSNP	T/C		Intron
hCV2828103	dbSNP Celera	A/G A/G		Intron
hCV2828102	dbSNP Celera	T/A A/T		Intron
hCV2828101	Celera	T/A		Intron
hCV2828100	Celera	A/G		Intron
hCV2828099	Celera	C/T		Intron

SNP name	Source	Allele	Protein Variation	Location
hCV11439288	dbSNP	A/G G/A A/G A/G		Intron
hCV11439289	dbSNP HGBASE	G/C C/G G/C C/G C/G		Intron
hCV2828097	Celera	C/A		Intron
hCV2828096	Celera	C/A		Intron
hCV2828095	Celera	C/T		Intron
hCV11669963	Celera	C/G		Intron
hCV2828094	Celera	C/T		Intron
hCV11669964	Celera	G/A		Intron
hCV11669965	Celera	A/G		Intron
hCV11669967	Celera	A/G		Intron
hCV11669968	Celera	A/G		Intron
hCV11439290	dbSNP	G/T		Intron
hCV11439291	dbSNP	A/G		Intron
hCV9478400	dbSNP	C/T		Intron
hCV7606003	dbSNP	G/C		Intron
hCV16210093	dbSNP	T/C		Intron
hCV2381366	dbSNP	C/T T/C C/T C/T		Intron
hCV2828091	dbSNP Celera	C/T T/C C/T C/T C/T		Intron
hCV11439294	dbSNP	C/G		Intron
hCV2828090	Celera	G/C		Intron
hCV2828089	dbSNP Celera	A/T A/T		Intron
hCV2828088	Celera	A/G		Intron
hCV2828087	Celera	T/C		Intron
hCV2828086	dbSNP Celera	A/C C/A		Intron
hCV16271265	dbSNP	A/G		Intron
hCV2828084	Celera	T/C		Intron
hCV11669971	Celera	A/-		Intron
hCV2828082	Celera	T/G		Intron
hCV2828081	Celera	C/T		Intron
hCV16261553	dbSNP	C/T		Intron
hCV7605998	dbSNP	G/A A/G		Intron
hCV9478310	dbSNP	G/C C/G		Intron
hCV16261554	dbSNP	A/G		Intron
hCV15845773	dbSNP	C/G		Intron
hCV7605997	dbSNP	C/A A/C		Intron
hCV2381364	dbSNP	T/C C/T C/T C/T C/T C/T		Intron
hCV7605993	dbSNP	A/G G/A		Intron

SNP name	Source	Allele	Protein Variation	Location
hCV7605992	dbSNP	A/G		Intron
hCV11669973	Celera	-/A		Intron
hCV7605991	dbSNP	T/C		Intron
hCV7605987	dbSNP	C/T		Intron
hCV15816829	dbSNP	T/C		Intron
hCV2381363	dbSNP	T/G G/T T/G		Intron
hCV7605980	dbSNP	C/A		Intron
hCV7605979	dbSNP	A/G		Intron
hCV2828079	dbSNP Celera	T/C C/T		Intron
hCV11669974	Celera	-/A		Intron
hCV11439309	dbSNP	T/C C/T C/T C/T		Intron
hCV7605972	dbSNP Celera	T/C C/T		Intron
hCV7605971	dbSNP	T/A		Intron
hCV2828078	Celera	G/C		Intron
hCV11669976	Celera	T/C		Intron
hCV2828077	Celera	C/T		Intron
hCV11669977	Celera	G/T		Intron
hCV2381361	dbSNP	C/T T/C T/C		Intron
hCV2381360	dbSNP	A/T T/A A/T		Intron
hCV11439314	dbSNP	T/C		Intron
hCV2828076	dbSNP Celera	T/A T/A		Intron
hCV2828074	Celera	T/A		Intron
hCV7605963	dbSNP Celera	C/G C/G		Intron
hCV7605957	dbSNP	A/C		Intron
hCV2828072	Celera	C/T		Intron
hCV2828071	Celera	A/G		Intron
hCV16016767	dbSNP	G/A		Intron
hCV7605956	dbSNP	G/T G/T		Intron
hCV7605955	dbSNP	C/A A/C		Intron
hCV2828070	dbSNP Celera	T/C C/T T/C		Intron
hCV2828069	dbSNP Celera	T/C T/C		Intron
hCV2828068	dbSNP Celera	G/A G/A G/A		Intron
hCV16261555	dbSNP	G/A		Intron
hCV16271253	dbSNP	A/G		Intron
hCV16261562	dbSNP	T/C		Intron
hCV7605948	dbSNP	T/C C/T		Intron
hCV7605947	dbSNP	C/G C/G		Intron
hCV16271271	dbSNP	C/G		Intron
hCV11669982	Celera	G/-		Intron
hCV11669983	Celera	A/C		Intron

SNP name	Source	Allele	Protein Variation	Location
hCV11669985	Celera	-/A		Intron
hCV15784638	dbSNP	AAAA/-		Intron
hCV2828065	dbSNP Celera	C/T C/T C/T		Intron
hCV2828064	dbSNP Celera	A/G G/A		Intron
hCV2828063	dbSNP Celera	C/G C/G		Intron
hCV9478268	dbSNP	C/T		Intron
hCV2828062	dbSNP Celera	G/A A/G		Intron
hCV16261563	dbSNP	A/G		Intron
hCV16261564	dbSNP	A/G		Intron
hCV16271266	dbSNP	C/T		Intron
hCV11669986	Celera	-/A		Intron
hCV2828060	dbSNP Celera	C/A A/C A/C		Intron
hCV2828059	dbSNP Celera	T/C T/C T/C		Intron
hCV2828058	dbSNP Celera	G/C C/G G/C		Intron
hCV2828057	dbSNP Celera	C/T C/T		Intron
hCV2828056	dbSNP Celera	C/T C/T		Intron
hCV2828055	dbSNP Celera	C/A A/C		Intron
hCV2828054	dbSNP Celera	A/T A/T		Intron
hCV16271272	dbSNP	T/C		Intron
hCV16261571	dbSNP	G/A G/A		Intron
hCV16261572	dbSNP	G/A		Intron
hCV16261573	dbSNP	G/C		Intron
hCV15784665	dbSNP	-/CTA		Intron
hCV16016733	dbSNP	A/G		Intron
hCV11669989	dbSNP Celera	T/C C/T T/C		Intron
hCV11669990	dbSNP Celera	T/C T/C C/T		Intron
hCV16261580	dbSNP	A/T		Intron
hCV16271273	dbSNP	A/G		Intron
hCV16261582	dbSNP	G/C		Intron
hCV11669992	Celera	G/T		Intron
hCV15845774	dbSNP	T/C T/C		Intron
hCV16016736	dbSNP	C/T		Intron
hCV2828045	Celera	C/T		Intron
hCV2828044	Celera	A/G	His(CAC)244Tyr(TAC)	Mis-sense Mutation
hCV2828043	dbSNP Celera	T/G G/T		Intron
hCV2828042	Celera	C/T		Intron
hCV2828041	Celera	G/A		Intron
hCV11439320	dbSNP	A/G A/G		Intron
hCV2828040	dbSNP Celera	G/A A/G		Intron

SNP name	Source	Allele	Protein Variation	Location
hCV11669993	Celera	T/A		Intron
hCV2828039	Celera	A/C		Intron
hCV16018201	dbSNP	G/A		Intron
hCV11669994	Celera	G/A		Intron
hCV2828038	Celera	G/A		Intron
hCV2828037	Celera	A/G		Intron
hCV2828036	dbSNP Celera	G/A A/G		Intron
hCV2828035	dbSNP Celera	T/C T/C T/C		Intron
hCV11669995	dbSNP Celera	A/G G/A		Intron
hCV11439321	dbSNP	G/C G/C		Intron
hCV11439324	dbSNP	C/G C/G		Intron
hCV7605946	dbSNP	T/C T/C C/T C/T		Intron
hCV2828033	Celera	C/G		Intron
hCV2828032	Celera	A/G		Intron
hCV2381355	dbSNP	G/C C/G G/C C/G		Intron
hCV2381354	dbSNP	A/G G/A G/A A/G		Intron
hCV16016737	dbSNP	G/A		Intron
hCV16016738	dbSNP	A/G		Intron
hCV2381353	dbSNP	C/T C/T C/T T/C		Intron
hCV16018237	dbSNP	T/C		Intron
hCV2381352	dbSNP	C/T C/T T/C C/T		Intron
hCV2381351	dbSNP	T/C C/T C/T T/C		Intron
hCV15864249	dbSNP	A/C		Intron
hCV11439333	dbSNP	C/A		Intron
hCV11439334	dbSNP	A/C A/C		Intron
hCV2381349	dbSNP	T/C T/C T/C T/C		Intron
hCV2828031	dbSNP Celera	C/T T/C T/C T/C T/C		Intron
hCV2828030	dbSNP Celera	C/T C/T C/T C/T		Intron
hCV2828029	Celera	C/T		Intron
hCV2381348	dbSNP	C/T C/T C/T		Intron
hCV2381347	dbSNP	A/T A/T T/A		Intron
hCV2828028	Celera	C/G		Intron
hCV16018247	dbSNP	T/A		Intron
hCV16018248	dbSNP	G/C		Intron
hCV2828027	Celera	A/G		Intron
hCV16016748	dbSNP	A/T		Intron
hCV16016749	dbSNP	A/G		Intron
hCV16018249	dbSNP	C/T		Intron
hCV9606709	dbSNP	C/T C/T C/T C/T		Intron

SNP name	Source	Allele	Protein Variation	Location
		C/T		
hCV2828026	dbSNP Celera	C/T C/T		Intron
hCV16016750	dbSNP	G/C		Intron
hCV9606713	dbSNP	G/A G/A		Intron
hCV16016754	dbSNP	G/C		Intron
hCV2828025	Celera	G/A		Intron
hCV9606714	dbSNP	T/C		Intron
hCV2828024	Celera	G/A		Intron
hCV2381346	dbSNP	C/T T/C T/C T/C		Intron
hCV2381345	dbSNP	G/A A/G A/G G/A		Intron
hCV2828023	Celera	T/A		Intron
hCV2828022	Celera	T/A		Intron
hCV2381344	dbSNP Celera	A/T A/T A/T T/A A/T		Intron
hCV2381343	dbSNP	C/T C/T C/T C/T		Intron
hCV2381342	dbSNP	C/G C/G C/G G/C		Intron
hCV16018211	dbSNP	C/T		Intron
hCV2381341	dbSNP	C/G G/C C/G G/C G/C		Intron
hCV11669997	Celera	-/A		Intron
hCV2828020	Celera	G/A		Intron
hCV11439337	dbSNP	A/T		Intron
hCV2828019	Celera	A/G		Intron
hCV11669998	Celera	A/-		Intron
hCV2828017	Celera	C/A		Intron
hCV2828016	Celera	C/G		Intron
hCV2828015	Celera	C/G		Intron
hCV2828014	Celera	G/A		Intron
hCV2828013	Celera	C/T		Intron
hCV2828012	Celera	T/C		Intron
hCV15944296	dbSNP	T/G		Intron
hCV9605371	dbSNP	C/T		Intron
hCV2381340	dbSNP	C/T C/T C/T T/C C/T		Intron
hCV2828011	Celera	G/T		Intron
hCV2828010	Celera	A/G		Intron
hCV2828009	Celera	C/T		Intron
hCV2828008	Celera	A/G		Intron
hCV11670003	Celera	C/G		Intron
hCV7605903	dbSNP	C/A		Intron

SNP name	Source	Allele	Protein Variation	Location
hCV7605890	dbSNP	C/T		Intron
hCV2828002	Celera	A/G		Intron
hCV7605889	dbSNP	C/G		Intron
hCV2828001	Celera	C/T		Intron
hCV2828000	Celera	G/A		Intron
hCV2827999	Celera	A/G		Intron
hCV2827998	Celera	T/C		Intron
hCV2827997	Celera	G/C		Intron
hCV2827996	Celera	C/G		Intron
hCV2827995	Celera	-/G		Intron
hCV11670006	Celera	-/G		Intron
hCV2827993	Celera	C/G		Intron
hCV2827992	Celera	A/C		Intron
hCV2827991	Celera	A/G		Intron
hCV2827990	Celera	G/A		Intron
hCV2827989	Celera	G/A		Intron
hCV16080952	dbSNP	A/G		Intron
hCV2827988	dbSNP Celera	G/A A/G		Intron
hCV2827987	Celera	G/A		Intron
hCV11670008	dbSNP Celera	T/G T/G		Intron
hCV11670009	Celera	T/-		Intron
hCV2827984	Celera	G/T		Intron
hCV2827983	Celera	G/A		Intron
hCV11670011	Celera	C/T		Intron
hCV11670012	Celera	T/A		Intron
hCV11670013	Celera	A/G		Intron
hCV2827979	Celera	A/G		Intron
hCV11670014	Celera	C/T		Intron
hCV2827977	Celera	A/T		Intron
hCV2827976	Celera	G/A		Intron
hCV2827975	Celera	T/A		Intron
hCV2827974	Celera	T/A		Intron
hCV2827973	Celera	C/G		Intron
hCV2827972	Celera	A/G		Intron
hCV2827971	Celera	C/A		Intron
hCV11439338	dbSNP	A/G		Intron
hCV2381339	dbSNP	C/T C/T T/C C/T		Intron
hCV2827970	Celera	T/C		Intron
hCV2827969	Celera	T/A		Intron
hCV7605880	dbSNP	T/C T/C		Intron

SNP name	Source	Allele	Protein Variation	Location
hCV7605879	dbSNP	A/G G/A		Intron
hCV2827968	Celera	T/C		Intron
hCV2827967	Celera	G/C		Intron
hCV2827966	Celera	C/G		Intron
hCV2381338	dbSNP	A/G G/A A/G		Intron
hCV2827964	Celera	A/C		Intron
hCV2827963	dbSNP Celera	C/T C/T		Intron
hCV11439341	dbSNP	C/T		Intron
hCV2827962	Celera	A/G		Intron
hCV2827961	dbSNP Celera	C/T T/C		Intron
hCV11670022	Celera	-/A		Intron
hCV2827959	Celera	G/A		Intron
hCV2827958	Celera	T/C		Intron
hCV2827957	Celera	C/G		Intron
hCV2827956	Celera	T/G		Intron
hCV2827955	Celera	G/C		Intron
hCV2827954	Celera	T/C		Intron
hCV2827953	Celera	G/C		Intron
hCV15815639	dbSNP	C/A		Intron
hCV16142119	dbSNP	T/A		Intron
hCV2827952	Celera	C/T		Intron
hCV15816830	dbSNP	T/C		Intron
hCV1004253	dbSNP	T/G T/G		Intron
hCV9606740	dbSNP	C/T		Intron
hCV3189734	dbSNP Celera	C/T T/C		Intron
hCV9606733	dbSNP	A/G		Intron
hCV3189733	Celera	C/G		Intron
hCV3189732	dbSNP Celera	T/A T/A T/A T/A A/T		Intron
hCV1004252	dbSNP	C/A A/C A/C C/A C/A		Intron
hCV1004251	dbSNP	A/T A/T T/A T/A A/T T/A		Intron
hCV11670025	Celera	G/A		Intron
hCV3189731	Celera	T/C		Intron
hCV11670028	Celera	-/A		Intron
hCV3189730	Celera	G/T		Intron
hCV8560814	dbSNP Celera	A/G G/A		Intron
hCV11670031	Celera	A/G		Intron
hCV11670032	Celera	G/A		Intron

SNP name	Source	Allele	Protein Variation	Location
hCV11439346	dbSNP	C/T		Intron
hCV3189728	Celera	G/C		Intron
hCV9606725	dbSNP	C/G		Intron
hCV3189727	Celera	C/A		Intron
hCV9606724	dbSNP	C/A		Intron
hCV9606723	dbSNP	T/C		Intron
hCV9606719	dbSNP	T/G		Intron
hCV16142120	dbSNP	G/C		Intron
hCV16142127	dbSNP	T/A		Intron
hCV3189726	Celera	T/C		Intron
hCV3189725	Celera	C/T		Intron
hCV9606718	dbSNP	C/G		Intron
hCV3189724	dbSNP Celera	C/T T/C		Intron
hCV2950480	Celera	G/T		Intron
hCV11670036	Celera	-/A		Intron
hCV3189723	Celera	T/A		Intron
hCV2950479	Celera	C/T		Intron
hCV7605776	dbSNP	C/T		Intron
hCV3189722	Celera	C/T		Intron
hCV2950478	Celera	C/G		Intron

[053] The aggrecanase molecules provided also include factors encoded by sequences similar to those of SEQ ID NO: 1, but which include modifications or deletions that are naturally occurring, for example, allelic variations in the nucleotide sequence which may result in amino acid changes in the protein or artificially engineered proteins. For example, synthetic proteins may wholly or partially duplicate continuous sequences of the amino acid residues of SEQ ID NO: 2. These sequences, by virtue of sharing primary, secondary, or tertiary structural and conformational characteristics with aggrecanase proteins may possess biological properties in common therewith. It is known, for example that numerous conservative amino acid substitutions are possible without significantly modifying the structure and conformation of a protein, thus maintaining the biological properties of the protein. For example, it is recognized that conservative amino acid substitutions may be made among amino acids with basic side chains, such

as lysine (Lys or K), arginine (Arg or R) and histidine (His or H); amino acids with acidic side chains, such as aspartic acid (Asp or D) and glutamic acid (Glu or E); amino acids with uncharged polar side chains, such as asparagine (Asn or N), glutamine (Gln or Q), serine (Ser or S), threonine (Thr or T), and tyrosine (Tyr or Y); and amino acids with nonpolar side chains, such as alanine (Ala or A), glycine (Gly or G), valine (Val or V), leucine (Leu or L), isoleucine (Ile or I), proline (Pro or P), phenylalanine (Phe or F), methionine (Met or M), tryptophan (Trp or W) and cysteine (Cys or C). Thus, these modifications and deletions of the native aggrecanase may be employed as biologically active substitutes for naturally-occurring aggrecanase and in the development of inhibitors or other proteins for therapeutic purposes. It can be readily determined whether a given variant of aggrecanase maintains the biological activity of aggrecanase by subjecting both aggrecanase and the variant of aggrecanase, as well as inhibitors thereof, to the assays described in the examples.

[054] Desired amino acid substitutions (whether conservative or non-conservative) can be determined by those skilled in the art at the time such substitutions are desired. For example, amino acid substitutions can be used to identify important amino acid residues of the proteins or polypeptides of the invention, or to increase or decrease the activity of the aggrecanases of the invention described. Exemplary amino acid substitutions are set forth in Table 3.

Table 3: Amino Acid Substitutions

Original Residues	Exemplary Substitutions	More Conservative Substitutions
Ala (A)	Val, Leu, Ile	Val
Arg (R)	Lys, Gln, Asn	Lys
Asn (N)	Gln	Gln
Asp (D)	Glu	Glu
Cys (C)	Ser, Ala	Ser
Gln (Q)	Asn	Asn
Gly (G)	Pro, Ala	Ala

His (H)	Asn, Gln, Lys, Arg	Arg
Ile (I)	Leu, Val, Met, Ala, Phe, Norleucine	Leu
Leu (L)	Norleucine, Ile, Val, Met, Ala, Phe	Ile
Lys (K)	Arg, 1, 4 Diamino-butyric Acid, Gln, Asn	Arg
Met (M)	Leu, Phe, Ile	Leu
Phe (F)	Leu, Val, Ile, Ala, Tyr	Leu
Pro (P)	Ala	Gly
Ser (S)	Thr, Ala, Cys	Thr
Thr (T)	Ser	Ser
Trp (W)	Tyr, Phe	Tyr
Tyr (Y)	Trp, Phe, Thr, Ser	Phe
Val (V)	Ile, Met, Leu, Phe, Ala, Norleucine	Leu

[055] In certain embodiments, conservative amino acid substitutions also encompass non-naturally occurring amino acid residues which are typically incorporated by chemical peptide synthesis rather than by synthesis in biological systems.

[056] Other specific mutations of the sequences of aggrecanase proteins described include modifications of glycosylation sites. These modifications may involve O-linked or N-linked glycosylation sites. For instance, the absence of glycosylation or presence of only partial glycosylation can result from amino acid substitutions or deletions at asparagine-linked glycosylation recognition sites. Asparagine-linked glycosylation recognition sites comprise tripeptide sequences which are recognized specifically by appropriate cellular glycosylation enzymes. These tripeptide sequences usually are either asparagine-X-threonine or asparagine-X-serine, where X can be any amino acid. A variety of amino acid substitutions or deletions at one or both of the first or third amino acid positions of a glycosylation recognition site (and/or amino acid deletion at the second position) results in non-glycosylation at the modified tripeptide sequence. Additionally, bacterial expression of aggrecanase-related proteins will also result in production of a non-glycosylated protein, even if the glycosylation sites are left unmodified.

IV. Novel Aggrecanase Nucleotide Sequences

[057] Nucleic acid sequences within the scope of the invention include isolated DNA and RNA sequences that hybridize to the native aggrecanase DNA sequences disclosed under conditions of moderate to high stringency. Stringent conditions or conditions of high stringency generally refer to hybridization and washing conditions that employ higher temperature and lower salt concentrations. Additionally, inclusion of formamide also increases stringency. For example, hybridization conditions at 60-65°C in the absence of formamide or at 42°C with 50% formamide, are both high stringency conditions.

[058] Still a further aspect of the invention are DNA sequences encoding aggrecanase proteins having aggrecanase proteolytic activity or other disclosed or yet undiscovered activities of aggrecanase. Such sequences include nucleotide sequence illustrated in SEQ ID NO: 1, and DNA sequences which, but for the degeneracy of the genetic code, are identical to the DNA sequence of SEQ ID NO: 1 and encode an aggrecanase protein, for example, including the amino acid sequence of SEQ ID NO: 2, or a variant thereof.

[059] Further included in the present invention are DNA sequences which hybridize under high to moderate stringent conditions with the DNA sequence of SEQ ID NO: 1 and encode a protein having the ability to cleave aggrecan. In one embodiment, DNA sequences include those which hybridize under high stringent conditions (see Maniatis *et al.*, *Molecular Cloning (A Laboratory Manual)*, Cold Spring Harbor Laboratory, at 387-389 (1982)). Such stringent conditions comprise, for example, 0.1X SSC, 0.1% SDS, at 65°C. DNA sequences identified by hybridization include, for example, DNA sequences that encode a protein which is at least about 80% identical, at least about 90% identical, or at least about 95% identical to the sequence set forth in SEQ ID NO: 2. DNAs that are equivalents to the DNA of

SEQ ID NO: 1 will also hybridize under moderately stringent conditions to the DNA sequence encoding the peptide sequence of SEQ ID NO: 2.

[060] Conditions of moderate stringency are known in the art, and are defined by Sambrook *et al. Molecular Cloning: A Laboratory Manual*, 2 ed. Vol. 1, Cold Spring Harbor Press. (1989). In one embodiment, for example, conditions of moderate stringency include use of a prewashing solution of 5X SSC/0.5% SDS, 1.0 mM EDTA (pH 8.0) and hybridization conditions of about 55°C-60°C temperature and washing overnight in 5X SSC overnight at about 55°C. The skilled artisan will recognize that the conditions may be adjusted as necessary according to factors such as the length and composition of the nucleic acid sequences.

[061] Finally, allelic or other variations of the sequences of SEQ ID NO: 1, encoding the amino acid sequence of SEQ ID NO: 2, or peptide sequence variants of SEQ ID NO: 2, that have aggrecanase activity, are also included in the present invention. Additionally, the present invention includes fragments of the DNA sequence shown in SEQ ID NO: 1 and variants of SEQ ID NO: 1, encoding a protein with aggrecanase activity.

[062] Similarly, DNA sequences which encode aggrecanase proteins comprising the sequence set forth in SEQ ID NO: 2 but which differ from SEQ ID NO: 1 in codon usage because of the degeneracies of the genetic code or because of allelic variations (naturally-occurring base changes in the species population which may or may not result in an amino acid change) also encode the novel factors described. Variations in the DNA sequence of SEQ ID NO: 1 which are caused by point mutations or by induced modifications (including insertion, deletion, and substitution) to enhance the activity, half-life or production of the proteins encoded by them are also encompassed by the invention. The DNA sequences of the present invention are useful, for example, as probes for the detection of mRNA encoding aggrecanase in a given cell population. Thus, the present invention includes methods of detecting or diagnosing diseases and genetic disorders involving

aggrecanase proteins, or disorders involving cellular, organ or tissue disorders in which aggrecanase is irregularly transcribed or expressed. Antisense DNA sequences may also be used for preparing vectors for gene therapy applications. Antisense DNA sequences are also useful in *in vivo* methods involving a cell or an organism, for example, introducing an antisense DNA sequence for aggrecanase into a cell in order to study the interaction of the antisense DNA with the endogenous aggrecanase sequences, and further in order to test the capacity of a promoter operatively linked to the antisense DNA sequence in a vector as a measure of how much antisense DNA is produced in a cell.

[063] A further aspect of the invention includes vectors comprising a DNA sequence as described above in operative association with an expression control sequence therefor. These vectors may be employed in a novel process for producing an aggrecanase protein of the invention in which a cell line transformed with a DNA sequence encoding an aggrecanase protein in operative association with an expression control sequence therefor, is cultured in a suitable culture medium and an aggrecanase protein is recovered and isolated therefrom. This process may employ a number of known cells both prokaryotic and eukaryotic as host cells for expression of the protein. The vectors may be used in gene therapy applications. In such use, the vectors may be transfected into cells of a patient *ex vivo*, and the cells may be reintroduced into a patient. Alternatively, the vectors may be introduced into a patient *in vivo* through targeted transfection.

V. Production of Aggrecanase Proteins

[064] Another aspect of the present invention provides methods for producing novel aggrecanase proteins. In one embodiment, a method of the present invention involves culturing a suitable cell line, which has been transformed with a DNA sequence, for example, the sequence set forth in SEQ ID NO: 1, and translating the DNA into an aggrecanase protein of the invention, set forth in SEQ ID NO: 2, under the control of known regulatory

sequences. The transformed host cells are cultured and the aggrecanase proteins recovered and isolated from the culture medium. The isolated expressed proteins are substantially free from other proteins with which they are co-produced as well as from other contaminants. The recovered isolated protein is contemplated to exhibit proteolytic aggrecanase activity comprising aggrecan cleavage. Thus, the proteins of the invention may be further characterized by the ability to demonstrate aggrecanase proteolytic activity in an assay which determines the presence of an aggrecan-degrading molecule. These assays or the development thereof is within the knowledge of one skilled in the art. Such assays may involve contacting an aggrecan substrate with an aggrecanase molecule and monitoring the production of aggrecan fragments (see for example, Hughes *et al.*, *Biochem J* 305: 799-804 (1995); Mercuri *et al.*, *J Biol. Chem* 274:32387-32395 (1999)). Suitable cells or cell lines may be mammalian cells, such as Chinese hamster ovary cells (CHO). The selection of suitable mammalian host cells and methods for transformation, culturing, amplification, screening, product production and purification are known in the art. (See, e.g., Gething and Sambrook, *Nature*, 293:620-625 (1981); Kaufman *et al.*, *Mol Cell Biol*, 5(7):1750-1759 (1985); Howley *et al.*, U.S. Patent 4,419,446.) Another suitable mammalian cell line, which is described in the accompanying examples, is the monkey kidney COS-1 cell line. The mammalian CV-1 cells may also be used.

[065] Bacterial cells may also be used as suitable hosts for expression of the proteins or polypeptides of the invention. For example, the various strains of *E. coli* (e.g., HB101, MC1061) are well-known as host cells in the field of biotechnology. Various strains of *B. subtilis*, *Pseudomonas*, other bacilli and the like may also be employed in the methods of the invention. For expression of the protein in bacterial cells, DNA encoding the propeptide of an aggrecanase is generally not necessary.

[066] Many strains of yeast cells known to those skilled in the art may also be available as host cells for expression of the proteins or polypeptides of

the present invention. Additionally, where desired, insect cells may be utilized as host cells in the method of the present invention. See, e.g., Miller *et al.*, *Genetic Engineering*, 8:277-298 (Plenum Press 1986).

[067] Another aspect of the present invention provides vectors for use in a method of expression of these novel aggrecanase proteins. In one embodiment, vectors of the invention contain full length DNA sequences described which encode the novel factors of the invention. Additionally, the vectors contain appropriate expression control sequences permitting expression of the aggrecanase protein sequences. Alternatively, vectors incorporating modified sequences as described above are also embodiments of the present invention. Additionally, the sequence of SEQ ID NO: 1 or other sequences encoding aggrecanase proteins could be manipulated to express composite aggrecanase proteins. Thus, the present invention includes chimeric DNA molecules that encode a recombinant protein including an aggrecanase protein comprising a fragment of SEQ ID NO: 2 linked to a different aggrecanase protein. Such a recombinant or fusion protein can be produced by linking the DNA encoding a fragment of the aggrecanase molecule set forth in SEQ ID NO: 2 in frame with the DNA encoding a different aggrecanase protein. The DNA encoding the aggrecanase protein set forth in SEQ ID NO: 2 or a fragment or variant thereof can be linked either 3' or 5' to the DNA encoding a different aggrecanase. Vectors used for the expression of aggrecanase molecules of the invention may be employed in a method of transforming cell lines and usually contain selected regulatory sequences capable of directing the replication and expression of aggrecanase molecules in operative association with DNA sequences of the invention. Regulatory sequences for such vectors are known to those skilled in the art and may be selected depending upon the host cells. Such selection is routine and does not form part of the present invention.

[068] One skilled in the art can construct mammalian expression vectors by employing a sequence comprising, for example, SEQ ID NO: 1 or

other DNA sequences encoding aggrecanase-related proteins or other modified sequences and known vectors, such as, for example, pCD (Okayama *et al.*, *Mol Cell Biol*, 2:161-170 (1982)), pJL3, pJL4 (Gough *et al.*, *EMBO J*, 4:645-653 (1985)) and pMT2 CXM. In addition, one skilled in the art can employ a suitable expression vector for expressing a recombinant form of the aggrecanase protein, for example, rA18FS, in an expression system of choice.

[069] The construction of vectors may involve modification of the aggrecanase-related DNA sequences. For instance, aggrecanase cDNA can be modified by removing the non-coding nucleotides on the 5' and 3' ends of the coding region. The deleted non-coding nucleotides may or may not be replaced by other sequences known to be beneficial for expression. These vectors are transformed into appropriate host cells for expression of aggrecanase or aggrecanase-related proteins. Additionally, the sequence of SEQ ID NO: 1 or other sequences encoding aggrecanases or aggrecanase-related proteins can be manipulated to express a mature aggrecanase or aggrecanase-related protein by deleting aggrecanase encoding propeptide sequences and replacing them with sequences encoding complete propeptides of other aggrecanase proteins.

[070] One skilled in the art can manipulate the sequence of SEQ ID NO: 1 by eliminating or replacing the mammalian regulatory sequences flanking the coding sequence with bacterial sequences to create bacterial vectors for intracellular or extracellular expression by bacterial cells. For example, the coding sequences could be further manipulated (e.g., ligated to other known linkers or modified by deleting non-coding sequences therefrom or altering nucleotides therein by other known techniques). The modified aggrecanase-related coding sequence could then be inserted into a known bacterial vector using procedures such as described in Taniguchi *et al.*, *Proc. Natl. Acad. Sci. USA*, 77:5230-5233 (1980). This exemplary bacterial vector could then be transformed into bacterial host cells and an aggrecanase-

related protein expressed thereby. For a strategy for producing extracellular expression of aggrecanase-related proteins in bacterial cells, see, e.g., European patent application EPA 177,343.

[071] Similar manipulations can be performed for the construction of an insect vector (see, e.g. procedures described in published European patent application EPA 155,476) for expression in insect cells. A yeast vector could also be constructed employing yeast regulatory sequences for intracellular or extracellular expression of the factors of the present invention by yeast cells. (See, e.g., procedures described in published PCT application WO 86/00639 and European patent application EPA 123,289.)

[072] A method for producing high levels of a aggrecanase-related protein of the invention in mammalian, bacterial, yeast or insect host cell systems may involve the construction of cells containing multiple copies of the heterologous aggrecanase-related gene. The heterologous gene is linked to an amplifiable marker, e.g., the dihydrofolate reductase (DHFR) gene for which cells containing increased gene copies can be selected for propagation in increasing concentrations of methotrexate (MTX) according to the procedures of Kaufman and Sharp, *J Mol Biol*, 159:601-629 (1982). This approach can be employed with a number of different cell types.

[073] For example, a plasmid containing a DNA sequence for an aggrecanase-related protein of the invention in operative association with other plasmid sequences enabling expression thereof and the DHFR expression plasmid pAdA26SV(A)3 (Kaufman and Sharp, *Mol Cell Biol* 2:1304 (1982)) can be co-introduced into DHFR-deficient CHO cells, DUKX-BII, by various methods including calcium phosphate coprecipitation and transfection, electroporation or protoplast fusion. DHFR expressing transformants are selected for growth in alpha media with dialyzed fetal calf serum, and subsequently selected for amplification by growth in increasing concentrations of MTX (e.g. sequential steps in 0.02, 0.2, 1.0 and 5 μ M MTX) as described in Kaufman *et al.*, *Mol Cell Biol.*, 5:1750 (1983). Transformants are cloned, and

biologically active aggrecanase expression is monitored by the assays described above. Aggrecanase protein expression should increase with increasing levels of MTX resistance. Aggrecanase proteins are characterized using standard techniques known in the art such as pulse labeling with ^{35}S methionine or cysteine and polyacrylamide gel electrophoresis. Similar procedures can be followed to produce other related aggrecanase-related proteins.

[074] Aggrecanase proteins of the invention can also be expressed as fusion proteins comprising the protein sequence, for example, the sequence set forth in SEQ ID NO: 2 or a fragment or a variant thereof, and for example, a tag, i.e., a second protein or one or more amino acids, from about 2 to 50 amino acids, or from about 50 to about 100 amino acids, which are added to the amino terminus of, the carboxy terminus of, or any point within the amino acid sequence of an aggrecanase protein, or a fragment or variant thereof. Typically, such amino acid tags are made to stabilize the resulting fusion protein or to simplify purification of an expressed recombinant form of the corresponding aggrecanase protein or a fragment or a variant of such protein, including for example, a truncated form of an aggrecanase protein of the invention. Such tags are known in the art. Representative examples of such tags include sequences which encode a series of histidine residues, the epitope tag FLAG, the Herpes simplex glycoprotein D, beta-galactosidase, maltose binding protein, streptavidin tag or glutathione S-transferase.

VI. Generation of Antibodies

[075] The isolated proteins of the present inventions may be used to generate antibodies, either monoclonal or polyclonal, to aggrecanase and/or other aggrecanase-related proteins, using methods of antibody production that are generally known in the art. Thus, the present invention also includes antibodies to aggrecanase or other related proteins. The antibodies include both antibodies that block aggrecanase activity and antibodies that do not. The antibodies may be useful for detection and/or purification of aggrecanase

or related proteins, or for inhibiting or preventing the effects of aggrecanase. Aggrecanases of the invention or portions thereof may be utilized to prepare antibodies that specifically bind to aggrecanase.

[076] Antibodies can be made, for example, via traditional hybridoma techniques (Kohler and Milstein, *Nature* 256:495-499 (1975)), recombinant DNA methods (for example, U.S. Pat. No. 4,816,567), or phage display techniques using antibody libraries (Clackson *et al.*, *Nature* 352: 624-628 (1991); Marks *et al.*, *J. Mol. Biol.* 222:581-597 (1991)). For various antibody production techniques, see *Antibodies: A Laboratory Manual*, eds. Harlow *et al.*, Cold Spring Harbor Laboratory (1988).

[077] Proteins are known to have certain biochemical properties including sections which are hydrophobic and sections which are hydrophilic. The hydrophobic sections are most likely to be located in the interior of the structure of the folded protein while the hydrophilic sections are most likely to be located in the exterior of the structure of the folded protein. It is believed that the hydrophilic regions of a protein correspond to antigenic epitopes on the protein. The hydrophobicity of the protein set forth in SEQ ID NO: 2 was determined using the GCG program called plotstructure. The results, as depicted in Figure 9, indicated that the protein of SEQ ID NO: 2 has several regions that are hydrophilic and therefore, expected to be on the surface of the folded protein. For example, between amino acids 50 and 100, there is a region that is predicted to be hydrophilic as well as antigenic. Such antigenic regions can be employed for the generation of antibodies.

[078] Antibodies of the invention may be used in the treatment of the diseases described below. Antibodies can also be used in the assays and methods of detection described.

VII. Development of Inhibitors

[079] Various conditions such as osteoarthritis are known to be characterized by degradation of aggrecan. Therefore, an aggrecanase protein of the present invention which cleaves aggrecan may be useful for the

development of inhibitors of aggrecanase. The invention therefore provides compositions comprising an aggrecanase inhibitor. The inhibitors may be developed using an aggrecanase molecule in screening assays involving a mixture of aggrecan substrate with an inhibitor of aggrecanase activity followed by exposure to aggrecan. Inhibitors can be screened using high throughput processes, such as by screening a library of inhibitors. Inhibitors can also be made using three-dimensional structural analysis and/or computer aided drug design. The method may entail determination of binding sites for inhibitors based on the three dimensional structure of aggrecanase and aggrecan and developing molecules reactive with a binding site on aggrecanase or aggrecan. Candidate molecules are assayed for inhibitory activity. Additional standard methods for developing inhibitors of aggrecanase molecules are known to those skilled in the art. Assays for the inhibitors involve contacting a mixture of aggrecan and an inhibitor with an aggrecanase molecule followed by measurement of the degree of aggrecanase inhibition, for instance by detection and measurement of aggrecan fragments produced by cleavage at an aggrecanase susceptible site. Inhibitors may be proteins, antibodies or small molecules.

VIII. Disease Treatment and Diagnosis

[080] Inhibitors of aggrecanase activity may be used in the treatment of diseases described below. Inhibitors can also be used in the assays and methods of detection described. Various diseases that are contemplated as being treatable by using inhibitors of aggrecanases of the invention include, but are not limited to, osteoarthritis, cancer, inflammatory joint disease, rheumatoid arthritis, septic arthritis, periodontal diseases, corneal ulceration, proteinuria, coronary thrombosis from atherosclerotic plaque rupture, aneurysmal aortic disease, inflammatory bowel disease, Crohn's disease, emphysema, acute respiratory distress syndrome, asthma, chronic obstructive pulmonary disease, Alzheimer's disease, brain and hematopoietic malignancies, osteoporosis, Parkinson's disease, migraine, depression,

peripheral neuropathy, Huntington's disease, multiple sclerosis, ocular angiogenesis, macular degeneration, aortic aneurysm, myocardial infarction, autoimmune disorders, degenerative cartilage loss following traumatic joint injury, head trauma, dystrophic epidermolysis bullosa, spinal cord injury, acute and chronic neurodegenerative diseases, osteopenias, temporomandibular joint disease, demyelating diseases of the nervous system, organ transplant toxicity and rejection, cachexia, allergy, tissue ulcerations, restenosis, and other diseases characterized by altered aggrecanase activity or altered aggrecanase level.

[081] It is contemplated that inhibitors and antibodies of the invention that inhibit activity of aggrecanases and/or compounds that may lower expression of aggrecanases may be used in the treatment of any disease in a mammal that involves degradation of the extracellular matrix proteins, such as aggrecan, by aggrecanases and aggrecanase-related proteins.

IX. Administration

[082] Another aspect of the invention provides pharmaceutical compositions containing a therapeutically effective amount of at least one of aggrecanase antibodies and inhibitors, in a pharmaceutically acceptable vehicle. Aggrecanase-mediated degradation of aggrecan in cartilage has been implicated in osteoarthritis and other inflammatory diseases. Therefore, these compositions of the invention may be used in the treatment of diseases characterized by the degradation of aggrecan and/or an up regulation of aggrecanase activity or level of aggrecanases.

[083] The invention includes methods for treating patients suffering from conditions characterized by a degradation of aggrecan. These methods, according to the invention, entail administering to a patient needing such treatment, an effective amount of a composition comprising an aggrecanase antibody or inhibitor which inhibits the proteolytic activity of an aggrecanase enzyme.

[084] Antibodies and inhibitors of the present invention are useful to diagnose or treat various medical disorders in humans or animals. In one embodiment, the antibodies of the invention can be used to inhibit or reduce one or more activities associated with an aggrecanase protein, relative to an aggrecanase protein not bound by the same antibody. In one embodiment, antibodies and inhibitors of the invention can inhibit or reduce one or more of the activities of an aggrecanase molecule relative to the aggrecanase that is not bound by an antibody. In certain embodiments, an activity of an aggrecanase, when bound by one or more of the presently disclosed antibodies, is inhibited at least 50%, may be inhibited at least 60, 62, 64, 66, 68, 70, 72, 72, 76, 78, 80, 82, 84, 86, or 88%, may be inhibited at least 90, 91, 92, 93, or 94%, or may be inhibited at least 95% to 100% relative to the aggrecanase protein that is not bound by one or more of the presently disclosed antibodies.

[085] Generally, compositions of the present are administered to a patient so that antibodies or their binding fragments are administered at a dose ranging from about 1 µg/kg to about 20 mg/kg, about 1 µg/kg to about 10 mg/kg, about 1 µg/kg to about 1 mg/kg, about 10 µg/kg to about 1 mg/kg, about 10 µg/kg to about 100 µg/kg, about 100 µg to about 1 mg/kg, or about 500 µg/kg to about 1 mg/kg. Antibodies are administered as a bolus dose, to maximize the interval of time that the antibodies can circulate in the patient's body following their administration to the patient. Continuous infusion may also be used after an initial bolus dose.

[086] In another embodiment, the invention is directed to administration of inhibitors of aggrecanases, such as proteins and small molecules. The effective amount of an inhibitor is a dosage which is useful for reducing activity of aggrecanases to achieve a desired biological outcome. Generally, appropriate therapeutic dosages for administering an inhibitor may range, for example, from about 5 mg to about 100 mg, from about 15 mg to about 85 mg, from about 30 mg to about 70 mg, or from about 40 mg to about

60 mg. Inhibitors can be administered in one dose, or at intervals such as once daily, once weekly, or once monthly. Dosage schedules for administration of an aggrecanase inhibitor can be adjusted based on, for example, the affinity of the inhibitor for its aggrecanase target, the half-life of the inhibitor, and the severity of the patient's condition. Generally, inhibitors are administered as a bolus dose, to maximize their circulating levels. Continuous infusions may also be used after the bolus dose.

[087] Toxicity and therapeutic efficacy of such compounds can be determined by standard pharmaceutical procedures in cell culture or experimental animal models, e.g., for determining the LD₅₀ (the dose lethal to 50% of the population) and the ED₅₀ (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio LD₅₀/ED₅₀. Antibodies and inhibitors, which exhibit large therapeutic indices, are generally preferred.

[088] The data obtained from cell culture assays and animal studies can be used in formulating a range of dosages for use in humans. The dosage of such compounds may lie within a range of circulating concentrations that exhibit an ED₅₀ with little or no toxicity. The dosage may vary within this range depending upon the dosage form employed and the route of administration utilized. For any antibody or inhibitor used according to the present invention, a therapeutically effective dose can be estimated initially from cell culture assays. A dose may be formulated in animal models to achieve a circulating plasma concentration range that exhibits an IC₅₀ (*i.e.*, the concentration of the test antibody which achieves a half-maximal inhibition of symptoms) as determined by cell culture assays. Levels in plasma may be measured, for example, by high performance liquid chromatography. The effects of any particular dosage can be monitored by suitable bioassays. Examples of suitable bioassays include DNA replication assays, transcription-based assays, GDF protein/receptor binding assays, creatine

kinase assays, assays based on the differentiation of pre-adipocytes, assays based on glucose uptake in adipocytes, and immunological assays.

[089] Therapeutic methods of the invention include administering the aggrecanase inhibitor compositions topically, systemically, or locally as an implant or a device. The dosage regimen will be determined by the attending physician based on various factors which modify the action of the aggrecanase protein, the site of pathology, the severity of disease, the patient's age, sex, and diet, the severity of any inflammation, time of administration and other clinical factors. Generally, systemic or injectable administration will be initiated at a dose which is minimally effective, and the dose will be increased over a preselected time course until a positive effect is observed. Subsequently, incremental increases in dosage will be made limiting to levels that produce a corresponding increase in effect, while taking into account any adverse affects that may appear. The addition of other known factors, to a final composition, may also affect the dosage.

[090] Progress can be monitored by periodic assessment of disease progression. The progress can be monitored, for example, by X-rays, MRI or other imaging modalities, synovial fluid analysis, patient response, and/or clinical examination.

X. Assays and Methods of Detection

[091] The inhibitors and antibodies of the invention can be used in assays and methods of detection to determine the presence or absence of, or quantify aggrecanase in a sample. The inhibitors and antibodies of the present invention may be used to detect aggrecanase proteins, *in vivo* or *in vitro*. By correlating the presence or level of these proteins with a disease, one of skill in the art can diagnose the associated disease or determine its severity. Diseases that may be diagnosed by the presently disclosed inhibitors and antibodies are set forth above.

[092] Detection methods for use with antibodies are well known in the art and include ELISA, radioimmunoassay, immunoblot, western blot,

immunofluorescence, immuno-precipitation, and other comparable techniques. The antibodies may further be provided in a diagnostic kit that incorporates one or more of these techniques to detect a protein (e.g., an aggrecanase protein). Such a kit may contain other components, packaging, instructions, or other material to aid the detection of an aggrecanase protein, and instructions regarding use of the kit. When protein inhibitors are used in such diagnostic assays, protein-protein interaction assays can be employed

[093] Where antibodies and inhibitors are intended for diagnostic purposes, it may be desirable to modify them, for example, with a ligand group (such as biotin) or a detectable marker group (such as a fluorescent group, a radioisotope or an enzyme). If desired, the antibodies (whether polyclonal or monoclonal) may be labeled using conventional techniques. Suitable labels include fluorophores, chromophores, radioactive atoms, electron-dense reagents, enzymes, and ligands having specific binding partners. Enzymes are typically detected by their activity. For example, horseradish peroxidase can be detected by its ability to convert tetramethylbenzidine (TMB) to a blue pigment, quantifiable with a spectrophotometer. Other suitable binding partners include biotin and avidin or streptavidin, IgG and protein A, and the numerous receptor-ligand couples known in the art.

EXAMPLES

EXAMPLE 1: Isolation of DNA

[094] Potential novel aggrecanase family members were identified using a database screening approach. Aggrecanase-1 (*Science* 284:1664-1666 (1999)) has at least six domains: signal, propeptide, catalytic domain, disintegrin, tsp (thrombospondin), and c-terminal. The catalytic domain contains a zinc binding signature region, TAAHELGHVKF (SEQ. ID NO: 6) and a "MET turn" which are responsible for protease activity. Substitutions within the zinc binding region in the number of the positions still allow protease activity, but the histidine (H) and glutamic acid (E) residues must be

present. The thrombospondin domain of Aggrecanase-1 is also a critical domain for substrate recognition and cleavage. It is these two domains that determine our classification of a novel aggrecanase family member. The coding region of the aggrecanase-1 DNA sequence was used to query against the GeneBank ESTs focusing on human ESTs using TBLASTN. The resulting sequences were the starting point in an effort to identify a sequence for potential family members. A particular nucleotide sequence of the aggrecanase of the present invention, referred to as ADAMTS-18 or EST18, is depicted in Figures 1A and 1B (SEQ ID NO: 1).

[095] The virtual EST18 sequence is set forth in Figures 5A and 5B (SEQ ID NO: 5). Based on the initial virtual sequence, a set of PCR primers was designed to amplify approximately 1200 base pairs spanning the pro and catalytic domain of EST18. This primer set was used to screen cDNA molecules from different types of tissue to identify tissue sources for aggrecanase molecules. Once the tissue sources were identified, two overlapping fragments of EST18 were amplified by PCR using the cDNA molecule and the amplified fragments were cloned into vectors for sequencing. Cloned sequences differed from the predicted sequence therefore, multiple replicas of each reaction were cloned and sequenced from three independent tissue sources. Based on sequence analysis of all the clones, a consensus open reading frame (ORF) of 3219 base pairs was determined (SEQ ID NO: 3). It is contemplated that this 3219 bp ORF frame does not represent the full-length gene, as further described below. The obtained sequence may be utilized to screen for and isolate the full length sequence. Since the PCR conditions used to amplify the EST18 sequence promoted the introduction of errors, the 3219 bp ORF had to be constructed by amplifying multiple overlapping fragments, digesting them with specific restriction enzymes, followed by final ligation into the mammalian expression vector called pED.

[096] Specifically, marathon-readyTM cDNA, brain, stomach, and thymus (Clontech, Palo Alto, CA) was used as a template in all PCR cloning reactions. All the PCR reactions were carried out in a Perkin-Elmer 9600 thermocycler (Wellesley, MA) utilizing the following cycling parameters: 94°C for 30 sec, 5 cycles of 94°C for 5 sec, 72°C for 4 min, 5 cycles of 94°C for 5 sec, 70°C for 4 min, 30 cycles of 94°C for 5 sec, 68°C 4 min. Clontech's AdvantageTM GC2 polymerase was used with a final concentration of 0.5 M GC-melt according to the manufacturer's recommendations (Clontech, Palo Alto, CA). The various primer sets used for amplifying each fragment of the putative full-length nucleotide for EST18 are depicted in Figure 6A as the sequences set forth in SEQ ID NOs.: 9, 10, 11 and 12.

[097] PCR products were digested with different enzymes, as shown in Figure 6B, and then fractionated on a 1 or 1.5% agarose gel. DNA bands corresponding to the indicated digested sizes were recovered from the gel. Ligation reaction included equal molar ratios of the digested DNA fragments and the vector pED pre-digested with EcoRI and Sall. A particular cDNA construction using various amplification fragments was confirmed by DNA sequencing and is set forth in Figure 3. (SEQ ID NO: 3)

[098] The predicted amino acid sequence (SEQ ID NO: 4) of the aggrecanase of the present invention is set forth in Figure 4. The cloned sequence appears to have 3 TSP sub-motifs. A TSP sub-motif is described as about 50 amino acids, it starts with signature WXXXXW and contains six cysteine residues. The third sub-motif in the sequence set forth in Figure 4 consists of 41 amino acids, starts with WXXXXW and contains 4 cysteins. It is therefore contemplated that there are at least 10 additional amino acids, assuming that there are no additional TSP submotifs. The majority of aggrecanase of the invention is found in the three tissue sources: brain, stomach, and thymus.

[099] An aggrecanase molecule according to the invention as set forth in Figure 4 may be characterized as follows: The pre-pro region signal-sequence,

LLQALQLCCLCCA- (SEQ ID NO: 13

SVAAALASDSSSGASGLNDDYVFVTPVEVDSAGSYISHDILHNGRKKRSA

(SEQ ID NO: 14)

| (signal) | (mature peptide) 5 18

contains three conserved cysteine residues and a furin site. The catalytic domain is characterized by a typical zinc binding motif. It contains 5 conserved cysteine residues upstream of the zinc binding sequence and three residues downstream of the zinc binding sequence. It also contains a conserved methionine "Met-turn" downstream of the zinc binding sequence. The Disintegrin-like domain contains eight conserved cysteine residues. The TSP module contains a heparin binding domain (WXXWXXW); a CD36-binding motif (CSRTC GG) (SEQ ID NO: 15); and six conserved cysteine residues. The cysteine-rich domain is characterized as containing ten conserved cysteines. The spacer domain is characterized by TSP-repeats wherein two and one half have been cloned. The N-terminal portion of the aggrecanases can be cloned using the sequences described. The TSP sub-motifs start with signature WXXXXW and contain six cysteines. The third motif in Figure 4 has 4 cysteines.

[0100] The ADAMTS-18 nucleotide sequence was extended beyond the original sequence by 5' and 3' RACE. Thymus Marathon-Ready™ cDNA was purchased from Clontech (Palo Alto, CA) for use as a template in PCR cloning reactions. The antisense primer 5' TGGTATGATTCACGACGGAGAAGGG (SEQ ID NO: 16) was used in a first

round 5' RACE reaction and the sense primer 5' CGGGTCACCATCCTCACGTACTGTA (SEQ ID NO: 17) was used in the first round 3' RACE reaction, both in combination with the AP-1 end primers specific to the Marathon cDNAs. Clontech AdvantageTM GC2 polymerase reagents (Clontech, Palo Alto, CA) were used according to the manufacturer's recommendations. All amplifications were carried out in a Perkin-Elmer 9600 thermocycler (Perkin Elmer, Wellesley, MA). Cycling parameters were 94°C for 30 sec., 5 cycles of 94°C for 5 sec., 72°C for 4 mins., 5 cycles of 94°C for 5 sec, 70°C for 4 mins., 30 cycles of 94°C for 5 sec, 68°C for 4 min. The first round reactions were diluted 10 fold in TE, and 5µl of the reaction mixture was used as a template for a second round of PCR. The antisense primer 5' AACCTCGTGGTGGCAGACAAG (SEQ ID NO: 18) was used for second round 5' RACE and the sense primer 5' TCATTCCAGCTGGCGCCCGAAGCAT (SEQ ID NO: 19) was used for second round 3' RACE utilizing the identical parameters as described for the first round, except with the AP-2 end primers specific to the Marathon cDNAs.. Aliquots of each reaction were fractionated on a 1% agarose gel and then transfer to nitrocellulose for Southern analysis. The nitrocellulose membrane was prehybridized in Clontech ExpressHybTM (Clontech, Palo Alto, CA) for 30 min. at 37°C according to the manufacture recommendations. The membrane was then incubated with 1x10⁶ CPM of α-ATP end-labeled oligos 5' CTGCCTCTGCTGTGCGTCGGTCGC (SEQ ID NO: 11) (5' RACE) or 5' GATAACTTTCCCAGAGCGAAGATGC (SEQ ID NO: 20) (3' RACE) at 37°C for 1 hour. Unbound probe was removed by two washes at room temperature with 2X SSC/0.05% SDS followed by two additional washes at room temperature with 0.1X SSC/0.1% SDS. Duplicate agarose gels were run and the PCR products that corresponded with positive signals on the autoradiographs were excised out of the agarose gel and DNA was recovered from the gel matrix via BioRad's Prep-A-Gene DNA purification System. (Biorad, Hercules, CA). The recovered DNA was ligated into Stratagene's

PCR-Script[®] Amp Cloning (Stratagene, La Jolla, CA) according to the manufacturer's instructions.

[0101] An aliquot of the ligation mixtures were transformed into Gibco Technologies Electromax DH10B cells according to the manufacturer's instructions. (Carlsbad, CA). Plasmid DNA was subsequently isolated from the resulting recombinant bacteria and the DNA was sequenced. In one experiment, the 5' RACE reactions yielded a total of 1065 bases, 156 bases of the 5' UTR, followed by a methionine that initiated the 909 base pairs of an open reading frame ending in the sequence that is described as the second round antisense primer (SEQ ID NO: 18). The 3' RACE reactions produced a total of 2368 bases, 1358 bases of coding sequence beginning with the sequence described as the second round sense primer (SEQ ID NO: 19), ending with a translational stop codon followed by 1007 base pairs of 3' UTR.

EXAMPLE 2: EST18 Tissue Expression

[0102] A Clontech human multiple tissue expression array MTE[™] (Clontech Catalog #: 7776-1) was probed with a 533 base pair α -³²P dCTP-labeled cDNA probe according to the manufacturer's guidelines. Probe labeling and hybridization were performed as follows: 5 μ g of A18FS plasmid (described below) was digested with EcoRI enzyme in its optimal buffer according to the vendor's recommendations. The restriction digest was fractionated on a 1% agarose gel and a 533 base pair fragment encoding EST18 protein sequence including amino acid #1 (methionine) through amino acid #174 (asparagine) of SEQ ID NO: 2 was recovered from the agarose gel as outlined above. An α -³²P dCTP-labeled probe was made utilizing Amersham Pharmacia's Ready-To-Go kit (Catalog #: 27-9240-01, Pharmacia,). Briefly, 30 ng of heat-denatured DNA was incubated at 37°C for 15 minutes with 50 μ Ci of α -³²P dCTP and one labeling bead. Following the incubation, the reaction mix was applied to a pre-equilibrated Pharmacia NICK column (Catalog #: 17-0855-02) to remove unincorporated α -³²P dCTP from the

labeled probe. The desalted probe was assayed and 15×10^6 cpm was added to 5 ml of pre-warmed ExpressHyb. The hybridization mix was then transferred to a prehybridized MTE. Hybridization was allowed to proceed overnight with agitation at 65°C.

[0103] Probe detection: Following hybridization, the MTE was washed in a series of buffers accordingly to the manufacturer's guidelines. The MTE was then placed in a X-ray cassette with Kodak BioMax MS film (Kodak) and one intensifying screen. The cassette was then stored at -70°C. Individual films were developed after either 20 or 76 hours. The results after either exposure were identical. Expression was restricted to left and right cerebellum, corpus callosum and placenta.

EXAMPLE 3: Expression of a Truncated form the Aggrecanase Protein

[0104] A truncated form of protein encoded by the EST18 nucleotide sequence was expressed as a fusion protein. One such truncated protein, A18FS, refers to the first 650 amino acids, from amino acid #1 (methionine) to amino acid #650 (phenylalanine) encoded by the EST18 nucleotide sequence. The expression construct was generated in two steps. First, the 5' end of EST18 nucleotide sequence was modified to include the additional coding nucleotide sequence identified by 5' RACE. Second, the construct had an open reading frame, such that it ended at the codon for phenylalanine. A Streptavidin-Tag sequence was added to aid in purification of the recombinant protein.

[0105] Modification of the 5' end: The six synthetic oligonucleotides listed below were designed to anneal together to form a DNA sequence flanked by an EcoRI site on the 5' end and a SacII site on the 3' end. The cloned EST18 sequence was digested with EcoRI and SacII enzymes. The digested vector was fractionated on a 1% agarose gel and the recovered DNA was ligated with the synthetic oligonucleotides. The oligonucleotides are depicted below:

5' AATTCCCACCATGGAGTGCGCCCTCCTGCTCGCGTGTGCCT 3' (SEQ ID NO: 21);

5'
CCCACCATGGAGTGCGCCCTCCTGCTCGCGTGTGCCTTCCCGGCTGCG
3' (SEQ ID NO: 22);

5'
TCCCGGCTGCGGGTTCGGGCCCCGCCGAGGGGCCTGGCGGGACTGGGG
CGC
GTGGCCAAG 3' (SEQ ID NO: 23);

5'
GGTTCGGGCCCCGCCGAGGGGCCTGGCGGGACTGGGGCGCGTGGCCAA
GGC
GCTCCAGCT 3' (SEQ ID NO: 24);

5' GCGCTCCAGCTGTGCTGCCTCTGCTGTGCGTCGGTCGCCGC 3' (SEQ ID NO: 25); and

5' GTGCTGCCTCTGCTGTGCGTCGGTCGCC 3' (SEQ ID NO: 26).

An aliquot of the ligation mix was transformed into Gibco Life Technologies ElectroMax DH10B cells and the sequence of the recombinant plasmid was confirmed by sequencing.

A18FS truncation and Streptavidin-Tagging: A18FS was PCR amplified using the following primer pair

Forward primer 5' CTCGCGGTTGAGGACAACTCTTCG 3' (SEQ ID NO: 27)
and

Reverse primer

5'CCCTTGCAATGAAAATAGCTTGGATTTTGAAGCGCTTGGAGCCACCC
GCAGTTCGAAAAATAAGGCGGCCGCGCAAA 3' (SEQ ID NO: 28)

and the EST18 nucleotide sequence as template. The forward primer contained the unique restriction site BglII and the reverse primer contained the unique restriction sites NotI to allow for directional cloning into the pre-digested expression vector. The reverse primer also included the resulting

protein sequence GSAWSHPQFEK (SEQ ID NO: 29) that functions as an epitope tag.

[0106] PCR amplification was preformed in a 50 µl volume reaction containing: 5µl 10X PCR reaction buffer; 1 µl dNTP mix up to the final concentration of 0.2 mM; 10 pmoles of the forward primer (SEQ ID NO: 27; 10 pmoles of the reverse primer ((SEQ ID NO: 28); 1 ng of the EST18 full-length nucleotide template as depicted in SEQ ID NO: 1; 2.5 units of the Stratagene Pfu Turbo Hotstart polymerase (Catalog # 600320); and distilled H₂O up to 50 µl. Amplification reaction conditions were 94°C for 2 mins; 94°C for 15 secs; amplification at 70°C for 3 mins for a total of 22 cycles; and extension at 72°C for 5 mins followed by chilling at 4°C. The nucleotide sequence encoding the truncated form of aggrecanase protein including a Streptavidin tag is disclosed in SEQ ID NO: 7.

EXAMPLE 4: Expression of Aggrecanase in CHO cells

[0107] In order to produce murine, human or other mammalian aggrecanase-related proteins, the DNA encoding an aggrecanase protein is cloned into an appropriate expression vector and introduced into mammalian cells or other preferred eukaryotic or prokaryotic hosts, including insect host cell culture systems, using conventional genetic engineering techniques. Expression systems for biologically active recombinant human aggrecanase are contemplated to include stably transformed mammalian, insect, yeast or bacterial cells.

[0108] The mammalian expression vector pMT2 CXM is a derivative of p91023(b) (Wong *et al.*, *Science* 228:810-815 (1985)) and differs from the latter in that it contains an ampicillin resistance gene in place of a tetracycline resistance gene and further contains a XhoI site for insertion of cDNA molecules into the vector. The functional elements of pMT2 CXM have been described (Kaufman, *Proc. Natl. Acad. Sci. USA* 82:689-693 (1985)) and include adenovirus VA genes, the SV40 origin of replication including the 72 bp enhancer, the adenovirus major late promoter including a 5' splice site and

majority of the adenovirus tripartite leader sequence present on adenovirus late mRNAs, a 3' splice acceptor site, a DHFR insert, the SV40 early polyadenylation site (SV40), and pBR322 sequences needed for propagation in *E. coli*.

[0109] Plasmid pMT2 CXM was obtained by EcoRI digestion of pMT2-VWF, which has been deposited with the American Type Culture Collection (ATCC), Rockville, MD (USA) under accession number ATCC 67122. EcoRI digestion excises the cDNA insert present in pMT2-VWF, yielding pMT2 in linear form which can be ligated and used to transform *E. coli* HB 101 or DH-5 which are then resistant to ampicillin. Plasmid pMT2 DNA can be prepared by conventional methods. pMT2 CXM is then constructed using loopout/in mutagenesis technique (Morinaga, *et al.*, *Biotechnology* 84: 636 (1984)). This removes bases 1075 to 1145 relative to the Hind III site near the SV40 origin of replication and enhancer sequences of pMT2. In addition it inserts the following sequence: 5' CATGGGCAGCTCGAG 3' (SEQ. ID NO: 30) at nucleotide 1145. This sequence contains the recognition site for the restriction endonuclease Xho I. A derivative of pMT2CXM, termed pMT23, contains recognition sites for the restriction endonucleases PstI, Eco RI, Sall and XhoI. Plasmid pMT2 CXM and pMT23 DNA may be prepared by conventional methods.

[0110] pEMC2 β 1 derived from pMT21 may also be suitable in practice of the invention. pMT21 was derived from pMT2 which is derived from pMT2-VWF. As described above, EcoRI digestion excises the cDNA insert present in pMT-VWF, yielding pMT2 in linear form which subsequently can be ligated and used to transform *E. Coli* HB 101 or DH-5 resulting in ampicillin resistance. Plasmid pMT2 DNA can be prepared by conventional methods.

[0111] pMT21 was derived from pMT2 through the following two modifications. First, 76 bp of the 5' untranslated region of the DHFR cDNA, including a stretch of 19 G residues from G/C tailing for cDNA cloning, is

deleted. In this process, a XhoI site was inserted to obtain the following sequence immediately upstream from DHFR:

5' CTGCAGGCGAGCCTGAATTCCTCGAGCCATCATG 3' (SEQ. ID NO: 31)

PstI

Eco RI XhoI

[0112] Second, a unique ClaI site was introduced by digestion with EcoRV and XbaI, treatment with Klenow fragment of DNA polymerase I, and ligation to a ClaI linker (CATCGATG). This deletes a 250 bp segment from the adenovirus associated RNA (VAI) region but does not interfere with VAI RNA gene expression or function. pMT21 was digested with EcoRI and XhoI, and used to derive the vector pEMC2B1.

[0113] A portion of the EMCV leader was obtained from pMT2-ECAT1 (S.K. Jung, *et al.*, *J. Virol* 63:1651-1660 (1989)) by digestion with Eco RI and PstI, resulting in a 2752 bp fragment. This fragment was digested with TaqI yielding an Eco RI-TaqI fragment of 508 bp which was isolated by electrophoresis on low melting agarose gel. A 68 bp adapter and its complementary strand were synthesized with a 5' TaqI protruding end and a 3' XhoI protruding end which has the following sequence:

5

CGAGGTTAAAAAACGTCTAGGCCCCCGAACCACGGGGACGTGGTTTTTCCTT
T

TaqI

GAAAAACACGATTGC 3' (SEQ. ID NO: 32)

XhoI

[0114] This sequence matches the EMC virus leader sequence from nucleotide 763 to 827. It also changes the ATG at position 10 within the EMC virus leader to an ATT and was followed by a XhoI site. A three way ligation of the pMT21 Eco RI-XhoI fragment, the EMC virus EcoRI-TaqI fragment, and the 68 bp oligonucleotide adapter TaqI-XhoI adapter resulting in the vector pEMC2B1.

[0115] This vector contains the SV40 origin of replication and enhancer, the adenovirus major late promoter, a cDNA copy of the majority of the adenovirus tripartite leader sequence, a small hybrid intervening sequence, an SV40 polyadenylation signal and the adenovirus VA I gene, DHFR and β -lactamase markers and an EMC sequence, in appropriate relationships to direct the high level expression of the desired cDNA in mammalian cells.

[0116] In one example, the aggrecanase nucleotide sequence of the present invention set forth in SEQ ID NO: 1 may be cloned into the expression vector pED6 (Kaufman *et al.*, *Nucleic Acid Res* 19:44885-4490 (1991)). COS and CHO DUKX B11 cells were transiently transfected with the aggrecanase sequence of the invention (+/- co-transfection of PACE on a separate pED6 plasmid) by lipofection (LF2000, Invitrogen, Carlsbad, CA)). Duplicate transfections were performed for each gene of interest: (a) one for harvesting conditioned media for activity assay and (b) one for ^{35}S methionine/cysteine metabolic labeling.

[0117] On day one, media was changed to DME(COS) or alpha(CHO) media + 1% heat-inactivated fetal calf serum +/- 100 $\mu\text{g/ml}$ heparin for one set of transfections (a) to be harvested for activity assay. After 48 h (day 4), conditioned media was harvested for activity assays.

[0118] On day 3, the medium for cells of the duplicate set of transfections (b) was changed to MEM (methionine-free/cysteine free) media + 1% heat-inactivated fetal calf serum + 100 $\mu\text{g/ml}$ heparin + 100 $\mu\text{Ci/ml}$ ^{35}S -methionine/cysteine (RedivueTM Pro mix, Amersham, Piscataway, NJ). Following a 6 h incubation at 37°C, conditioned media was harvested and run on SDS-PAGE gels under reducing conditions. Proteins were visualized by autoradiography.

[0119] In another example, the aggrecanase nucleotide sequence of the present invention set forth in SEQ ID NO: 1 may be cloned into expression vector pHTop, a derivative of pED (Kaufman *et al.*, 1991 NAR 19:4485-4490)

in which the majority of the adenomajor late promoter was replaced by six repeats of the tet operator (described in Gossen *et al.*, 1992, *Proc. Natl. Acad. Sci. USA* 89:5547-5551). This vector contains the dihydrofolate reductase gene and when introduced in the cell line CHO/A2 (see description below) functions very efficiently and high expressors can be selected by isolating cells surviving in high methotrexate concentrations.

[0120] Similarly, the recombinant aggrecanase protein set forth in SEQ ID NO: 8 and as expressed using a method described can be cloned into a pHTop vector.

[0121] Establishment of CHO stable cell lines: The CHO/A2 cell line was derived from CHO DUKX B11 (Urlaub and Chasin, 1980, *Proc. Natl. Acad. Sci. USA* 77:4216-4220) by stably integrating a transcriptional activator (tTA), a fusion protein between the Tet repressor and the herpes virus VP16 transcriptional domain (Gossen *et al.*, 1992, *Proc. Natl. Acad. Sci. USA* 89: 5547-5551). A CHO cell line expressing extracellular ADAMTS-18 was established by transfecting (lipofection) pHTopADAMTS8-Streptavidin tagged DNA into CHO/A2 cells and selecting clones in 0.02, 0.05 and 0.01 μ M methotrexate.

[0122] Screening of CHO stable cell lines: Multiple clones were screened by Western Blot using a streptavidin HRP antibody. The best clone was determined by virtue of its high expression and was one which resulted from 0.02 μ M MTX selection and was chosen to be scaled up for roller bottle conditioned media production (4 Liters). The cell line was sent for large scale production.

EXAMPLE 5: Biological Activity of Expressed Aggrecanase

[0123] To measure the biological activity of the expressed aggrecanase-related proteins, for example, proteins obtained in Example 4 above, the proteins are recovered from the cell culture and purified by isolating the aggrecanase-related proteins from other proteinaceous materials with which they are co-produced as well as from other contaminants.

Purification is carried out using standard techniques known to those skilled in the art. The isolated protein may be assayed in accordance with the following assays:

[0124] Assays specifically to determine if the protein is an enzyme capable of cleaving aggrecan at the aggrecanase cleavage site:

[0125] 1: Fluorescent peptide assay: Expressed protein is incubated with a synthetic peptide which encompasses amino acids at the aggrecanase cleavage site of aggrecan. Either the N-terminus or the C-terminus of the synthetic peptide is labeled with a flourophore and the other terminus includes a quencher. Cleavage of the peptide separates the flourophore and quencher and elicits flourescence. From this assay it is determined that the expressed aggrecanase protein can cleave aggrecan at the aggrecanase site , and relative fluorescence is a determination the relative activity of the expressed protein.

[0126] 2. Neoepitope western: Expressed aggrecanase protein is incubated with intact aggrecan. After several biochemical manipulations of the resulting sample (dialysis, chondroitinase treatment, lyophilization and reconstitution) the sample is run on an SDS PAGE gel. The gel is incubated with an antibody that is specific to a site on aggrecan which is only exposed after aggrecanase cleavage. The gel is transferred onto nitrocellulose paper and developed using a secondary antibody (called a western assay) which subsequently results in a banding pattern indicative of products with a molecular weight consistent with aggrecanase generated cleavage products of aggrecan. This assay results in the finding that the expressed aggrecanase protein cleaved native aggrecan at the aggrecanase cleavage site, and also gives the molecular weight of the cleavage products. Relative density of the bands can give an indication of relative aggrecanase activity.

[0127] Assay to determine if an expressed protein can cleave aggrecan anywhere in the protein (not specific to the aggrecanase site):

[0128] 3. Aggrecan ELISA: Expressed protein is incubated with intact aggrecan which had been previously adhered to plastic wells. The wells are washed and then incubated with an antibody that detects aggrecan. The wells are developed with a secondary antibody. If the original amount of aggrecan remains in the wells, the antibody staining is dense. Whereas, if aggrecan was digested by aggrecanase activity of the expressed aggrecanase protein, the aggrecan comes off the plate and the subsequent staining of the aggrecan coated wells by the antibody is reduced. This assay tells whether an expressed protein is capable of cleaving aggrecan (anywhere in the protein, not only at the aggrecanase site) and can further determine relative aggrecan cleavage.

[0129] Protein analysis of the isolated proteins is conducted using standard techniques such as SDS-PAGE acrylamide (Laemmli, *Nature* 227:680 (1970)) stained with silver (Oakley, *et al.*, *Anal Biochem.* 105:361 (1980)) and by immunoblot (Towbin, *et al.*, *Proc. Natl. Acad. Sci. USA* 76:4350 (1979)). Using the above described assays, expressed aggrecanase-related proteins are evaluated for their activity and useful aggrecanase-related molecules are identified.

EXAMPLE 6: Aggrecanase Activity of ADAMTS-18

[0130] Bovine articular cartilage was incubated with isolated ADAMTS-18 for 16 h at 37°C in 50 mM Tris, pH 7.3, containing 100 mM NaCl and 5 mM CaCl₂. Digestion products were deglycosylated by incubation for 2 h at 37°C in the presence of chondroitinase ABC (Seikagaku America, Falmouth, MA; 1 mU/μg aggrecan), keratinase (Seikagaku, 1 mU/μg aggrecan) and keratanase II (Seikagaku; 0.02 mU/μg aggrecan). After separation by SDS-PAGE, digestion products were transferred to nitrocellulose and detected by Western immunoblotting with the neoepitope (monoclonal) antibody AGG-C1 which recognizes the C-terminal neoepitope sequence-NITEGE³⁷³ (SEQ ID NO: 33) generated by cleavage of the

aggrecanase-susceptible E³⁷³-A³⁷⁴ peptide bond located between the G1 and G2 domains of aggrecan. (Figure 10).

EXAMPLE 7: Preparation of Antibodies

[0131] An antibody against a novel aggrecanase molecule is prepared. To develop an antibody capable of inhibiting aggrecanase activity, a group of mice are immunized every two weeks with a novel aggrecanase protein mixed in Freund's complete adjuvant for the first two immunizations, and incomplete Freund's adjuvant thereafter. Throughout the immunization period, blood is sampled and tested for the presence of circulating antibodies. At week 9, an animal with circulating antibodies is selected, immunized for three consecutive days, and sacrificed. The spleen is removed and homogenized into cells. The spleen cells are fused to a myeloma fusion partner (cell line P3-x63-Ag8.653-]) using 50% PEG 1500 by an established procedure (Oi & Herzenberg, *Selected Methods in Cellular Immunology*, W. J. Freeman Co., San Francisco, CA, at 351 (1980)). The fused cells are plated into 96-well microtiter plates at a density of 2×10^5 cells/well. After 24 hours, the cells are subjected to HAT selection (Littlefield, *Science*, 145: 709 (1964)) effectively killing any unfused and unproductively fused myeloma cells.

[0132] Successfully fused hybridoma cells secreting anti-aggrecanase antibodies are identified by solid and solution phase ELISAs. Novel aggrecanase protein is prepared from CHO cells as described above and coated on polystyrene (for solid phase assays) or biotinylated plates (for a solution based assay). Neutralizing assays are also employed where aggrecan is coated on a polystyrene plate and biotin aggrecanase activity is inhibited by the addition of hybridoma supernatant. Results identify hybridomas expressing aggrecanase antibodies. These positive clones are cultured and expanded for further study. These cultures remain stable when expanded and cell lines are cloned by limiting dilution techniques and subsequently cryopreserved.

[0133] From these cell cultures, a panel of antibodies is developed that specifically recognize aggrecanase proteins. Isotype of the antibodies is determined using a mouse immunoglobulin isotyping kit (Zymed™ Laboratories, Inc., San Francisco, CA).

EXAMPLE 8: Method of Detecting Level of Aggrecanase

[0134] An anti-aggrecanase antibody prepared according to the invention as described, can be used to detect level of aggrecanases in a sample. An antibody can be used in an ELISA, for example, to identify the presence or absence, or quantify the amount of, an aggrecanase in a sample, to which the antibody binds. The antibody can be labeled with a fluorescent tag. In general, the level of aggrecanase in a sample can be determined using any of the assays disclosed.

EXAMPLE 9: Method of Treating a Patient

[0135] Antibodies developed according to methods disclosed can be administered to patients suffering from a disease or disorder related to the loss of aggrecan, or an increase in aggrecanase activity. Patients may need to take a composition of the invention as a once time administration or at intervals, such as once daily, until the symptoms and signs of their disease or disorder improve. For example, subsequent to the administration of a composition of the invention to a patient, loss of aggrecan decreases or ceases and degradation of articular cartilage decreases or ceases. It is expected that symptoms of osteoarthritis would be reduced or eliminated. This would show that compositions of the invention would be useful for the treatment of diseases or disorders related to the loss of aggrecan, or an increase in the levels and/or activity of aggrecanases. Antibodies can also be used with patients that are susceptible to osteoarthritis, such as those who have a family history or markers of the disease, but are asymptomatic. The following results would be expected for treatment of patients.

Patient's Condition	Route of Administration	Dosage	Frequency	Predicted Results
Osteoarthritis	Subcutaneous	500 µg/kg	Daily	Decrease in symptoms
"	"	1 mg/kg	Weekly	"
"	Intramuscular	500 µg/kg	Daily	"
"	"	1 mg/kg	Weekly	"
"	Intravenous	500 µg/kg	Daily	"
"	"	1 mg/kg	Weekly	"
Family History of Osteoarthritis	Subcutaneous	500 µg/kg	Daily	Prevention of condition
"	Intramuscular	500 µg/kg	Daily	"
"	Intravenous	500 µg/kg	Daily	"

[0136] The foregoing descriptions detail presently preferred embodiments of the present invention. Numerous modifications and variations in practice thereof are expected to occur to those skilled in the art upon consideration of these descriptions. Those modifications and variations are believed to be encompassed within the claims appended hereto. All of the documents cited in this application are incorporated by reference in their entirety. Additionally, all sequences cited in databases and all references disclosed are incorporated by reference in their entirety.

SEQUENCE LISTING

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<211> 1071

<212> PRT

<213> Homo sapiens

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```

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```

```

Gly Leu Asn Asp Asp Tyr Val Phe Val Thr Pro Val Glu Val Asp Ser
      35             40             45

```

```

Ala Gly Ser Tyr Ile Ser His Asp Ile Leu His Asn Gly Arg Lys Lys
      50             55             60

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```

Arg Ser Ala Gln Asn Ala Arg Ser Ser Leu His Tyr Arg Phe Ser Ala
      65             70             75             80

```

```

Phe Gly Gln Glu Leu His Leu Glu Leu Lys Pro Ser Ala Ile Leu Ser
      85             90             95

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```

Ser His Phe Ile Val Gln Val Leu Gly Lys Asp Gly Ala Ser Glu Thr
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Gln Lys Pro Glu Val Gln Gln Cys Phe Tyr Gln Gly Phe Ile Arg Asn
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Asp Ser Ser Ser Ser Val Ala Val Ser Thr Cys Ala Gly Leu Ser Gly
      130            135            140

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```

Leu Ile Arg Thr Arg Lys Asn Glu Phe Leu Ile Ser Pro Leu Pro Gln
      145            150            155            160

```

```

Leu Leu Ala Gln Glu His Asn His Ser Ser Pro Ala Gly His His Pro
      165            170            175

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His Val Leu Tyr Lys Arg Thr Ala Glu Glu Lys Ile Gln Arg Tyr Arg
      180            185            190

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Ile	Pro	His	Ala	Ser	Gln	Ser	Arg	Glu	Thr	Glu	Tyr	His	His	Arg	Arg	210	215	220	
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Pro	Pro	Thr	Glu	Asp	Thr	Tyr	Leu	Arg	Phe	Asp	Glu	Tyr	Gly	Ser	Ser	245	250	255	
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Ser	Leu	Ile	Leu	Leu	Glu	Gln	Glu	Pro	Gly	Gly	Leu	Leu	Ile	Asn	His	325	330	335	
His	Ala	Asp	Gln	Ser	Leu	Asn	Ser	Phe	Cys	Gln	Trp	Gln	Ser	Ala	Leu	340	345	350	
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Phe	Asp	Ile	Cys	Ser	Trp	Lys	Asn	Glu	Pro	Cys	Asp	Thr	Leu	Gly	Phe	370	375	380	
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Glu	Asp	Thr	Gly	Leu	Gly	Leu	Ala	Phe	Thr	Ile	Ala	His	Glu	Ser	Gly	405	410	415	
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Ala	Glu	Gly	Asn	Ile	Met	Ser	Pro	Thr	Leu	Thr	Gly	Asn	Asn	Gly	Val	435	440	445	
Phe	Ser	Trp	Ser	Ser	Cys	Ser	Arg	Gln	Tyr	Leu	Lys	Lys	Phe	Leu	Ser	450	455	460	
Thr	Pro	Gln	Ala	Gly	Cys	Leu	Val	Asp	Glu	Pro	Lys	Gln	Ala	Gly	Gln	465	470	475	480

Tyr	Lys	Tyr	Pro	Asp	Lys	Leu	Pro	Gly	Gln	Ile	Tyr	Asp	Ala	Asp	Thr
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Gln	Cys	Lys	Trp	Gln	Phe	Gly	Ala	Lys	Ala	Lys	Leu	Cys	Ser	Leu	Gly
			500					505					510		
Phe	Val	Lys	Asp	Ile	Cys	Lys	Ser	Leu	Trp	Cys	His	Arg	Val	Gly	His
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Arg	Cys	Glu	Thr	Lys	Phe	Met	Pro	Ala	Ala	Glu	Gly	Thr	Val	Cys	Gly
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Cys	Ser	Arg	Thr	Cys	Gly	Gly	Gly	Val	Lys	Phe	Gln	Glu	Arg	His	Cys
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Leu	Gly	Ser	Lys	Ala	Val	Ser	Asp	Ala	Cys	Gly	Val	Cys	Gly	Asp	Asn
705					710					715					720
Ser	Thr	Cys	Lys	Phe	Tyr	Lys	Gly	Leu	Tyr	Leu	Asn	Gln	His	Lys	Ala
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 Thr Gln Val Asn Ser Ser Phe Cys Ser Ala Lys Thr Lys Pro Val Thr
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 Glu Pro Lys Ile Cys Asn Ala Phe Ser Cys Pro Ala Tyr Trp Met Pro
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 915 920 925
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 930 935 940
 Val Leu His Ser Leu Cys Pro Val Ser Thr Pro Thr Gln Val Gln Ala
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<211> 3954

<212> DNA

<213> Homo sapiens

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WO 03/064622

PCT/US03/03058

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<210> 6

<211> 11

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Synthetic zinc
binding signature peptide sequence

<400> 6

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<210> 7

<211> 1986

<212> DNA

<213> Homo sapiens

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<210> 8

<211> 661

<212> PRT

<213> Homo sapiens

<400> 8

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```

```

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```

```

Gln Leu Cys Cys Leu Cys Cys Ala Ser Val Ala Ala Ala Leu Ala Ser
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```

```

Asp Ser Ser Ser Gly Ala Ser Gly Leu Asn Asp Asp Tyr Val Phe Val
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```

Thr Pro Val Glu Val Asp Ser Ala Gly Ser Tyr Ile Ser His Asp Ile
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```

```

Leu His Asn Gly Arg Lys Lys Arg Ser Ala Gln Asn Ala Arg Ser Ser
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```

```

Leu His Tyr Arg Phe Ser Ala Phe Gly Gln Glu Leu His Leu Glu Leu
          100                      105                      110

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Lys Pro Ser Ala Ile Leu Ser Ser His Phe Ile Val Gln Val Leu Gly
          115                      120                      125

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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	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	His	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	
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			
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
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		
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				
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	

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
 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
 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
 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
 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
 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
 Gly Ile 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 Gly Ser Ala Trp Ser His
 645 650 655
 Pro Gln Phe Glu Lys
 660

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<212> DNA
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<220>
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<220>
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<210> 12
<211> 25
<212> DNA
<213> Artificial Sequence

<220>
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<400> 12
ctattgaaag ggtctcgctt ctacg 25

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<210> 13
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 <212> PRT
 <213> Artificial Sequence

<220>
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 pre-pro signal peptide sequence

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 1 5 10

<210> 14
 <211> 50
 <212> PRT
 <213> Artificial Sequence

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 pre-pro signal peptide sequence

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 1 5 10 15

Leu Asn Asp Asp Tyr Val Phe Val Thr Pro Val Glu Val Asp Ser Ala
 20 25 30

Gly Ser Tyr Ile Ser His Asp Ile Leu His Asn Gly Arg Lys Lys Arg
 35 40 45

Ser Ala
 50

<210> 15
 <211> 7
 <212> PRT
 <213> Artificial Sequence

<220>
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 CD36-binding motif

<400> 15
 Cys Ser Arg Thr Cys Gly Gly
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<210> 16
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<220>
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<211> 25
<212> DNA
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<210> 18
<211> 22
<212> DNA
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<220>
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<220>
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<210> 20
<211> 25
<212> DNA
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<213> Artificial Sequence

<220>
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<220>
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<212> DNA
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<220>
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oligonucleotide

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<220>
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 oligonucleotide

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<210> 25
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<220>
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<220>
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 <211> 25
 <212> DNA
 <213> Artificial Sequence

<220>
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 <213> Artificial Sequence

<220>
 <223> Description of Artificial Sequence: Primer

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 ataaggcggc cgccgcaaa 79

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 <212> PRT
 <213> Artificial Sequence

<220>
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 peptide sequence

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 1 5 10

<210> 30
 <211> 15
 <212> DNA
 <213> Artificial Sequence

<220>
 <223> Description of Artificial Sequence: Primer

<400> 30
 catgggcagc tcgag 15

<210> 31
 <211> 34
 <212> DNA
 <213> Artificial Sequence

<220>
 <223> Description of Artificial Sequence: Primer

<400> 31
 ctgcaggcga gcctgaattc ctcgagccat catg 34

<210> 32
<211> 68
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Primer

<400> 32
cgagggttaaa aaacgtctag gccccccgaa ccacggggac gtggttttcc tttgaaaaac 60
acgattgc 68

What is claimed is:

1. An isolated DNA molecule comprising a DNA sequence chosen from:
 - a) the sequence of SEQ ID NO: 1 from nucleotide #1-#3663;
 - b) fragments of SEQ ID NO: 1;
 - c) variants of SEQ ID NO: 1;
 - d) sequences which hybridize under stringent conditions with SEQ ID NO: 1; and
 - e) naturally occurring human allelic sequences and equivalent degenerative codon sequences of (a) to (d).
2. A vector comprising a DNA molecule of claim 1 in operative association with an expression control sequence therefor.
3. A host cell transformed with the DNA sequence of claim 1.
4. A host cell transformed with a DNA sequence of claim 2.
5. A method for producing an isolated human aggrecanase protein, said method comprising:
 - a) culturing a host cell transformed with a DNA molecule according to claim 1; and
 - b) recovering and purifying said aggrecanase protein encoded by the DNA molecule from the culture medium.
6. The method of claim 5, wherein said host cell is an insect cell.
7. An isolated aggrecanase protein comprising an amino acid sequence chosen from:
 - a) the amino acid sequence of SEQ ID NO: 2 from amino acid #1-#1221;
 - b) fragments of SEQ ID NO: 2; and
 - c) variants of aggrecanase proteins consisting of addition, substitution, and deletion mutants of the sequences of (a) to (b).
8. An isolated aggrecanase protein produced by the steps of
 - a) culturing a cell transformed with a DNA molecule according to claim 1; and

- b) recovering and purifying from said culture medium a protein comprising an amino acid sequence chosen from SEQ. ID NO: 2.
- 9. An antibody that binds to an isolated aggrecanase protein of claim 7.
- 10. The antibody of claim 9, wherein the antibody inhibits aggrecanase activity.
- 11. A method for identifying inhibitors of aggrecanase comprising
 - a) providing an aggrecanase protein chosen from SEQ ID NO: 2 or a fragment thereof;
 - b) combining the aggrecanase protein with a potential inhibitor; and
 - c) evaluating whether the potential inhibitor inhibits aggrecanase activity.
- 12. The method of claim 11 wherein the method further comprises evaluating the aggrecanase protein in a three dimensional structural analysis prior to combining with the potential inhibitor.
- 13. The method of claim 11 wherein the method further comprises evaluating the aggrecanase protein in a computer aided drug design program prior to combining with the potential inhibitor.
- 14. A pharmaceutical composition for inhibiting the proteolytic activity of aggrecanase, wherein the composition comprises an antibody according to claim 9 and a pharmaceutical carrier.
- 15. A method for inhibiting aggrecanase in a mammal comprising administering to said mammal an amount of the composition of claim 14 effective to inhibit aggrecanase activity.
- 16. The method of claim 15, wherein the composition is administered intravenously, subcutaneously, or intramuscularly.
- 17. The method of claim 15, wherein the composition is administered at a dosage of about 500 µg/kg to about 1 mg/kg.

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FIG. 1A

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AACCCCTTGCAATGAAAATAGCTTGATTTTCGGGCCCAACAGTGTGCAGAAATATAACAGCAAAACCTTTCCGTGGATGGTT
CTACCAGTGGAACCCCTATACAAAAGTGGAAGAGGAAGATCGATGCAAACTGTACTGCAAGGCTGAGAACTTTGAAATTTT
TTTTTGCAATGTCCGGCAAGTGAAAGATGGAACCTCCCTGCTCCCAACACAGAAATGATGTTTGTAATTGACGGGTTTGT
GAACTAGTGGGATGTGATCATGAACTAGGCTCTAAAGCAGTTTCAGATGCTTGTGGCGTTTGCAAAAGTGATAATTCAAC
TTGCAAGTTTATAAAGCCCTGTACCTCAACCAGCATAAAGCAAAATGAATATTAATCCGGTGGTCATCATCCAGCTGGCG
CCCGAAGCATCGAAATCCAGGAGCTGCAGGTTTCCTCCAGTTACCTCGCAGTTCGAAGCCTCAGTCAAAAGTATTACCTC
ACCGGGGCTGGAGCATCGACTGGCCCTGGGAGTTCCCTCCCTGCTGGGACCACTGTTGAATACCAAGCGCTCTTCAACCG
CCCGAACGTCTGTACGCGCCAGGCCACAAAATGAGACGCTGGTCTTTGAAATCTGATGCAAGGCAAAATCCAGGGA
TAGCTTGGAAGTATGCACCTTCCCAAGGTCAATGAATGGAACCTCACCAAGCAAAAGACCTGCCCTATACCTGGAGTATC
GTGCAGTCAGAGTGCTCCGTCTCCTGTGGTGAGGTTACATAAATGTAAAGGCCATTGCTTGCGAGATCAAAATACTCA
AGTCAATTCCCTCATTTCTGCAGTGCAAAACCAAGCCAGTAACCTGAGCCCAAAATCTGCAACGCTTTCCTGCCCGGCTT
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CAGCCATGCCCTGTCTCCACAATGGAGCCTTGGAACCTTGACCCCTGAGAGCCAGTGTCCAAAGACCTGTGGACGAGGTGAGGAAAGC
GTGAACCTCCTGTGCAAGGCTCTGCGCGAGAAACCTCCCGAGAGCCAGTGTACCAAGTCTCCCAAGACCTGAGCTGCAG
GAGGCTGTGTGCTTGGAACGATGCCCAAGAACAGCCGGCTACAGTGGTCTTCTGTTGGAGCGAGTGTCTGCAAC
CTGTGGTTTGGGTGTGAGGAAGAGGGAGATGAAGTGCAGCGAGAGGGCTTCCAGGGAAGCTGATAACTTTCCAGAGC
GAAGATGCCGTAAATATAAGAAACCAATCTGGACTTGGAAGAGACCTGCAACCGACGGCTTGCCAGCCCATCCAGTG
TACAACATGGTAGCTGGATGGTATTCAATTGCCGTGGCAGCAGTGACAGTCACTGTGGGGAGGGTCCAGACCCGGTC
AGTCCACTGTGTTCAGCAAGGCCGCCCTTCCCAAGTTGTCTGCTCCATCAGAAACCTCCGGTGCTACGAGCCTGTAATA
CAAACTTCTGTCCAGCTCCTGAAAAGAGAGAGGATCCATCCTGCGTAGATTCTTCAACTGGTGTACCTAGTTCCTCAG
CATGGTGTCTGCAACCAACAAAGTTTTACGGAAAACAAATGCTGCAAGTCAATGCCACAAGGAAGATC

FIG. 1B

Full-length EST18 protein sequence: SEQ ID NO: 2

MECALLLACAFPAAGSGPPRGLAGLRVAKALQLCCLCCASVAAALASDSSGASGLNDDYVFVTPVEVDSAGSYISHDI
LHNGRKKRSAQNARSSLHYRFSAFGQELHLELKPSSAILSSHEIVQVLGKDGASETQKPEVQQCFYQGFIRNDSSSVAVS
TCAGLSGLIRTRKNEFLISPLPQLLAQEHNHSSPAGHHPHVLYKRTAEEKIQRYRGPYSGRNPYGPSPSHIPHASQSRE
TEYHHRRLQKHFCGRRKKYAPKPTEDTYLRFDEYGSGRPRRSAGKSQKGLNVETLVVADKKMVEKHGKGNVTYILT
VMNMVSGLFKDGTTIGSDINVVVSLILLEQEPGGLINHHADQSLNSFCQWQSAALIGKNGKRHDHAILLTGFDICSWKNE
PCDTLGEAPISGMSKYRSC TINEDTGLGLAFTIAHESGHNEFGMIHDGEGNPCRKAEGNIMSPTLTGNNGVFSWSSCSRQ
YLKKFLSTPQAGCLVDEPKQAGQYKYPDKLPQQIYDADTQCKWQFGAKAKLCSLGFVKDICKSLWCHRVGHRCE TKFMPA
AEGTVCGLSMWCRRQGQCVKFGELGPRPIHGQWSAWSKWSKSECSRTC GGGVKFQERHCNNPKPQYGGIFCPCGSSRIYQLCNI
NPCNENSLDFRAQQCAEYNSKPFGRWFYQWKPYTKVEEEDRCKLYCKAENFEFFAMSGKVKGDTPCSPNRNDVCIDGVC
ELVGCDELGSKAVSDACGVCKGDNSTCKFYKGLYLNQHKANEYYPVVIIPAGARSTFIQELQVSSSYLAVRSLSQYYL
TGGWSIDWPGEFFAGTTFEYQRSFNRPERLYAPGPTNETLVFEILMQGNPGIAWKYALPKVMNGTTPATKRPAYTWSI
VQSECSVSCGGYINVKAICLRDQNTQVNSSFCSAKTKPVTEPKICNAFSCPAYWMPGEWSTCSKACAGGQSRKIQCQVQ
KKPFQKEEAVLHSLCPVSTPTQVQACNSHACPPQWSLGPWSQCSKTCGRGVRKRELLCKGSAETLPESQCTSLPRPELQ
EGCVLGRCPKNSRLQWVASSWSECSATCGLGVRKREMKCSEKGFQGLITFFERRCRNIKKPNDLLEETCNRRACPAHPV
YNMVAGWYSLPWQQCTVTTCGGGVQTRS VHCVQQGRPSSCLLHQKPPVLRACNTNFCPAPEKREDPSCVDFFNWCHLVPQ
HGVCNHNKFGKQCKSCTRKI

FIG. 2

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ATGTCACCTTTTCTTGCAGGCGCTCCAGCTGTGCTGCCCTCTGCTGCGTGGTCGCCGGCCCTTAGCCAGTGACAG
CAGCAGCGCGCCAGCGGATTAAATGATGATTACGTCTTTGTCCAGCCAGTAGAAGTAGACTCAGCCGGGTCAATATATTT
CACACGACATTTTGCACAACGGCAGGAAAAGCGATCGGCGCAGAAATGCCAGAAGCTCCCTGCACCTACCGATTTTCAGCA
TTTGGACAGGAACTGCACCTTAGAACTTAAGCCCTCGCGGATTTTGAGCAGTCACCTTTATTGTCCAGGTACTTGGAAAAGA
TGGTGCTTCAGAGACTCAGAAACCCGAGGTGCAGCAATGCTTCTATCAGGGATTTATCAGAAATGACAGCTCCTCCTCTG
TCGCTGTCTACGTGTGCTGGCTTGTCAAGTTTAAATAAGGACACGAAAATAATGAATTCCTCATCTCGCCATTACCTCAG
CTTCTGCCCCAGGAACACAACACAGCTCCCCCTCGGGTCACCATCTCACGTACTGTACAAAAGGACAGCAGAGGAGAA
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GAATCCCTGCAGAAAGGCTGAAGGCAATATCATGTCTCCACACTGACCGGGGTGCTAGTGGATGGAATTTTCATGGTCTTCTT
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GTTCCAGGAGACACTGCAATAACCCCAAGCCTCAGTATGGTGGCATAATCTGTCCAGGTTCTAGCCGTATTTATCAGC
TGTGCAATATTAAACCTTGCAAATGAAAATAGCTTGGATTTTCCGGCCCCAACAGTGTGCAGAAATATAACAGCAACCTTTC
CGTGGATGTTCTACCAGTGGAAACCTTATACAAAAGTGGAAAGAGATCGATGCAAACTGTACTGCAAGGCTGAGAA
CTTTGAATTTTCTTGTGCAATGTCCGGCAAGTGAAAGATGGAACCTCCTGCTCCCCAAACAGAAATGATGTTTGTATTG

FIG. 3A

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ACGGGGTTTGTGAAC TAGTGGGATGTGATCATGAAC TAGGCTCTAAAGCAGTTTCAGATGCTTGTGGCGTTTGCAAAGGT
GATAATTCAACTTGCAAGTTTATAAAGGCCCTGTACCTCAACCAGCATAAAGCAAATGAATATATATCCGGTGGTCATCAT
TCCAGCTGGCGCCGAAGCATCGAAATCCAGGAGCTGCAGGTTTCCCTCCAGTTACCTCGCAGTTCGAAAGCCTCAGTCAAAA
AGTATTACCTCACCGGGGCTGGAGCATCGACTGGCTGGGAGTTCCCCCTTCGCTGGGACCACTGTTTGAATACCAAGCGC
TCTTTC AACCGCCCGGAACGTCTGTACGCGCCAGGCCACAAATGAGACGCTGGTCTTTGAAATTTCTGATGCAAGGCAA
AAATCCAGGATAGCTTGGAAAGTATGCAC TTTCCCAAGTCA TGAAATGGAAC TCCACG CCAAAAAGACCTGCCCTATA
CCTGGAGTATCGTGCAGTCAGAGTGCTCCGTCTCCTGTGGTGAGGTTACATAAAATGTAAAGGCCATTGTGCTTGCGAGAT
CAAAATACTCAAGTCAATTCCCTCATTTCTGCAGTGCAAAAACCAAGCCAGTAACTGAGCCCAAATCTGCAACGCTTCTC
CTGCCCGGCTTACTGGATGCCAGGTGAATGGAGTACATGTAGCAAGGCCCTGTGCTGGAGGCCAGCAGAGCCGAAAGATCC
AGTGTGTGCAAAAGAGCCCTTCCAAAAGGAGAGTGTGCA TTTCTCTCTG TCCAGT GAGCACACCCACTCAGGTC
CAAGCCTGCAACAGCCATGCCCTGTCCCTCCACAATGGAGCC TTTGGAC CCTGGTCTCAGTGTCCAAAGACCTGTGGACGAGG
GGTGAGGAAGCGTGAACTCCCTCTGCAAGGCTCTGCGCAGAAACCC TCCCGAGAGCCAGTG TACCAGTCTCCCCAGAC
CTGAGCTGCAGGAGGCTGTGTGCTTGGACGATGCCCCAAAGAACAGCCGCTACAGTGGGTGCTTCTTCGTGGAGCCGAG
TGTTCTGCAACCTGTGGTTTGGGTGTGAGGAAGAGGGAGATGAAGTGCAGCCGAGAGGGCTTCCAGGGAAGCTGATAAC
TTTCCAGAGCGAAGATGC

FIG. 3B

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MSPFLLQALQLCCLCCASVAAALASDSSGASGLNDDYVFVTPVEVDSAGSYISHDILHNGRKKRSAQNARSSLHYRFS
FGQELHLELKPSAILSSHFI VQVLGKDGASETQKPEVQQCIFYQGFIRNDSSSVAVSTCAGLSGLIRTRKNEFLISPLPQ
LLAQEHNHSSPAGHHPVLYKRTAEKIQRYRGYPGSGRNYPGYSPSHIPHASQSRTEYHHRRLQKQHF CGRKKYAPK
PPTEDTYLRFDEYGSGRPRRSAGKSQKGLNVELVADKKMVEKHGKGNVTYI LTVNMVSGLFKDG TIGSDINVVVV
SLILLEQEPGLLINHHADQSLNSFCQWQSA LIGKNGKRHDHAILLTGFDICSWKNEPCDTLGFAPI SGMCSKYRSC TIN
EDTGLGLAFTIAHESGHNFGMIHDGEGNPCRKAEGNIMSP TLTGNNGVFSWSSCSRQY LKKFLSTPQAGCLVDEPKQAGQ
YKYPDKLPGQIYDADTQCKWQFGAKAKLCSLGFVKDICKSLWCHRVGHRCE TKFMPAAEGTVCGLSMWCRCRQGQCVKFGL
GPRPIHGQWSAWSKWSECSRTC GGGVKFQERHCNNPKPQYGGIFCPGSSRIYQLCNINPCNENSLDFRAQQCAEYNSKPF
RGWFYQWKPYTKVEEEDRCKLYCKAENFEFFAMSGKVKGDTGPCSPNRNDVCIDGVCELVGCDHELGSKAVSDACGVCG
DNSTCKFYKGLYLNQHKANEYYPVVIIPAGARSIEIQELQVSSSYLAVRSLSQKYLLTGGSIDWPGEFFPAGTTFEYQR
SFNRPERLYAPGPTNETLVFEILMQGNP GIAWKYALPKVMNGTPPATKRPAYTWSIVQSECSVSCGGYINVKAI CLR D
QNTQVNSSFCSAKTKPVTEPKICNAFSCPAYWMPGEWSTCSKACAGGQQRKIQC VQKKPFQKEEAVLHSLCPVSTPTQV
QACNSHACPPQWSLGPWSQC SKTCGRGVRKKRELLCKGSAETLPESQCTSLPRPELQEGCVLGRCPKNSRLQWVASSWSE
CSATCGLGVRKREMKCSEKGFQGKLITFPERRC

FIG. 4

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ATGTCACCTTTTCTTGCAGGCGCTCCAGCTGTGCTGCCTCTGCTGTGCGTCGGTCGCCGGCCCTTAGCCAGTGACAG
CAGCAGCGGCCAGCGGATTAAATGATGGTTTCGTATTTCGCCCCCATCCCAAGAGGGCCCTTCGCAGCACTTTGACC
CTCCCTTCCCCCAAAGAGAGAAAAGATGGAAAAGCGCACCCCTAACCTGGCAGATTACGTCCTTGTACCGCCAGTAGAA
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CTCCCTGCACCTACCGATTTCAGCAATTGGACAGGAACCTGCACTTAGAACTTAAGCCCTCGCGATTTCAGCAGTCACCT
TTATTGTCAGGTACTTGGAAAAGATGGTGCTTCAGAGACTCAGAAAACCCGAGGTGCAGCAATGCTTCTATCAGGGATT
ATCAGAAAATGACAGCTCCTCCTCTGTCGCTGTGCTACGTGTGCTGGCTTGATGATCCCCAAGGAAATTAACCTTGATGGA
TGCCATTGCTTTGTAATGTCCCGGAGACCAGGCATTCTATAAATCTAACAAAGCTTCATGCGTCTACATGGCTTTGAAA
TGGGAAAACCTGTATTTCAAATGCGAAATTGCATTTCAGCAGCACCTGTTTAATAAAGGAAAAGAAAGCTTCACCTATGGGGA
CTCAGAGTCATTGTCCTCAAGGTGCTGAACAGGACCTTCAGTGGAAAACGAGACTGCCCTGAACCTCTCTGGGAGATTGT
TTTGCTTTGTGGAAATGCATCACACCATCTCATGGCTTTACATAATGAATTCCCTCATCTCGCCATTACCTCAGCTTCTGGC
CCAGGAACACAACTACAGCTCCCCTGCGGTCAACCATCCTCACGTACTGTACAAAAGGACAGCAGAGGAGAAAGATCCAGC
GGTACCGTGGCTACCCGGCTCTGGCCGGAAATTATCCTGGTTACTCCCAAGTCACATTCCCCATGCATCTCAGAGTCGA
GAGACAGAGTATCACCATCGAAGGTGCAAAAAGCAGCATTTTGTGGACGACGCAAGAAATGTATTTCTCTCTCAACTG
TCCTATCCAGATATTCTCTAATATCCCTTCCAAATGCTCTTCGTTCATCGTAGATGCTCCCAAGCTCCACAGAGGAC
ACCTATCTAAGGTTTGATGAATATGGGAGCTCTGGCGACCCAGAGATCAGCTGGAAAATCACAAAAGGCCCTCAATGT
GAAACCCCTCGTGGTGGCAGACAAAGAAAATGGTGGAAAAGCATGGCAAGGAAATGTCACCAATACATCTCACAGTAA
TGAACATGGTTTCTGGCCATTATAAGATGGGACTATTGGAAAGTGACATAAACGTGGTTGTGGTGAGCCATAATTCTCTG
GAACAAGAACCTGGAGGATTATTGATCAACCATCATGCAGACCAGTCTCTGAATAGTTTTTGTCAAATGGCAGTCTGCCCT
CATTGGAAGAATGGCAAGAGACATGATCATGCCATCTTACTAACAGGATTTGATATTGTTCCTTGGAAGAAATGAACCAT
GTGACACTCTAGGGTTTGCCCCCATCAGTGAATGTGCTCTAAGTACCGAAGTTGTACCATCAATGAGGACACAGGACTT
GGCCTTGCCCTTCACCATCGCTCATGAGTCAGGGCACAACTTTGGTATGATTACGACGGAGAAGGAAATCCCTGCAGAAA
GGCTGAAGGCAATATCATGTCTCCACACTGACCGGAAACAAATGGAGTGTTCATGGTCTTCCTGCAGCCGCCAGTATC
TCAAGAAAATTCCTCAGCACACCTCAGCGGGTGCTAGTGGATGAGCCCAAGCAAGCAGGACAGTATAAATATCCGGAC
AAACTACCAGGACAGATTTATGATGCTGACACACAGTGTAATGGCAAATTTGGAGCAAAAGCCAAAGTTATGCAGCCCTTGG
TTTTGTGAAGTGGTGTCCGCAAGGCCAGTGCCGTAAAGTTTGGGGAGCTCGGGCCCCGCCCATCCACGGCCAGTGGTCCG

FIG. 5A

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CCTGGTCGAAGTGGTCAGAAATGTTCCCGGACATGTGGTGGAGGAGTCAAGTTCAGGAGAGACACTGCAATAACCCCAAT
AACAAATCAACCAGAGTTTTACTGTTTGCAATATAAAGTCCATGTGCACCGAAGGTATGGTGGCAGAAACCAAACA
CAGCAGAGGAGTCATTTCTACGGGACTGTGATGATCCAGCCTCAGTATGGTGGCTTATTTCTGTCCAGGTTCTAGCCGTA
TTTATCAGCTGTGCAATATTAAACCTTGCAATGAAAATAGCTTGGATTTTCGGGCTCAACAGTGTGCAGAAATATAACAGC
AAACCTTTCCGTGGATGGTTCTACCGAGTGGAAACCCATATACAAAGTGGAAGAGGAAGATCGATGCAAACTGTACTGCAA
GGCTGAGAACTTTGAAATTTTTTTTGGCAATGTCCGGCAAGTGAAAGATGGAACCTCCTGCTCCCAAAACAAATGATG
TTTGTAATTGACGGGTTTGTAACCTAGTGGGATGTGATCATGAACCTAGGCTCTAAAGCAGTTTCAGATGCTTGTGGCCGT
TGCAAAAGTGATAATTCAACTTGCAAGTTTATAAAAGCCCTGTACCTCAACCAGCATAAAGCAAAATGAATATTATCCGGT
GGTCCTCATTCAGCTGGCGCCCGAAGCATCGAAATCCAGGAGCTGCAGGTTTCTCCAGTTACCTCGCAGTTCGAAAGCC
TCAGTCAAAAGTATTACCTCACCGGGGCTGGAGCATCGACTGGCCTGGGAGTTCCCTTCGCTGGGACCACTTGTGAA
TACCAGCGCTCTTTCAACCGCCCGGAACGTCTGTACGCGCCAGGGCCCAAAATGAGACGCTGGTCTTTGAAAGTAAAGCCC
CTTCTGTGTAATTCAGTTCTCAGTGCTTCTTGCTACATTTATATCGTATGGATATCCCTCAGGGGTAAGGTCAGCAAAGG
TTCTCTCACTAGAGGAATGGATTAAATCTGAGACAAACCTTGCAAGGAAGAAACAACAGCAACCATCTACTGGCTGGATG
CCAGGTGAATGGAGTACATGCAGCAAGTCCCTGTGTGGAGGCCAGCAGAGCCGAAAGATCCAGTGTGTGCAAAAGAAAGCC
CTTCCAAAAGGAGAGCAGTGTGCAATTCCTCTGTCCAGTAAGCACACCCACTCAGGTCCAAGCTTGCAACAGCCATG
CCTGCCCTCCACAATGGAGCCTTGGAACCTGGTCTCAGTGTCCAAAGACCTGTGGACGAGGGGTAGGAAGCGTGAACTC
CTCTGCAAGGCTCTGCCGCAGAAACCCCTCCCAGAGCCAGTGTACCACTCTCCCAGACCTGAGCTGCAGGAGGGCTG
TGTGCTTGGAACGATGCCCCAAAGAACAGCCGGCTACAGTGGTCTGCTTCTTGAGCGAGTGTCTTGCAACCTGTGGTT
TGGGTGTGAGGAAGAGGAGATGAAGTGCAGCGAAGGCTTCCAGGGAAGCTGATAACTTTCCAGAGCGGAAGATGC
CGTAATATTAAAGAAACCAAAATCTGGACTTGGAAGAGACCTTGCAACCGAGGGCTTGCCAGCCCATCCAGTGTAACAAT
GGTAGCTGGATGGTATTCAATTGCCGTGGCAGTGCACAGTCACCTGTGGGGAGGGGTCCAGACCCGGTCAGTCCACT
GTGTTCAAGCAAGGCCGCCCTTCCCTCAAGTTGTCTGCTCCATCAGAAACCTCCGGTGCTACGAGCCTGTAAATACAAACTTC
TGTCCAGCTCCTGAAAAGAGAGATCTTAATTCCCTTGAATACCTCTATGGTCTCCACTGGTGTGAGGTCACACCTAAG
ACGGTTTTCGTCAGTCACCCCTGGATCTGGGTGA

FIG. 5B

PCR products (base pairs).

Fragment 1	undigested 1762 bp	digested 353 bp
5' primer TAAATCGAATTCCCACCATGTCACCTTTTCTCTTGCAGGCG	(SEQ ID NO: 9)	
3' primer CCGGGAACATTCTGACCACTTCGAC	(SEQ ID NO: 10)	
Fragment 2	1762 bp	293 bp
5' primer TAAATCGAATTCCCACCATGTCACCTTTTCTCTTGCAGGCG	(SEQ ID NO: 9)	
3' primer CCGGGAACATTCTGACCACTTCGAC	(SEQ ID NO: 10)	
Fragment 3	3189 bp	187 bp
5' primer CTGCCCTCTGCTGTGCGTCGGTCGC	(SEQ ID NO: 11)	
3' primer GCATCTTCGCTCTGGGAAAGTTATC	(SEQ ID NO: 12)	
Fragment 4	1762 bp	667 bp
5' primer TAAATCGAATTCCCACCATGTCACCTTTTCTCTTGCAGGCG	(SEQ ID NO: 9)	
3' primer CCGGGAACATTCTGACCACTTCGAC	(SEQ ID NO: 10)	
Fragment 5	3189 bp	1796 bp
5' primer CTGCCCTCTGCTGTGCGTCGGTCGC	(SEQ ID NO: 11)	
3' primer GCATCTTCGCTCTGGGAAAGTTATC	(SEQ ID NO: 12)	

FIG. 6A

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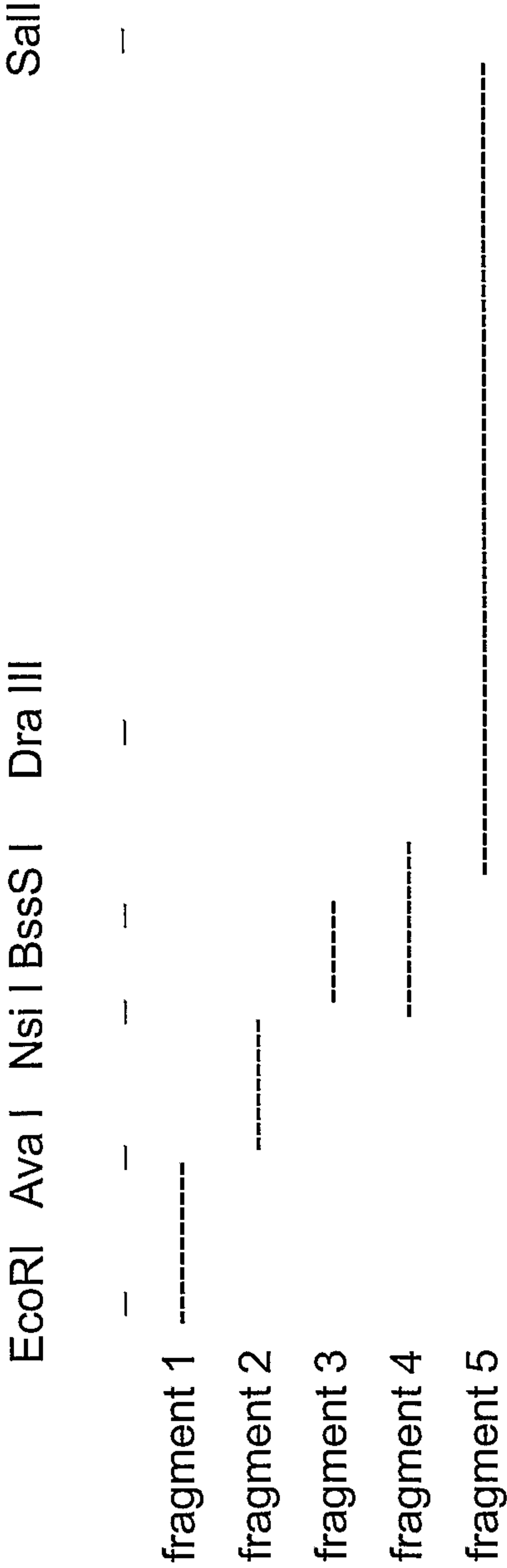


FIG. 6B

Truncated EST18 nucleotide sequence plus Streptavidin-Tag: SEQ ID NO: 7

ATGGAGTGCGCCCTCCTGCTCGCGTGTGCCCTTCCGGCTGCGGGTTCCGGCCCGGAGGGCCCTGGCGGACTGGGGCG
CGTGGCCAAAGCGCTCCAGCTGTGCTGCCCTCTGCTGTGCTCGGTCCGGCTTAGCCAGTGACAGCAGCAGCGGCG
CCAGCGGATTAAATGATGATTACGTCTTTGTACGCCAGTAGAAGTAGACTCAGCCGGGTCAATATATTTCACACGACATT
TTGCACAACGGCAGGAAAGCGATCGCGCAGAAAGCTCCCTGCACCTACCGATTTCAGCATTTGGACAGGA
ACTGCACCTTAGAACTTAAGCCCTCGGCGATTTTGAGCAGTCACCTTTATTGTCCAGGTACTTGGAAGAGATGGTCTTCAG
AGACTCAGAAACCCGAGGTGCAGCAATGCTTCTATCAGGGATTATCAGAAATGACAGCTCCTCCTCGTGTGTGTCT
ACGTGTGCTGGCTTGTCAAGTTTAATAAGGACACGAAAAATGAATTCTCATCTCGCCATTACCTCAGCTTCTGGCCCA
GGAACACAACACAGCTCCCTGCGGTCACCATCCTCACGTA CTGTACAAAAGGACAGCAGAGGAGAAATCCAGCGGT
ACCGTGGCTACCCGGCTCTGGCCGGAAATTAATCCTGTTACTCCCAAGTCACATTCCCCATGCACTCAGAGTCGAGAG
ACAGAGTATCACCATCGAAGTTGCAAAAGCAGCATTTTGTGGACGCGCAAGAAATATGCTCCCAAGCTCCACACAGA
GGACACCTATCTAAGTTTGATGAATATGGGAGCTCTGGCGACCCAGAAAGATCAGCTGGAATAATCACAAAAGGCCCTCA
ATGTGGAACCCCTCGTGGTGGCAGACAAAGAAATGGTGGAAAGCATGGCAAGGAAATGTACCCACATACATCTCACACA
GTAATGAACATGGTTTCTGGCCATTATTAAAGATGGACTATTGGAAAGTGACATAAACGTGGTTGTGTGAGCCCTAAATCT
TCTGGAACAAGAACCTGGAGGATTATTGATCAACCATCATGCAGACCAGTCTCTGAATAGTTTGTGTCAAATGGCAGTCTG
CCCTCATTTGGAAAGAAATGGCAAGAGACATGATCATGCCATCTTACTAACAGGATTTGATATTGTCTTGGAAAGAAATGAA
CCATGTGACACTCTAGGGTTTGGCCCCCATCAGTGGAATGTGCTCTAAGTACCGAAGTTGTACCATCAATGAGGACACAGG
ACTTGGCCTTGCCCTTCACCATCGCTCATGAGTCAGGGCACAACTTTGGTATGATTTCACGACGGAGAGGAAATCCCTGCA
GAAAGGCTGAAGGCAATATCATGTCTCCACACTGACCGGAAACAATGGAGTGTTCATGGTCTTCTTGCAGCCGCCAG
TATCTCAAGAAATTCCCTCAGCACACCTCAGGCGGGTGTCTAGTGGATGAGCCCAAGCAAGCAGACAGTATAAATATCC
GGACAAACTACCAGGACAGATTATGATGCTGACACACAGTGTAAATGGCAATTTCAGAGCAAAAGCCAAGTTATGCAGCC
TTGGTTTGTGAAGGATATTTGCAAAATCAGTTTGGTGCCACCGAGTAGGCCACAGGTGTGAGACCAAGTTTATGCCCGCA
GCAGAAAGGACCGTTTGTGGCTTGAGTATGTGGTGTCCGCAAGGCCAGTGCGTTAAAGTTTGGGAGCTCGGGCCCCGGCC
CATCCACGGCCAGTGGTCCGCTGGTCGAAGTGGTCAGAAATGTTCCCGACATGTGGTGGAGGAGTCAAGTTCCAGGAGA
GACACTGCAATAACCCCAAGCCCTCAGTATGGTGGCATATTCTGTCCAGGTTCTAGCCGTATTATCAGCTGTGCAATATT
AACCCTTGCAATGAAAAATAGCTTGGATTTTGGAAGCGCTTGAGCCACCCGAGTTCGAAAAATAA

FIG. 7

Truncated EST18 protein sequence plus Streptavidin-Tag: SEQ ID NO: 8

MECALLLACAFPAAGSGPPRGLAGLGRVAKALQLCCLCCASVAAALASDSSSGASGLNDDYVFVTPVEVDSAGSYISHDI
LHNGRKKRSAQNARSSLHYRFSAFGQELHLELKPSAILSSHFIQVLGKDGASETQKPEVQQCFYQGFIRNDSSSSVAVS
TCAGLSGLIRTRKNEFLISPLPQLLAQEHNHSSPAGHHPHVLYKRTAEKKIQRYGYPGSGRNYPGYSPSHIPHASQSRE
TEYHHRRLQKHFCGRRKKYAPKPPTEDTYLRFDEYGSGRPRRSAGKSQKGLNVEITLVADKKMVEKHGKGNVTYYILT
VMNMVSGLFKDGTTIGSDINVVVVSLILLEQEPGGLLINHHADQSLNSFCQWQSALIGKNGKRHDHAILLTGFDICSWKNE
PCDTLGFAPISGMC SKYRSTINEDTGLGLAFTIAHESGHNFMIHDGEGNPCRKAEGNIMSPTLTGNNGVFSWSSCSRQ
YLKKFLSTPQAGCLVDEPKQAGQYKYPDKLPGQIYDADTQCKWQFGAKAKLCSLGFVKDICKSLWCHRVGHRCE TKFMPA
AEGTVCGLSMWCRCRQGQCVKFGELGPRPIHGQWSAWSKWSKSECSRTC GGGVKFQERHCNNPKPQYGGIFCPGSSRIYQLCNI
NPCNENSLDFGSAWSHPQFEK

FIG. 8

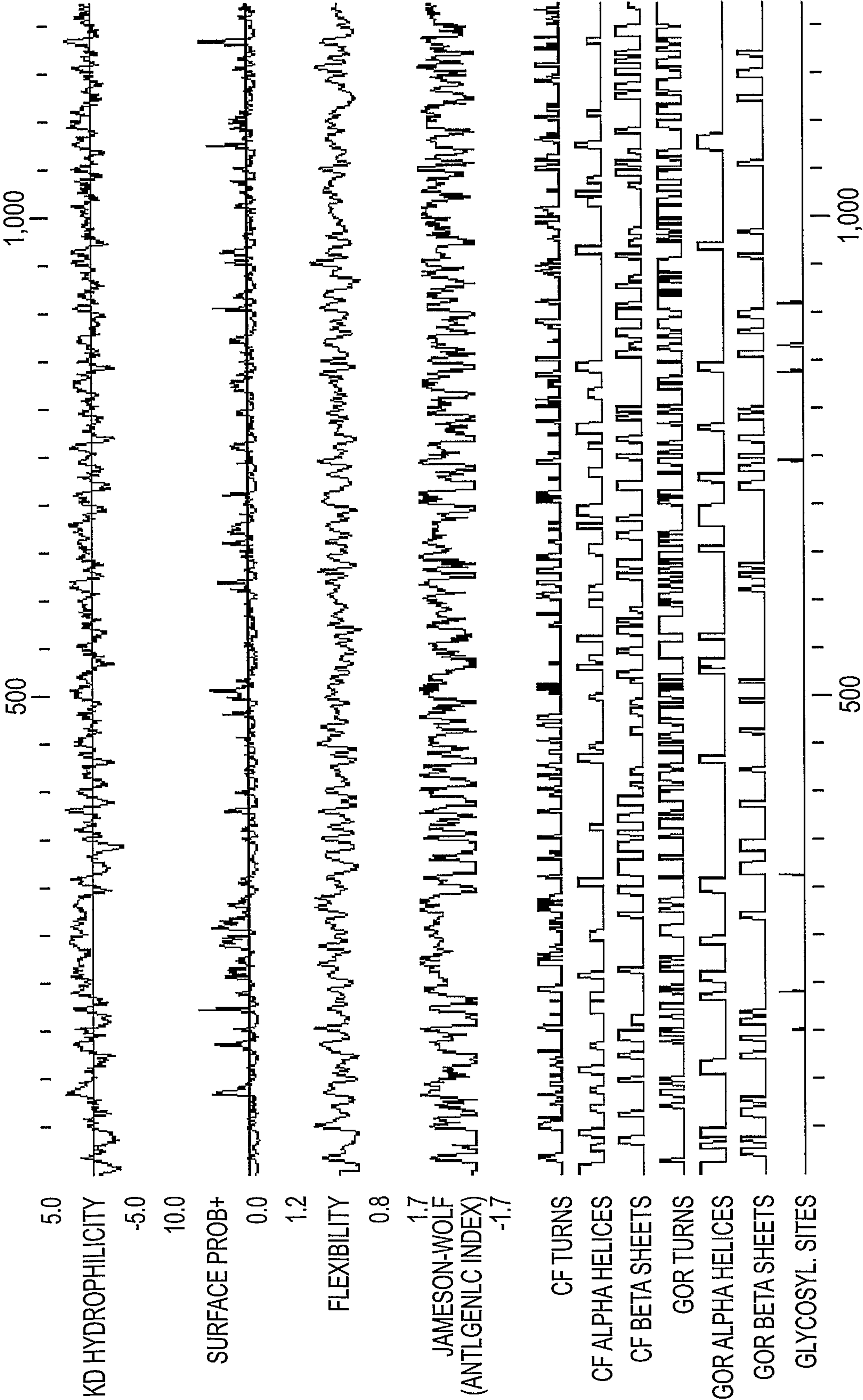


FIG. 9

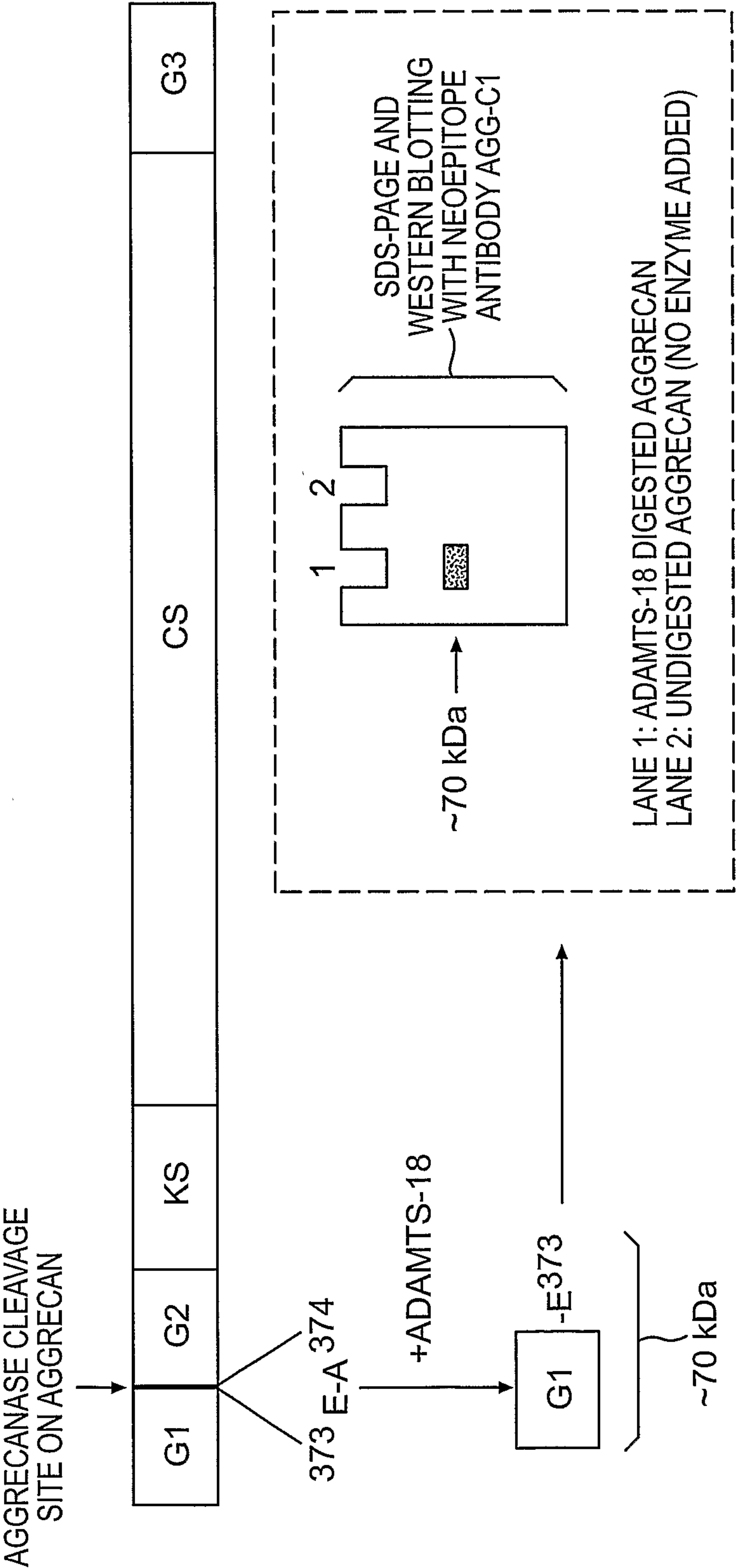


FIG. 10