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(54) Title: NOVEL SERINE PROTEASE CAPABLE OF SELECTIVE CLEAVAGE OF INSULIN-LIKE GROWTH FACTOR BINDING PROTEIN

(57) Abstract

The present invention is directed to novel nucleic acids and proteins which have sequence identity to an ASP05 protease, and the use of such compositions in assays, as well as in the diagnosis and treatment of disease.

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NOVEL SERINE PROTEASE CAPABLE OF SELECTIVE CLEAVAGE OF INSULIN -LIKE GROWTH FACTOR BINDING PROTEIN

FIELD OF THE INVENTION

The invention relates to methods and compositions of a novel serine protease termed "ASP05," capable of selective cleavage of insulin-like growth factor binding protein ("IGFBP") including nucleic acids, proteins and antibodies. The invention also relates to the use of such a novel ASP05 protease in assays for selective cleavage of IGFBP, and the methods of screening for modulators of the enzyme. The invention further relates to the use of antibodies directed against such a novel ASP05 protein in diagnosis of disease.

BACKGROUND OF THE INVENTION

Insulin-like growth factor binding proteins (IGFBPs) play a critical role in IGF biology. Cellular proteases that selectively cleave the IGFBPs mediate these activities. Such proteolysis is known to alter affinities of the IGFBPs for IGF and has been reported to either agonize or antagonize IGF function in various systems.

Insulin-like growth factor-l (IGF-I) and IGF-II are synthesized and secreted by a variety of cells and act as autocrine and paracrine growth factors to regulate differentiation, proliferation and function of many types of cells, including those derived from brain, blood, bone, liver, muscle, kidney. and the nervous system (Jones, J. I *et al. Endocrine Reviews 16* 3-34 (1995)).

IGF binding proteins (IGFBPs) are indispensable components of the IGF system; by tightly binding the IGF molecules they alter their bioavailability to target cells (Jones *et al* supra, Cohen *et al., Horm. Metab. Res. 26* 81-84 (1994)). The IGFBP family consists of six distinct proteins, termed IGFBP-1 to -6, each with high binding affinity for both IGF-1 and IGF-2 (Cohen *et al.* supra, Oh *et al. J. Biol. Chem. 271* 30322-30325 (1996) , Kim *et al. Proc Natl. Acad. Sci. USA, 94* 12981-12986 (1997)). Amino acid sequences of IGFBP's display between 40-60% similarity and encode between 11-18 highly conserved cysteine residues. Among the members of IGFBP family, IGFBP7, which is the most recently identified, has the lowest amino acid sequence similarity and the least conserved cysteine residues (Oh *et al.* supra, Kim *et al.* supra)

Metabolism of IGFBPs affects their interaction with the IGFs and, therefore, alters the action of IGFs. There are a number of reports describing identification of proteases which cleave IGFBPs. It has been reported that IGFBP-1 is a potential physiological substrate of matrix metalloprotease stromelysin-3 (Manes *et al. J. Biol. Chem., 272* 25706-25712 (1997)); a protease activity for IGFBP-3 has been demonstrated in human breast cancer cells, MCF-7 (Salahifar *et al., Endocrinology 138* 1683-1690 (1997)); IGFBP-4 was found degraded in normal and transformed human fibroblasts (Conover *et al., J. Clin. Invest. 91* 1129-1137 (1993)); and specific protease activities for IGFBP-3 and IGFBP-4 have been found in sera from patients after major heart surgery (Hughes *et al., J. Endocrinology 135* 135-145 (1992)). Human fibroblasts have been identified as a source of proteases that cleave IGFBP-5 (Nam *et al., Endocrinology 135* 1385-1391 (1994) Nam *et al., Endocrinology 137* 5530-5536 (1996)). It has also been demonstrated that a protease degrades IGFBPs in a 150kD complex during pregnancy and that the plasmin system is involved in dissociation of the IGFBP complex (Giudice *et al., J. Clin. Endocrinology and Metabolism 71* 806-816 (1990), Campbell *et al., Am. J. Physiol. 273* E996-E1004 (1997)). All these reports indicate that proteolysis of IGFBPs is an essential way to control the availability of IGFBPs, and thereby regulate the function of the IGF system.

Despite much work in this area over the last several years, the identity of the protease(s) that carry out such cleavage has remained unclear. The sequence of the ASP05 protease has also been recently published by others. (JP Application Serial No.: 9-1007980).

However, attempts to express the ASP05 protein from the native nucleic acid were unsuccessful. (Zumbrunn, J. & Trueb, B. FEBS Letters 398: (1996) pp.187-192).

Described herein is the identification and characterization of an IGFBP specific serine protease, "ASP05", which is a member of the serine protease family and which specifically cleaves IGFBP. The examples demonstrate the generation of a nucleic acid sequence which expresses a protein that is biologically active and capable of specific cleavage of IGFBP, designated herein as "ASP05".

Accordingly, it is an object of the invention to provide ASP05, enzymatically active ASP05 and related molecules. It is a further object of the invention to provide recombinant nucleic acids encoding ASP05, and expression vectors and host cells containing the nucleic acid encoding such an ASP05 protease. A further object of the invention is to provide methods for screening for such an ASP05 protease and antagonists thereof.

SUMMARY OF THE INVENTION

In accordance with the objects outlined above, the present invention provides for preparation of a nucleic acid that encodes an IGFBP specific serine protease, wherein the polypeptide is designated in the present application as "ASP05".

In one embodiment, the invention provides an isolated nucleic acid molecule comprising DNA encoding ASP05. The present invention also provides for ASP05 proteins and fragments thereof.

The invention provides methods of cleavage IGFBP, comprising adding an ASP05 protease to a IGFBP.

The present invention further provides methods for screening for a bioactive agent capable of inhibiting ASP05, ASP05-like proteins and methods for cleaving IGFBPs.

In a further aspect, the invention provides methods of modulating cleavage of IGFBP in a cell comprising administering to the cell an exogenous compound that binds to a serine protease capable of selective cleavage of IGFBP wherein the binding modulates the biological activity of said serine protease.

In a further aspect, the invention provides methods for determining the presence of an ASP05 protease in an individual. The activity of such an ASP05 in a tissue from a first individual may be measured and compared to the activity of such an ASP05 in a tissue from a second, unaffected individual or from a second tissue in the first individual. When the activity of such an ASP05 from said first individual is modified or different in comparison to the activity of the ASP05 in the second individual or the second tissue, the first individual is at risk for a disease condition associated with insulin metabolism.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 depicts the nucleotide sequence cDNA, nucleotides 1-1443 (SEQ ID NO:1), and the amino acid sequence 1-480 (SEQ ID NO:2) of a serine protease capable of selective cleavage of IGFBP, designated "ASP05". Nucleotides changed for expression of ASP05 are underlined. The arrow indicates the signal cleavage site. The IGFBP-like domain is underlined and the PSTI-domain is double underlined. The Catalytic domain is boxed with the catalytically essential Histidine (H), Aspartate (D) and Serine (S) residues circled.

Figures 2A-2B depict an alignment of the nucleic acid sequence which encodes the native protein (upper; SEQ ID NO:3) and the nucleic acid sequence which encodes the synthetic ASP05 protease (lower). Altered nucleotides in the synthetic ASP05 protease are underlined. The amino acid sequence encoded by both sequences is also presented.

Figure 3 depicts the results of an assessment of the autocleavage activity of ASP05 by Western blot analysis of purified ASP05 run on SDS-PAGE and detected by anti-ASP05 peptide antibody in conjunction with HRP-conjugated rabbit IgG antibody visualized by chemiluminescence. Lane 1, the protein (0.5 μg) purified with flag antibody affinity chromatography was run onto a 4-20% SDS-PAGE and stained with Commassie blue;

lanes 2 and 3, samples of same amount as in lane 1 were run on to a 4-20% SDS-PAGE and blotted with anti-ASP05 peptide antibody. Sample in lane 2 was not incubated at 37°C and sample in lane 3 was incubated at 37 °C for 15 hours before loading onto the gel. ST: molecular weight standard protein markers.

Figure 4A depicts the selective cleavage of IGFBP-5. IGFBP-1 to 6 were incubated with ASP05 and analyzed by Western blot with respective antibodies. "-" represents without ASP05 control, "+" with ASP05.

Figure 4B depicts the selective cleavage of IGFBP-5. IGFBP-1 to 6 were incubated with ASP05 and analyzed by Western ligand blot with ^{125}1-IGF-I, BP 1-6 represents IGFBP 1-6, respectively, "-" represents IGFBP 1-6, without ASP05 and "+" represents IGFBP 1-6, respectively, with ASP05.

Figure 5 depicts the effect of protease inhibitors on degradation of IGFBP-5 by ASP05. ASP05 was incubated with the protease inhibitors for 1 hour prior to addition of IGFBP-5, the products were analyzed with Western blot with IGFBP-5 antibody. Lane 1, no ASP05; lane 2, with ASP05, lane 3, aprotinin, 1.5 μM; lane 4, antipain, 370 μM; lane 5, 3, 4-DCI, 1 mM; lane 6, E64, 140 μM; lane 7, leupeptin, 5 μM; lane 8, pepstatin A, 5 μM; lane 9, PMSF, 5 mM; lane 10, TLCK, 675 μM; lane 11, TPCK, 1.4 mM and lane 12, EDTA, 10 mM.

Figure 6 depicts the nucleotide sequence of the C-terminal domain of ASP05 and a histidine tag useful for purification, as shown in the Examples.

Figure 7 depicts the amino acid sequence of the C-terminal domain of ASP05, plus a histidine tag.

Figure 8 depicts the selective cleavage of IGFBP-5 by the C-terminal fragment of ASP05. IGFBP-1 to 6 were respectively incubated with the fragment at 37 °C for two hours and then analyzed with Western blot with antibodies indicated on top of each blot. "-" represents the samples that were not treated with the fragment, "+" with the fragment.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a novel IGFBP specific serine protease termed herein as ASP05, capable of specifically cleaving IGFBP. The ASP05 protease of the present invention may be prepared by recombinant or synthetic methods or isolated from a variety of sources. In particular, the wild-type ASP05 nucleotide sequence contains a G-C rich N-terminus, leading to difficulties in expressing the ASP05 protein. Accordingly, the present invention provides ASP05 nucleic acids that have decreased G-C content, in particular decreased G-C regions in the N-terminal portion of the nucleic acid coding sequence, such that the expression of functional (i.e., enzymatically active) ASP05 may be accomplished.

Accordingly, the present invention provides ASP05 proteins and nucleic acids.

An ASP05 protein of the present invention may be identified in several ways. "Protein" in this sense includes proteins, polypeptides, and peptides. An ASP05 nucleic acid or an ASP05 protein is initially identified by substantial nucleic acid and/or amino acid sequence homology to the sequences shown in Figure 1. Such homology can be based upon the overall nucleic acid or amino acid sequence.

Homology in this context means sequence similarity or identity, with identity being preferred. This homology will be determined using standard techniques known in the art, such as the Best Fit sequence program described by Devereux *et al.*, *Nucl. Acid Res. 12*:387-395 (1984), preferably using the default settings, or the BLASTX program (Altschul et al., J. Mol. Biol. *215*, 403-410). The alignment may include the introduction of gaps in the sequences to be aligned. In addition, for sequences which contain either more or fewer amino acids than the protein shown in Figures 1, 2, and 7, it is understood that the percentage of homology will be determined based on the number of homologous amino acids in relation to the total number of amino acids. Thus, for example, homology of sequences shorter than that shown in the Figures, as discussed below, will be determined using the number of amino acids in the shorter sequence.

Similarity is determined using standard techniques known in the art, including, but not limited to, the algorithm of Smith & Waterman, Adv. Appl. Math. 2:482 (1981), by the algorithm of Needleman & Wunsch. J. Mol. Biol. 1970. 48:443, by the search for similarity method of Pearson & Lipman. 1988. PNAS USA 85:2444, by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Drive, Madison, WI), or the Best Fit sequence program described by Devereux *et al.* Nucl. Acid Res. 1984. 12:387-395.

In a preferred embodiment, percent identity or similarity is calculated by FastDB based upon the following parameters: mismatch penalty of 1.0; gap penalty of 1.0; gap size penalty of 0.33, joining penalty of 30.0. ("Current Methods in Comparison and Analysis", Macromolecule Sequencing and Synthesis, Selected Methods and Applications, pp. 127-149, 1998. Alan R. Liss, Inc.)

Another example of a useful algorithm is PILEUP. PILEUP creates a multiple sequence alignment from a group of related sequences using progressive, pairwise alignments. It can also plot a tree showing the clustering relationships used to create the alignment. PILEUP uses a simplification of the progressive alignment method of Feng and Doolittle. J. Mol. Evol. 1987. 35:351-360; the method is similar to that described by Higgins and Sharp. 1989. CABIOS 5:151-153. Useful PILEUP parameters including a default gap weight of 3.00, a default gap length weight of 0.10, and weighted end gaps.

An additional example of a useful algorithm is the BLAST algorithm, described in Altschul *et al.* J. Mol. Biol. 1990. 215:403-410 and Karlin *et al.*, PNAS USA 1993. 90:5873-5787. A particularly useful BLAST program is the WU-BLAST-2 program which was obtained from Altschul *et al.*, Methods in Enzymology. 1996. 266: 460-480; [http://blast.wustl/edu/blast/ README.html]. WU-BLAST-2 uses several search parameters, most of which are set to the default values. The adjustable parameters are set with the following values: overlap span =1, overlap fraction = 0.125, word threshold (T) = 11. The HSP S and HSP S2 parameters are dynamic values and are established by the program itself depending upon the composition of the particular sequence and composition

of the particular database against which the sequence of interest is being searched; however, the values may be adjusted to increase sensitivity.

In an alternative embodiment, percent amino acid sequence identity is determined manually. In percent identity calculations relative weight is not assigned to various manifestations of sequence variation, such as, insertions, deletions, substitutions, *etc.* Only identities are scored positively (+1) and all forms of sequence variation given a value of "0", which obviates the need for a weighted scale or parameters as described above for sequence similarity calculations. Therefore, percent identity represents a highly rigorous method of comparing sequences.

Percent sequence identity can be calculated, for example, by dividing the number of matching identical residues by the total number of residues of the "shorter" sequence in the aligned region and multiplying by 100. The "longer" sequence is the one having the most actual residues in the aligned region.

As used herein, a protein is an "ASP05 protein" preferably if (i) its IGFBP binding domain has a greater than 65%, typically greater than 80% and preferably greater than 90%, overall sequence identity to amino acids 30 to 104 of the sequence shown in Figure 1 (SEQ ID NO:2), and/or (ii) its catalytic domain has a greater than 65%, typically greater than 80% and preferably greater than 90%, overall sequence identity to amino acids 161 to 371 of the sequence shown in Figure 1 (SEQ ID NO:2). In some embodiments the sequence identity will be as high as about 90 to 95 or 98%.

An ASP05 protein may be identified by the presence of one or more important domains, including the catalytic domain, the IGFBP domain, the PSTI-like domain, and a PDZ-like domain, the last three of which can be identified on the basis of homology to known proteins.

In a preferred embodiment, an ASP05 protease comprises a serine protease catalytic domain. Expression of such a catalytic domain results in a functional ASP05 protease. This domain, corresponding to amino acids 161 to 371 of the sequence depicted in Figure

1, has an active site putatively comprising a histidine at position 220, an aspartic acid at position 250, and a serine at position 328. Thus, in a preferred embodiment, an ASP05 protein may be identified by at least about 65% identity to amino acids 161 to 371 of the Figure 1 sequence, with at least about 80% being preferred and at least about 90% being especially preferred. In some embodiments the identity will be as high as 90% to 95% or 98%.

In a preferred embodiment, an ASP05 protein may be identified by the enzymatic activity associated with this catalytic domain. Thus, the present invention provides enzymatically active ASP05. By "enzymatically active" herein is meant that the ASP05 will selectively cleave IGFBP-5. By "selectively cleave" herein is meant IGFBP-5 is cleaved under conditions that generally will not substantially cleave other IGFBPs. A representative assay for IGFBP-5 cleavage is shown in the Examples.

In a preferred embodiment, an ASP05 protein may be identified as comprising an N-terminal domain related to the IGFBPs. This domain corresponds to amino acids 30 to 104 of the sequence depicted in Figure 1. Thus, in a preferred embodiment, an ASP05 protein may be identified by at least about 65% identity to amino acids 30 to 104 of the Figure 1 sequence, with at least about 80% being preferred and at least about 90% being especially preferred. In some embodiments the identity will be as high as 90% to 95% or 98%.

In a preferred embodiment, an ASP05 protein comprises an pancreatic secretory trypsin inhibitor (PSTI)-like domain. This PSTI-like domain corresponds to amino acids 105 to 160 of the sequence depicted in Figure 2. Thus, in a preferred embodiment, an ASP05 protein also includes a PSTI-like domain that has at least about 65% identify to amino acids 105 to 160 of the Figure 2 sequence, with at least about 80% being preferred and at least about 90% being especially preferred. In some embodiments the homology will be as high as 93% to 95% or 98%.

ASP05 proteins of the present invention may be shorter or longer than the amino acid sequences shown in the Figures. Thus, in a preferred embodiment, included within the

definition of ASP05 proteins are portions or fragments of the sequences depicted in the Figures. As outlined herein, ASP05 deletion mutants can be made, including, but not limited to, the deletion of amino acids 106 - 480 and 1- 105. A preferred ASP05 fragment is the catalytic domain of ASP05, which selectively cleaves IGFBP, as shown herein. A further preferred ASP05 fragment is the binding domain of ASP05, which may be required for the regulation of ASP05 activity. A further preferred ASP05 fragment is the binding domain of ASP05, combined with the catalytic domain of ASP05. A further preferred ASP05 fragment is the PSTI-like protease inhibitor domain, of ASP05, which may be required for modulating the proteolytic activity of ASP05. A further preferred ASP05 fragment is the C-terminal PDZ-like domain of ASP05. Fragments may comprise combinations of any domains, with preferred embodiments comprising at least the catalytic domain, and the catalytic domain and the binding domain.

As further outlined herein, ASP05 fusion proteins can be made including, but not limited to, the fusion of amino acids 160 to 480 to a heterologous sequence such as a signal sequence, purification tags, and other proteins. Additional preferred fusion proteins comprise the catalytic domain of ASP05 fused to a heterologous sequence domain.

In a preferred embodiment, the ASP05 proteins are derivative or variant ASP05 proteins. That is, as outlined more fully below, the derivative ASP05 peptide will contain at least one amino acid substitution, deletion or insertion, with amino acid substitutions being particularly preferred. The amino acid substitution, insertion or deletion may occur at any residue within the ASP05 peptide. As outlined below, particularly preferred substitutions are made within the catalytic domain or binding domain of the ASP05 protein.

In addition, as is more fully outlined below, ASP05 proteins can be made that are longer than those depicted in the figures, for example, by the addition of epitope or purification tags, the additign of other fusion sequences, etc.

In a preferred embodiment, ASP05 proteins may also be identified as being encoded by ASP05 nucleic acids, as defined below. Thus, ASP05 proteins are encoded by nucleic

acids that will hybridize to the sequence depicted in Figure 1, or its complement, as outlined herein.

In a preferred embodiment, when the ASP05 protein is to be used to generate antibodies, the ASP05 protein must share at least one epitope or determinant with the full length protein shown in Figure 1. By "epitope" or "determinant" herein is meant a portion of a protein which will generate and/or bind an antibody. Thus, in most instances, antibodies made to a smaller ASP05 protein will be able to bind to the full length protein. In a preferred embodiment, the epitope is unique; that is, antibodies generated to a unique epitope show little or no cross-reactivity. In a preferred embodiment, the antibodies are generated to the catalytic portion of the ASP05 molecule, *i.e.* to all or some of the region from amino acid numbers 106 to 480.

In a preferred embodiment, the antibodies to ASP05 are capable of reducing or eliminating the biological function of ASP05, as is described below. That is, the addition of anti-ASP05 antibodies (either polyclonal or preferably monoclonal) to cells or body fluids comprising an ASP05 protein may reduce or eliminate the ASP05 activity. For example, the cleavage of IGFBP may be reduced or eliminated; that is, when the ASP05 catalytic function is reduced or eliminated, all or some of the IGFBP remains intact. Generally, at least a 20% decrease in activity is preferred, with at least about 50% being particularly preferred, at least about 75% being further preferred, and about a 95-100% decrease being especially preferred.

The ASP05 antibodies of the invention specifically bind to ASP05 proteins. By "specifically bind" herein is meant that the antibodies bind to the protein with a binding constant in the range of at least 10^{-6}-10^{-8} M, with a preferred range being 10^{-7} -10^{-9} M.

In the case of the nucleic acid, the overall homology of the nucleic acid sequence is commensurate with amino acid homology but takes into account the degeneracy in the genetic code and codon bias of different organisms. Accordingly, the nucleic

acid sequence homology may be either lower or higher than that of the protein sequence.

Nucleic acid similarity can be determined using, for example, BLASTN (Altschul *et al.* 1990. J. Mol. Biol. 147:195-197). BLASTN uses a simple scoring system in which matches count +5 and mismatches -4. To achieve computational efficiency, the default parameters have been incorporated directly into the source code.

Thus a nucleic acid is an "ASP05 nucleic acid" if (i) the nucleotide sequence encoding for the IGFBP binding domain has a greater than 75%, typically greater than 85% and preferably greater than 95%, overall sequence identity to nucleotides 90 to 314 of the polynucleotide sequence shown in Figure 1 (SEQ ID: NO:1) and/or (ii) the nucleotide sequence encoding for the catalytic domain has a greater than 75%, typically greater than 85% and preferably greater than 95%, overall sequence to nucleotides 481 to 1113 of the nucleotide sequence shown in Figure 1 (SEQ ID: NO:2). In some embodiments the identity will be as high as about 90 to 95 or 98%.

Accordingly, a preferred embodiment provides a recombinant nucleic acid encoding for an insulin-like growth factor binding protein (IGFBP) degrading protease, wherein (i) the nucleic acid encodes for a catalytic domain of the IGFBP degrading protease having greater than 65% sequence identity to amino acids 161 to 371 of the amino acid sequence shown in Figure 1 (SEQ ID: NO:2) and/or (ii) the nucleic acid encodes for a IGFBP binding domain of the IGFBP degrading protease having greater than 65% sequence identity to amino acids 30 to 104 of the amino acid sequence shown in Figure 1 (SEQ ID: NO:2) and (iii) the first approximately 500 nucleotides of the nucleotide sequence of the nucleic acid has less than 75% total GC content. In some embodiments the identity will be as high as about 90 to 95 or 98%.

As outlined herein, the wild-type or native nucleic acid sequence has an N-terminal G-C rich region. This has been shown to make expression difficult. For example,

Zumbrunn, *et al.* (*supra*) report that they were unable to express the ASP05 protein, and therefore could not confirm its biological activities. The present invention enables the production of the ASP05 protein, and in particular enzymatically active protein, by decreasing the G-C content of the gene.

Accordingly, in a preferred embodiment, in the case of the nucleic acid, when comparing the nucleotide sequence for ASP05 to the native nucleic acid sequence of Figure 2, there is preferably a decrease in GC content over the entire coding sequence of greater than 10%, with decreases of greater than 20% being preferred and decreases of greater than 30% being particularly preferred. In a preferred embodiment, the decrease is in the N-terminal region of the gene. Furthermore, the nucleic acid is an ASP05 nucleic acid wherein the first approximately 500 nucleotides of the nucleotide sequence has less than 75%, typically less than 60% and preferably less than 50%, total GC content.

In a preferred embodiment, an ASP05 nucleic acid encodes an ASP05 protein. As will be appreciated by those in the art, due to the degeneracy of the genetic code, an extremely large number of nucleic acids may be made, all of which encode the ASP05 proteins of the present invention. Thus, having identified a particular amino acid sequence, those skilled in the art could make any number of different nucleic acids, by simply modifying the sequence of one or more codons in a way which does not change the amino acid sequence of ASP05.

In one embodiment, the nucleic acid identity is determined through hybridization studies. Thus, for example, nucleic acids which hybridize under high stringency to the nucleic acid sequences shown in Figure 1 or its complement and have ASP05 biological activity (in particular, catalytic biological activity is preferred) is considered an ASP05 gene.

High stringency conditions are known in the art; *see* for example Maniatis *et al.*, Molecular Cloning: A Laboratory Manual, 2d Edition, 1989, and Short Protocols in Molecular Biology, ed. Ausubel, *et al.*, Hames and Higgins, eds. *Nucleic Acid*

Hybridization, A Practical Approach, IL press, Washington, D.C., 1985; Berger and Kimmel eds. *Methods in Enzymology, Vol.* 52, *Guide to Molecular Cloning Techniques*, Academic press Inc., New York, N.Y., 1987; and Bothwell, Yancopoulos and Alt, eds, *Methods for Cloning and Analysis of Eukaryotic Gene*, Jones and Bartlett Publishers, Boston, Mass. 1990, which are hereby expressly incorporated by reference in their entirety.

The choice of hybridization conditions will be evident to one skilled in the art and will generally be guided by the purpose of the hybridization, the type of hybridization (DNA-DNA, DNA-RNA, RNA-RNA, oligonucleotide-DNA *etc.*), and the level of desired relatedness between the sequences. Methods for hybridization are well established in the literature. For example, one of ordinary skill in the art realizes that the stability of nucleic acid duplexes will decrease with an increased number and proximity of mismatched bases; thus, the stringency of hybridization may be used to maximize or minimize the stability of such duplexes. Hybridiziation stringency can be altered by, for example, adjusting the temperature of hybridization solution; adjusting the percentage of helix-destabilizing agents, such as, formamide, in the hybridization solution; and adjusting the temperature and salt concentration of the wash solutions. In general, the stringency of hybridization is adjusted during the post-hybridization washes by varying the salt concentration and/or the temperature. Stringency of hybridization may be increased, for example, by: i) increasing the percentage of formamide in the hybridization solution; ii) increasing the temperature of the wash solution; or iii) decreasing the ionic strength of the wash solution. High stringency conditions may involve high temperature hybridization (*e.g.* 65°C-68°C in aqueous solution containing 4-6X SSC, or 42°C in 50% formamide) combined with high temperature (e.g., 5°C-25°C below the T_m) and a low salt concentration (e.g., 0.1X SSC) washes. Reduced stringency conditions may involve lower hybridization temperatures (*e.g.*, 35°C-42°C in 20-50% formamide) with intermediate temperature (*e.g.*, 40°C-60°C) washes in a higher salt concentration (e.g., 2-6X SSC). Moderate stringency condtions, which may involve hybridization at a temperature between 50°C-55°C and washes in 0.1X SSC, 0.1% SDS at between 50°C and 55°C, may be used (*see* Maniatis and

Ausubel, *supra*). In a preferred embodiment, nucleic acids which hybridize to the nucleic acids herein have the biological activity as described herein.

The ASP05 proteins and nucleic acids of the present invention are preferably recombinant. As used herein, "nucleic acid" may refer to either DNA or RNA, or molecules which contain both deoxy- and ribonucleotides. The nucleic acids include genomic DNA, cDNA and oligonucleotides including sense and anti-sense nucleic acids. Such nucleic acids may also contain modifications in the ribose-phosphate backbone to increase stability and half life of such molecules in physiological environments.

The nucleic acid may be double stranded, single stranded, or contain portions of both double stranded or single stranded sequence. As will be appreciated by those in the art, the depiction of a single strand ("Watson") also defines the sequence of the other strand ("Crick"); thus the sequence depicted in Figure 1 also includes the complement of the sequence. By the term "recombinant nucleic acid" herein is meant nucleic acid, originally formed *in vitro*, in general, by the manipulation of nucleic acid by endonucleases, in a form not normally found in nature. Thus an isolated ASP05 nucleic acid, in a linear form, or an expression vector formed *in vitro* by ligating DNA molecules that are not normally joined, are both considered recombinant for the purposes of this invention. Similarly, fragments of the full length sequence, optionally attached to regulatory sequences, are considered recombinant. Furthermore, altered nucleic acid sequences, such as those described herein that have altered nucleotide sequences (e.g., lower G-C content), are also recombinant. It is understood that once a recombinant nucleic acid is made and reintroduced into a host cell or organism, it will replicate non-recombinantly, *i.e.* using the *in vivo* cellular machinery of the host cell rather than *in vitro* manipulations; however, such nucleic acids, once produced recombinantly, although subsequently replicated non-recombinantly, are still considered recombinant for the purposes of the invention.

Similarly, a "recombinant protein" is a protein made using recombinant techniques, *i.e.* through the expression of a recombinant nucleic acid as depicted above. A recombinant protein is distinguished from naturally occurring protein by at least one or more characteristics. For example, the protein may be isolated or purified away from some or all of the proteins and compounds with which it is normally associated in its wild type host, and thus may be substantially pure. For example, an isolated protein is unaccompanied by at least some of the material with which it is normally associated in its natural state, preferably constituting at least about 0.5%, more preferably at least about 5% by weight of the total protein in a given sample. A substantially pure protein comprises at least about 75% by weight of the total protein, with at least about 80% being preferred, and at least about 90% being particularly preferred. The definition includes the production of an ASP05 protein from one organism in a different organism or host cell. Furthermore, the protein may be made at a significantly higher concentration than is normally seen, through the use of a inducible promoter or high expression promoter, such that the protein is made at increased concentration levels. Furthermore, the protein may be in a form not normally found in nature, as in the addition of an epitope tag or amino acid substitutions, insertions and deletions, as discussed below. In addition, the recombinant protein may be enzymatically active.

Once identified, the polypeptides comprising the biologically active sequences may be prepared in accordance with conventional techniques, such as synthesis (for example, use of a Beckman Model 990 peptide synthesizer or other commercial synthesizer).

Also included within the definition of ASP05 proteins of the present invention are amino acid sequence variants. These variants fall into one or more of three classes: substitutional, insertional or deletional variants. These variants ordinarily are prepared by site specific mutagenesis of nucleotides in the DNA encoding the ASP05 protein, using cassette or PCR mutagenesis or other techniques well known in the art, to produce DNA encoding the variant, and thereafter expressing the DNA in recombinant cell culture as outlined above. However, variant ASP05 protein

fragments having up to about 100-150 residues may be prepared by *in vitro* synthesis using established techniques. Amino acid sequence variants are characterized by the predetermined nature of the variation, a feature that sets them apart from naturally occurring allelic or interspecies variation of the ASP05 protein amino acid sequence. The variants typically exhibit the same qualitative biological activity as the naturally occurring analogue, although variants can also be selected which have modified characteristics as will be more fully outlined below.

While the site or region for introducing an amino acid sequence variation is predetermined, the mutation per se need not be predetermined. For example, in order to optimize the performance of a mutation at a given site, random mutagenesis may be conducted at the target codon or region and the expressed ASP05 variants screened for the optimal combination of desired activity. Techniques for making substitution mutations at predetermined sites in DNA having a known sequence are well known, for example, M13 primer mutagenesis and PCR mutagenesis. Screening of the mutants is done using assays of ASP05 protein activities; for example, for binding domain mutations, competitive binding studies such as are outlined in the Examples may be done.

Amino acid substitutions are typically of single residues; insertions usually will be on the order of from about 1 to 20 amino acids, although considerably larger insertions may be tolerated. Deletions range from about 1 to about 20 residues, although in some cases deletions may be much larger. For example, a preferred variant comprises the deletion of the catalytic domain, leaving only the binding domain of ASP05. Additional preferred variants comprise the catalytic domain alone or a soluble receptor (*i.e.* the binding domain).

Substitutions, deletions, insertions or any combination thereof may be used to arrive at a final derivative. Generally these changes are done on a few amino acids to minimize the alteration of the molecule. However, larger changes may be tolerated in certain circumstances. When small alterations in the characteristics of the ASP05

protein are desired, substitutions are generally made in accordance with the following chart:

Chart I

Original Residue	Exemplary Substitutions
Ala	Ser
Arg	Lys
Asn	Gln, His
Asp	Glu
Cys	Ser
Gln	Asn
Glu	Asp
Gly	Pro
His	Asn, Gln
Ile	Leu, Val
Leu	Ile, Val
Lys	Arg, Gln, Glu
Met	Leu, Ile
Phe	Met, Leu, Tyr
Ser	Thr
Thr	Ser
Trp	Tyr
Tyr	Trp, Phe
Val	Ile, Leu

Substantial changes in function or immunological identity are made by selecting substitutions that are less conservative than those shown in Chart I. For example, substitutions may be made which more significantly affect: the structure of the polypeptide backbone in the area of the alteration, for example the alpha-helical or beta-sheet structure; the charge or hydrophobicity of the molecule at the target site; or the bulk of the side chain. The substitutions which in general are expected to produce the greatest changes in the polypeptide's properties are those in which (a) a hydrophilic residue, *e.g.* seryl or threonyl, is substituted for (or by) a hydrophobic residue, *e.g.*, leucyl, isoleucyl, phenylalanyl, valyl or alanyl; (b) a cysteine or proline is substituted for (or by) any other residue; (c) a residue having an electropositive side chain, *e.g.*, lysyl, arginyl, or histidyl, is substituted for (or by) an electronegative residue, *e.g.*, glutamyl or aspartyl; or (d) a residue having a bulky

side chain, *e.g.*, phenylalanine, is substituted for (or by) one not having a side chain, *e.g.*, glycine.

The variants typically exhibit the same qualitative biological activity and will elicit the same immune response as the naturally-occurring analogue, although variants also are selected to modify the characteristics of the ASP05 proteins as needed. Alternatively, the variant may be designed such that the biological activity of the ASP05 protein is altered. For example, glycosylation sites, and more particularly one or more O-linked or N-linked gylcosylation sites may be introduced, altered or removed.

For example, it may be desirable to make ASP05 proteins that bind substrates or inhibitors but do not cleave them. Thus, as substitutions of the active site sequences, for this and other purposes, may be done.

In an alternative embodiment, a library of variants is generated by an entirely, non-specific, random mutagenesis method. These techniques are known in the art and do not require the selection of a specific cite or region to be altered. For example, DNA shuffling as described by Stemmer. *Nature 370*:389-391 (1994) and Stemmer. *PNAS USA 91*:10747-10751 (1994)) can be used to produce variants which are cloned, expressed, and screened for a desired property. For example, the biochemical activity of an ASP05 protein can be increased or decreased as needed.

Covalent modifications of ASP05 polypeptides are included within the scope of this invention. One type of covalent modification includes reacting targeted amino acid residues of an ASP05 polypeptide with an organic derivatizing agent that is capable of reacting with selected side chains or the N-or C-terminal residues of an ASP05 polypeptide. Derivatization with bifunctional agents is useful, for instance, for crosslinking ASP05 to a water-insoluble support matrix or surface for use in the method for purifying anti-ASP05- antibodies or screening assays, as is more fully described below. Commonly used crosslinking agents include, *e.g.*, 1,1-bis(diazo-acetyl)-2-phenylethane, glutaraldehyde, N-hydroxysuccinimide esters, for example,

esters with 4-azidosalicylic acid, homobifunctional imidoesters, including disuccinimidyl esters such as 3,3'-dithiobis(succinimidylpropionate), bifunctional maleimides such as bis-N-maleimido-1,8-octane and agents such as methyl-3-[(p-azidophenyl)dithio]propioimidate.

Other modifications include deamidation of glutaminyl and asparaginyl residues to the corresponding glutamyl and aspartyl residues, respectively, hydroxylation of proline and lysine, phosphorylation of hydroxyl groups of seryl or threonyl residues, methylation of the "-amino groups of lysine, arginine, and histidine side chains [T.E. Creighton, Proteins: Structure and Molecular Properties, W.H. Freeman & Co., San Francisco, pp. 79-86 (1983)], acetylation of the N-terminal amine, and amidation of any C-terminal carboxyl group.

Another type of covalent modification of the ASP05 polypeptide included within the scope of this invention comprises altering the native glycosylation pattern of the polypeptide. "Altering the native glycosylation pattern" is intended for purposes herein to mean deleting one or more carbohydrate moieties found in native sequence ASP05 polypeptide, and/or adding one or more glycosylation sites that are not present in the native sequence ASP05 polypeptide.

Addition of glycosylation sites to ASP05 polypeptides may be accomplished by altering the amino acid sequence thereof. The alteration may be made, for example, by the addition of, or substitution by, one or more serine or threonine residues to the native sequence ASP05 polypeptide (for O-linked glycosylation sites). The ASP05 amino acid sequence may optionally be altered through changes at the DNA level, particularly by mutating the DNA encoding the ASP05 polypeptide at preselected bases such that codons are generated that will translate into the desired amino acids.

Another means of increasing the number of carbohydrate moieties on the ASP05 polypeptide is by chemical or enzymatic coupling of glycosides to the polypeptide. Such methods are described in the art, *e.g.*, in WO 87/05330 published 11

September 1987, and in Aplin and Wriston, CRC Crit. Rev. Biochem., pp. 259-306 (1981).

Removal of carbohydrate moieties present on the ASP05 polypeptide may be accomplished chemically or enzymatically or by mutational substitution of codons encoding for amino acid residues that serve as targets for glycosylation. Chemical deglycosylation techniques are known in the art and described, for instance, by Hakimuddin, *et al.*, Arch. Biochem. Biophys., **259**:52 (1987) and by Edge, *et al.*, Anal. Biochem., **118**:131 (1981). Enzymatic cleavage of carbohydrate moieties on polypeptides can be achieved by the use of a variety of endo-and exo-glycosidases as described by Thotakura, *et al.*, Meth. Enzymol., **138**:350 (1987).

Another type of covalent modification of ASP05 comprises linking the ASP05 polypeptide to one of a variety of nonproteinaceous polymers, *e.g.*, polyethylene glycol, polypropylene glycol, or polyoxyalkylenes, in the manner set forth in U.S. Patent Nos. 4,640,835; 4,496,689; 4,301,144; 4,670,417; 4,791,192; or 4,179,337.

ASP05 polypeptides of the present invention may also be modified in a way to form chimeric molecules comprising an ASP05 polypeptide fused to another, heterologous (including synthetic) polypeptide or amino acid sequence. In one embodiment, such a chimeric molecule comprises a fusion of an ASP05 polypeptide with a tag polypeptide which provides an epitope to which an anti-tag antibody can selectively bind. The epitope tag is generally placed at the amino-or carboxyl-terminus of the ASP05 polypeptide. Alternatively, the epitope may be placed internally, preferably in hydrophilic regions so as to be on the surface of the ASP05 polypeptide and accessible to antibody. The presence of such epitope-tagged forms of an ASP05 polypeptide can be detected using an antibody against the tag polypeptide. Also, provision of the epitope tag enables the ASP05 polypeptide to be readily purified by affinity purification using an anti-tag antibody or another type of affinity matrix that binds to the epitope tag. In an alternative embodiment, the chimeric molecule may comprise a fusion of an ASP05 polypeptide with another protein or polypeptide, for example, an immunoglobulin

or a particular region of an immunoglobulin. For a bivalent form of the chimeric molecule, such a fusion could be to the Fc region of an IgG molecule or GST fusions. Similarly, chimeric nucleic acids encoding the chimeric proteins may be made as outlined herein.

Various tag polypeptides and their respective antibodies are well known in the art. Examples include poly-histidine (poly-his) or poly-histidine-glycine (poly-his-gly) tags; the flu HA tag polypeptide and its antibody 12CA5 [Field, *et al.*, Mol. Cell Biol., **8**:2159-2165 (1988)]; the c-myc tag and the 8F9, 3C7, 6E10, G4, B7, and 9E10 antibodies thereto [Evan, *et al.*, Molecular and Cellular Biology, **5**:3610-3616 (1985)]; and the Herpes Simplex virus glycoprotein D (gD) tag and its antibody [Paborsky, *et al.*, Protein Engineering, **3**(6):547-553 (1990)]. Other tag polypeptides include the Flag-peptide [Hopp, *et al.*, BioTechnology, **6**:1204-1210 (1988)]; the KT3 epitope peptide [Martin, *et al.*, Science, **255**:192-194 (1992)]; tubulin epitope peptide [Skinner, *et al.*, J. Biol. Chem., **266**:15163-15166 (1991)]; and the T7 gene 10 protein peptide tag [Lutz-Freyermuth, *et al.*, Proc. Natl. Acad. Sci. USA, **87**:6393-6397 (1990)].

Also included with the definition of ASP05 protein are other ASP05 proteins of the ASP05 family, and ASP05 proteins from other organisms, which are cloned and expressed as outlined below. Thus, probe or degenerate polymerase chain reaction (PCR) primer sequences may be used to find other related ASP05 proteins from humans or other organisms. As will be appreciated by those in the art, particularly useful probe and/or PCR primer sequences include the unique areas of the ASP05 nucleic acid sequence. Thus, useful probe or primer sequences may be designed to: all or part of the sequence of the catalytic domain, or all or part of the unique binding domain, or combinations. As is generally known in the art, preferred PCR primers are from about 15 to about 35 nucleotides in length, with from about 20 to about 30 being preferred, and may contain modified nucleotides, for example inosine, as needed. The conditions for the PCR reaction are well known in the art.

Once the ASP05 nucleic acid is identified, it can be cloned and, if necessary, its constituent parts recombined to form the entire ASP05 nucleic acid. Once isolated from its natural source, *e.g.*, contained within a plasmid or other vector or excised therefrom as a linear nucleic acid segment, the recombinant ASP05 nucleic acid can be further-used as a probe to identify and isolate other ASP05 nucleic acids. It can also be used as a "precursor" nucleic acid to make modified or variant ASP05 nucleic acids and proteins.

Using the nucleic acids of the present invention which encode an ASP05 protein, a variety of expression vectors are made. The expression vectors may be either self-replicating extrachromosomal vectors or vectors which integrate into a host genome. Generally, these expression vectors include transcriptional and translational regulatory nucleic acid operably linked to the nucleic acid encoding the ASP05 protein. The term "control sequences" refers to DNA sequences necessary for the expression of an operably linked coding sequence in a particular host organism. The control sequences that are suitable for prokaryotes, for example, include a promoter, optionally an operator sequence, and a ribosome binding site. Eukaryotic cells are known to utilize promoters, polyadenylation signals, and enhancers.

Nucleic acid is "operably linked" when it is placed into a functional relationship with another nucleic acid sequence. For example, DNA for a presequence or secretory leader is operably linked to DNA for a polypeptide if it is expressed as a preprotein that participates in the secretion of the polypeptide; a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence; or a ribosome binding site is operably linked to a coding sequence if it is positioned so as to facilitate translation. Generally, "operably linked" means that the DNA sequences being linked are contiguous, and, in the case of a secretory leader, contiguous and in reading phase. However, enhancers do not have to be contiguous. Linking is accomplished by ligation at convenient restriction sites. If such sites do not exist, the synthetic oligonucleotide adaptors or linkers are used in accordance with conventional practice. The transcriptional and translational

regulatory nucleic acid will generally be appropriate to the host cell used to express the ASP05 protein; for example, transcriptional and translational regulatory nucleic acid sequences from *Bacillus* are preferably used to express the ASP05 protein in *Bacillus*. Numerous types of appropriate expression vectors, and suitable regulatory sequences are known in the art for a variety of host cells.

In general, the transcriptional and translational regulatory sequences may include, but are not limited to, promoter sequences, ribosomal binding sites, transcriptional start and stop sequences, translational start and stop sequences, and enhancer or activator sequences. In a preferred embodiment, the regulatory sequences include a promoter and transcriptional start and stop sequences.

Promoter sequences encode either constitutive or inducible promoters. The promoters may be either naturally occurring promoters or hybrid promoters. Hybrid promoters, which combine elements of more than one promoter, are also known in the art, and are useful in the present invention.

In addition, the expression vector may comprise additional elements. For example, the expression vector may have more than one replication system, thus allowing it to be maintained in more than one organism, for example, for expression in mammalian or insect cells and for cloning and amplification in a procaryotic host. Furthermore, for integrating expression vectors, the expression vector contains at least one sequence homologous to the host cell genome, and preferably two homologous sequences which flank the expression construct. The integrating vector may be directed to a specific locus in the host cell by selecting the appropriate homologous sequence for inclusion in the vector. Constructs for integrating vectors are well known in the art.

In addition, in a preferred embodiment, the expression vector contains a selectable marker gene to allow the selection of transformed host cells. Selection genes are well known in the art and will vary with the host cell used.

A preferred expression vector system is a retroviral vector system such as is generally described in PCT/US97/01019 and PCT/US97/01048, both of which are hereby expressly incorporated by reference.

The ASP05 proteins of the present invention are produced by culturing a host cell transformed with an expression vector containing nucleic acid encoding an ASP05 protein, under the appropriate conditions to induce or cause expression of the ASP05 protein. The conditions appropriate for ASP05 protein expression will vary with the choice of the expression vector and the host cell, and will be easily ascertained by one skilled in the art through routine experimentation. For example, the use of constitutive promoters in the expression vector will require optimizing the growth and proliferation of the host cell, while the use of an inducible promoter requires the appropriate growth conditions for induction. In addition, in some embodiments, the timing of the harvest is important. For example, the baculoviral systems used in insect cell expression are lytic viruses, and thus harvest time selection can be crucial for product yield.

The ASP05 proteins of the present invention are "capable of being expressed" in appropriate host cells, resulting in biologically active protein. In a preferred embodiment the ASP05 proteins are expressed in Baculovirus-infected insect cells. Bac-to-Bac Baculovirus Expression System (GibcoBRL, Gaithersburg, MD) is used to express the protein. The ASP05 cDNA tagged with flag epitope (Eastman Kodak, New Haven. CT) is ligated into pFASTBac1 vector, which is then transformed into DH1 OBac competent cells to generate recombinant bacmid. The bacmid DNA is transfected into *Spodoptera frugiperda* ("Sf9") insect cells (ATCC CRL 1711) to produce recombinant baculovirus. High titer stock is used to infect sf9 insect cells and the expressed protein is purified by affinity chromatography using an agarose-linked antibody specific for the Flag epitope tag engineered into the C-terminal end of the expressed ASP05. Fractions containing the eluted and tagged ASP05 polypeptide are pooled and dialyzed against buffer, such as MES or HEPES buffer.

Appropriate host cells include yeast, bacteria, archebacteria, fungi, and insect and animal cells, including mammalian cells, for example primary cells, including stem cells, including, but not limited to bone marrow stem cells. Of particular interest are SF9 cells, *Drosophila melangaster* cells, *Saccharomyces cerevisiae* and other yeasts, *E. coli*, *Bacillus subtilis*, C129 cells, 293 cells, Neurospora, BHK, CHO, COS, and HeLa cells, fibroblasts, Schwanoma cell lines, immortalized mammalian myeloid and lymphoid cell lines, Jukat cells, human cells and other primary cells.

Alternatively, purification of the IgG tagged (or Fc tagged) ASP05 polypeptide can be performed using known chromatography techniques, including for instance, Protein A or protein G column chromatography.

In another preferred embodiment, the ASP05 proteins are expressed in mammalian cells. Mammalian expression systems are also known in the art, and include retroviral systems. A mammalian promoter is any DNA sequence capable of binding mammalian RNA polymerase and initiating the downstream (3') transcription of a coding sequence for ASP05 protein into mRNA. A promoter will have a transcription initiating region, which is usually placed proximal to the 5' end of the coding sequence, and a TATA box, using a located 25-30 base pairs upstream of the transcription initiation site. The TATA box is thought to direct RNA polymerase II to begin RNA synthesis at the correct site. A mammalian promoter will also contain an upstream promoter element (enhancer element), typically located within 100 to 200 base pairs upstream of the TATA box. An upstream promoter element determines the rate at which transcription is initiated and can act in either orientation. Of particular use as mammalian promoters are the promoters from mammalian viral genes, since the viral genes are often highly expressed and have a broad host range. Examples include the SV40 early promoter, mouse mammary tumor virus LTR promoter, adenovirus major late promoter, herpes simplex virus promoter, the CMV promoter, a retroviral LTR promoter, mouse maloney leukemia virus LTR, or pBabeMN.

Typically, transcription termination and polyadenylation sequences recognized by mammalian cells are regulatory regions located 3' to the translation stop codon and thus, together with the promoter elements, flank the coding sequence. The 3' terminus of the mature mRNA is formed by site-specific post-translational cleavage and polyadenylation. Examples of transcription terminator and polyadenlytion signals include those derived form SV40.

The methods of introducing exogenous nucleic acid into mammalian hosts, as well as other hosts, is well known in the art, and will vary with the host cell used. Techniques include dextran-mediated transfection, calcium phosphate precipitation, polybrene mediated transfection, protoplast fusion, electroporation, viral infection, encapsulation of the polynucleotide(s) in liposomes, and direct microinjection of the DNA into nuclei.

In a preferred embodiment, ASP05 proteins are expressed in bacterial systems. Bacterial expression systems are well known in the art.

A suitable bacterial promoter is any nucleic acid sequence capable of binding bacterial RNA polymerase and initiating the downstream (3') transcription of the coding sequence of ASP05 protein into mRNA. A bacterial promoter has a transcription initiation region which is usually placed proximal to the 5' end of the coding sequence. This transcription initiation region typically includes an RNA polymerase binding site and a transcription initiation site. Sequences encoding metabolic pathway enzymes provide particularly useful promoter sequences. Examples include promoter sequences derived from sugar metabolizing enzymes, such as galactose, lactose and maltose, and sequences derived from biosynthetic enzymes such as tryptophan. Promoters from bacteriophage may also be used and are known in the art. In addition, synthetic promoters and hybrid promoters are also useful; for example, the *tac* promoter is a hybrid of the *trp* and *lac* promoter sequences. Furthermore, a bacterial promoter can include naturally occurring promoters of non-bacterial origin that have the ability to bind bacterial RNA polymerase and initiate transcription.

In addition to a functioning promoter sequence, an efficient ribosome binding site is desirable. In *E. coli*, the ribosome binding site is called the Shine-Delgarno (SD) sequence and includes an initiation codon and a sequence 3-9 nucleotides in length located 3 - 11 nucleotides upstream of the initiation codon.

The expression vector may also include a signal peptide sequence that provides for secretion of the ASP05 protein in bacteria. The signal sequence typically encodes a signal peptide comprised of hydrophobic amino acids which direct the secretion of the protein from the cell, as is well known in the art. The protein is either secreted into the growth media (gram-positive bacteria) or into the periplasmic space, located between the inner and outer membrane of the cell (gram-negative bacteria).

The bacterial expression vector may also include a selectable marker gene to allow for the selection of bacterial strains that have been transformed. Suitable selection genes include genes which render the bacteria resistant to drugs such as ampicillin, chloramphenicol, erythromycin, kanamycin, neomycin and tetracycline. Selectable markers also include biosynthetic genes, such as those in the histidine, tryptophan and leucine biosynthetic pathways.

These components are assembled into expression vectors. Expression vectors for bacteria are well known in the art, and include vectors for *Bacillus subtilis, E. coli, Streptococcus cremoris*, and *Streptococcus lividans*, among others.

The bacterial expression vectors are transformed into bacterial host cells using techniques well known in the art, such as calcium chloride treatment, electroporation, and others.

In a preferred embodiment, ASP05 protein is produced in yeast cells. Yeast expression systems are well known in the art, and include expression vectors for *Saccharomyces cerevisiae*, *Candida albicans* and *C. maltosa*, *Hansenula polymorpha*, *Kluyveromyces fragilis* and *K. lactis*, *Pichia guillerimondii* and *P.*

pastoris, *Schizosaccharomyces pombe*, and *Yarrowia lipolytica*. Preferred promoter sequences for expression in yeast include the inducible GAL1,10 promoter, the promoters from alcohol dehydrogenase, enolase, glucokinase, glucose-6-phosphate isomerase, glyceraldehyde-3-phosphate-dehydrogenase, hexokinase, phosphofructokinase, 3-phosphoglycerate mutase, pyruvate kinase, and the acid phosphatase gene. Yeast selectable markers include ADE2, HIS4, LEU2, TRP1, and ALG7, which confers resistance to tunicamycin; the neomycin phosphotransferase gene, which confers resistance to G418; and the CUP1 gene, which allows yeast to grow in the presence of copper ions.

The ASP05 protein may also be made as a fusion protein, using techniques well known in the art. Thus, for example, for the creation of monoclonal antibodies, if the desired epitope is small, the ASP05 protein may be fused to a carrier protein to form an immunogen. Alternatively, the ASP05 protein may be made as a fusion protein to increase expression, to facilitate purification or for other reasons. For example, when the ASP05 protein is an ASP05 peptide, the nucleic acid encoding the peptide may be linked to other nucleic acid for expression purposes. In a preferred embodiment, a nucleic acid encoding a protease cleavage site is placed between and in frame with the nucleic acid carrier protein and the ASP05 polypeptide. The protease cleavage site can be used, for example, to facilitate purification of the expressed ASP05 polypeptide or for other purposes. Preferably the protease cleavage site is not found in the ASP05 polypeptide or the ASP05 polypeptide is not substantially digested by the protease that binds to and digests at the cleavage site.

In one embodiment, the ASP05 nucleic acids, proteins and antibodies of the invention are labeled. By "labeled" herein is meant that a compound has at least one element, isotope or chemical compound attached to enable the detection of the compound. In general, labels fall into three classes: a) isotopic labels, which may be radioactive or heavy isotopes; b) immune labels, which may be antibodies or antigens; and c) colored or fluorescent dyes. The labels may be incorporated into the compound at any position.

In a preferred embodiment, the ASP05 protein is purified or isolated after expression. ASP05 proteins may be isolated or purified in a variety of ways known to those skilled in the art depending on what other components are present in the sample. Standard purification methods include electrophoretic, molecular, immunological and chromatographic techniques, including ion exchange, hydrophobic, affinity, and reverse-phase HPLC chromatography, and chromatofocusing. For example, the ASP05 protein may be purified using a standard antiASP05- antibody column. Ultrafiltration and diafiltration techniques, in conjunction with protein concentration, are also useful. For general guidance in suitable purification techniques, see Scopes, R., Protein Purification, Springer-Verlag, NY (1982). The degree of purification necessary will vary depending on the use of the ASP05 protein. In some instances no purification will be necessary.

Once expressed and purified if necessary, the ASP05 proteins and nucleic acids are useful in a number of applications.

In a preferred embodiment, modified ASP05, and cells containing the modified proteins, are made. In one embodiment, non-human animals (preferably transgenic) are made that contain modified ASP05; similarly, "knock-out" animal models and ASP05 transgenic animals that contain an inducible promoter may be made.

In a preferred embodiment, the ASP05 proteins, nucleic acids, modified ASP05 proteins and cells containing the native or modified ASP05 proteins are used in screening assays. Identification of this important serine protease permits the design of drug screening assays for compounds that modulate ASP05 biological activity, including proteolytic activity, binding, etc.

Screens may be designed to first find candidate agents that can bind to ASP05, and then these agents may be used in assays that evaluate the ability of the candidate agent to modulate ASP05 activity. Thus, as will be appreciated by those in the art, there are a number of different assays which may be run; binding assays and activity assays. Of particular importance in these embodiments is that the catalytic

portion of ASP05 is mainly responsible for the selective cleavage of IGFBP. Further, IGFBP may be present in body fluids as well as in tissues *in vivo*. Accordingly, candidate agents may be added directly to cells without the need to target the agents to cells when assaying for cleavage. Accordingly, the catalytic domain of ASP05 may be used independently as a basis for biological assays.

Thus, in a preferred embodiment, the methods comprise combining an ASP05 protein and a candidate bioactive agent, and determining the binding of the candidate agent to the ASP05 protein. Preferred embodiments utilize the human ASP05 (or portions, as outlined herein, such as the catalytic or binding domain), although other mammalian ASP05 proteins may also be used in either case, including rodents (mice, rats, hamsters, guinea pigs, etc.), farm animals (cows, sheep, pigs, horses, etc.) and primates. These latter embodiments may be preferred in the development of animal models of human disease. In some embodiments, as outlined herein, variant or derivative ASP05 proteins may be used, including deletion ASP05 proteins as outlined above.

Furthermore, included within the definition of ASP05 proteins are portions of ASP05 proteins; that is, either the full-length protein may be used, or functional portions thereof. In a preferred embodiment, the catalytic domain of ASP05 may be used with or without the binding region. In an additional preferred embodiment, the binding domain of ASP05 may be used with or without the catalytic region. In addition, the assays described herein may utilize either isolated ASP05 proteins or cells comprising the ASP05 proteins, as required by the specific assay.

Generally, in a preferred embodiment of the methods herein, the ASP05 and the candidate agent are placed together in sample receiving areas or one of them may be bound to an insoluble support (*e.g.*, a microtiter plate, an array, etc.). The insoluble support may be made of any composition to which the ASP05 or the candidate agent can be bound, and is otherwise compatible with the overall method of screening. The surface of such supports may be solid or porous and of any convenient shape. Examples of suitable insoluble supports include microtiter

plates, arrays, membranes and beads. These are typically made of glass, plastic (*e.g.*, polystyrene), polysaccharides, nylon or nitrocellulose, teflon™, etc. Microtiter plates and arrays are especially convenient because a large number of assays can be carried out simultaneously, using small amounts of reagents and samples. The particular manner of binding of the composition is not crucial so long as it is compatible with the reagents and overall methods of the invention, maintains the activity of the composition and is nondiffusable. Preferred methods of binding include the use of antibodies (which do not sterically block the protease sequence when the ASP05 protein is bound to the support), direct binding to "sticky" or ionic supports, chemical crosslinking, the synthesis of the ASP05 protease on the surface, etc. Following binding of the ASP05 protein, excess unbound material is removed by washing. The sample receiving areas may then be blocked through incubation with bovine serum albumin (BSA), casein or other innocuous protein or other moiety.

A candidate bioactive agent, defined below, is added to the assay. Novel binding agents include specific antibodies, non-natural binding agents identified in screens of chemical libraries, peptide analogs, etc. Of particular interest are screening assays for agents that have a low toxicity for human cells. A wide variety of assays may be used for this purpose, including labeled *in vitro* protein-protein binding assays, electrophoretic mobility shift assays, immunoassays for protein binding, functional assays (phosphorylation assays, etc.) and the like.

The term "candidate bioactive agent" or "exogeneous compound" as used herein describes any molecule, *e.g.*, protein, oligopeptide, small organic molecule, polysaccharide, polynucleotide, etc., with the capability of directly or indirectly modulating the biological activity of the ASP05 protein. Generally a plurality of assay mixtures are run in parallel with different agent concentrations to obtain a differential response to the various concentrations. Typically, one of these concentrations serves as a negative control, *i.e.*, at zero concentration or below the level of detection.

Candidate agents encompass numerous chemical classes, though typically they are organic molecules, preferably small organic compounds having a molecular weight of more than 100 and less than about 2,500 daltons. Candidate agents comprise functional groups necessary for structural interaction with proteins, particularly hydrogen bonding, and typically include at least an amine, carbonyl, hydroxyl or carboxyl group, preferably at least two of the functional chemical groups. The candidate agents often comprise cyclical carbon or heterocyclic structures and/or aromatic or polyaromatic structures substituted with one or more of the above functional groups. Candidate agents are also found among biomolecules including peptides, saccharides, fatty acids, steroids, purines, pyrimidines, derivatives, structural analogs or combinations thereof. Particularly preferred are peptides.

Candidate agents are obtained from a wide variety of sources including libraries of synthetic or natural compounds. For example, numerous means are available for random and directed synthesis of a wide variety of organic compounds and biomolecules, including expression of randomized oligonucleotides. Alternatively, libraries of natural compounds in the form of bacterial, fungal, plant and animal extracts are available or readily produced. Additionally, natural or synthetically produced libraries and compounds are readily modified through conventional chemical, physical and biochemical means. Known pharmacological agents may be subjected to directed or random chemical modifications, such as acylation, alkylation, esterification, amidification to produce structural analogs.

In a preferred embodiment, the candidate bioactive agents are proteins. By "protein" herein is meant at least two covalently attached amino acids, which includes proteins, polypeptides, oligopeptides and peptides. The protein may be made up of naturally occurring amino acids and peptide bonds, or synthetic peptidomimetic structures. Thus "amino acid", or "peptide residue", as used herein means both naturally occurring and synthetic amino acids. For example, homo-phenylalanine, citrulline and noreleucine are considered amino acids for the purposes of the invention. "Amino acid" also includes imino acid residues such as proline and hydroxyproline. The side chains may be in either the (R) or the (S)

configuration. In the preferred embodiment, the amino acids are in the (S) or L-configuration. If non-naturally occurring side chains are used, non-amino acid substituents may be used, for example to prevent or retard *in vivo* degradations.

In a preferred embodiment, the candidate bioactive agents are naturally occuring proteins or fragments of naturally occuring proteins. Thus, for example, cellular extracts containing proteins, or random or directed digests of proteinaceous cellular extracts, may be used. In this way libraries of procaryotic and eucaryotic proteins may be made for screening against ASP05. Particularly preferred in this embodiment are libraries of bacterial, fungal, viral, and mammalian proteins, with the latter being preferred, and human proteins being especially preferred.

In a preferred embodiment, the candidate bioactive agents are peptides of from about 5 to about 30 amino acids, with from about 5 to about 20 amino acids being preferred, and from about 7 to about 15 being particularly preferred. The peptides may be digests of naturally occuring proteins as is outlined above, random peptides, or "biased" random peptides. By "randomized" or grammatical equivalents herein is meant that each nucleic acid and peptide consists of essentially random nucleotides and amino acids, respectively. Since generally these random peptides (or nucleic acids, discussed below) are chemically synthesized, they may incorporate any nucleotide or amino acid at any position. The synthetic process can be designed to generate randomized proteins or nucleic acids, to allow the formation of all or most of the possible combinations over the length of the sequence, thus forming a library of randomized candidate bioactive proteinaceous agents.

In one embodiment, the library is fully randomized, with no sequence preferences or constants at any position. In a preferred embodiment, the library is biased. That is, some positions within the sequence are either held constant, or are selected from a limited number of possibilities. For example, in a preferred embodiment, the nucleotides or amino acid residues are randomized within a defined class, for example, of hydrophobic amino acids, hydrophilic residues, sterically biased (either

small or large) residues, towards the creation of cysteines, for cross-linking, prolines for SH-3 domains, serines, threonines, tyrosines or histidines for phosphorylation sites, etc., or to purines, etc.

In a preferred embodiment, the candidate bioactive agents are nucleic acids. By "nucleic acid" or "oligonucleotide" or grammatical equivalents herein means at least two nucleotides covalently linked together. A nucleic acid of the present invention will generally contain phosphodiester bonds, although in some cases, as outlined below, nucleic acid analogs are included that may have alternate backbones, comprising, for example, phosphoramide (Beaucage, *et al.*, Tetrahedron, **49**(10):1925 (1993) and references therein; Letsinger, J. Org. Chem., **35**:3800 (1970); Sprinzl, *et al.*, Eur. J. Biochem., **81**:579 (1977); Letsinger, *et al.*, Nucl. Acids Res., **14**:3487 (1986); Sawai, *et al.*, Chem. Lett., 805 (1984), Letsinger, *et al.*, J. Am. Chem. Soc., **110**:4470 (1988); and Pauwels, *et al.*, Chemica Scripta, **26**:141 (1986)), phosphorothioate (Mag, *et al.*, Nucleic Acids Res., **19**:1437 (1991); and U.S. Patent No. 5,644,048), phosphorodithioate (Briu, *et al.*, J. Am. Chem. Soc., **111**:2321 (1989)), O-methylphophoroamidite linkages (see Eckstein, Oligonucleotides and Analogues: A Practical Approach, Oxford University Press), and peptide nucleic acid backbones and linkages (see Egholm, J. Am. Chem. Soc., **114**:1895 (1992); Meier, *et al.*, Chem. Int. Ed. Engl., **31**:1008 (1992); Nielsen, Nature, **365**:566 (1993); Carlsson, *et al.*, Nature, **380**:207 (1996), all of which are incorporated by reference)). Other analog nucleic acids include those with positive backbones (Denpcy, *et al.*, Proc. Natl. Acad. Sci. USA, **92**:6097 (1995)); non-ionic backbones (U.S. Patent Nos. 5,386,023; 5,637,684; 5,602,240; 5,216,141; and 4,469,863; Kiedrowshi, *et al.*, Angew. Chem. Intl. Ed. English, **30**:423 (1991); Letsinger, *et al.*, J. Am. Chem. Soc., **110**:4470 (1988); Letsinger, *et al.*, Nucleoside & Nucleotide, **13**:1597 (1994); Chapters 2 and 3, ASC Symposium Series 580, "Carbohydrate Modifications in Antisense Research", Ed. Y.S. Sanghui and P. Dan Cook; Mesmaeker, *et al.*, Bioorganic & Medicinal Chem. Lett., **4**:395 (1994); Jeffs, *et al.*, J. Biomolecular NMR, **34**:17 (1994); Tetrahedron Lett., **37**:743 (1996)) and non-ribose backbones, including those described in U.S. Patent Nos. 5,235,033 and 5,034,506, and Chapters 6 and 7, ASC Symposium Series 580, "Carbohydrate

Modifications in Antisense Research", Ed. Y.S. Sanghui and P. Dan Cook. Nucleic acids containing one or more carbocyclic sugars are also included within the definition of nucleic acids (see Jenkins, *et al.*, Chem. Soc. Rev., (1995) pp. 169-176). Several nucleic acid analogs are described in Rawls, C & E News, June 2, 1997, page 35. All of these references are hereby expressly incorporated by reference. These modifications of the ribose-phosphate backbone may be done to facilitate the addition of additional moieties such as labels, or to increase the stability and half-life of such molecules in physiological environments. In addition, mixtures of naturally occurring nucleic acids and analogs can be made. Alternatively, mixtures of different nucleic acid analogs, and mixtures of naturally occuring nucleic acids and analogs may be made. The nucleic acids may be single stranded or double stranded, as specified, or contain portions of both double stranded or single stranded sequence. The nucleic acid may be DNA, both genomic and cDNA, RNA or a hybrid, where the nucleic acid contains any combination of deoxyribo- and ribo-nucleotides, and any combination of bases, including uracil, adenine, thymine, cytosine, guanine, inosine, xathanine hypoxathanine, isocytosine, isoguanine, etc.

As described above generally for proteins, nucleic acid candidate bioactive agents may be naturally occuring nucleic acids, random nucleic acids, or "biased" random nucleic acids. For example, digests of procaryotic or eucaryotic genomes may be used as is outlined above for proteins.

In a preferred embodiment, the candidate bioactive agents are organic chemical moieties, a wide variety of which are available in the literature.

The determination of the binding of the candidate bioactive agent to the ASP05 may be done in a number of ways. In a preferred embodiment, the candidate bioactive agent is labelled, and binding determined directly. For example, this may be done by attaching all or a portion of the ASP05 to a solid support, adding a labelled candidate agent (for example a fluorescent label), washing off excess reagent, and

determining whether the label is present on the solid support. Various blocking and washing steps may be utilized as is known in the art.

By "labeled" herein is meant that the compound is either directly or indirectly labeled with a label which provides a detectable signal, *e.g.*, radioisotope, fluorescers, enzyme, antibodies, particles such as magnetic particles, chemiluminescers, or specific binding molecules, etc. Specific binding molecules include pairs, such as biotin and streptavidin, digoxin and antidigoxin, etc. For the specific binding members, the complementary member would normally be labeled with a molecule which provides for detection, in accordance with known procedures, as outlined above. The label can directly or indirectly provide a detectable signal.

In some embodiments, only one of the components is labeled. For example, the ASP05 protein (or proteinaceous candidate agent) may be labeled at tyrosine positions using ^{125}I, or with fluorophores. Alternatively, more than one component may be labeled with different labels; using ^{125}I for the ASP05 protein, for example, and a fluorophor for the candidate agents.

In a preferred embodiment, the binding of the candidate bioactive agent is determined through the use of competitive binding assays. In this embodiment, the competitor is a binding moiety known to bind to the target molecule, such as an antibody, peptide, binding partner, ligand, etc. Under certain circumstances, there may be competitive binding as between the bioactive agent and the binding moiety, with the binding moiety displacing the bioactive agent.

In one embodiment, the candidate bioactive agent is labeled. Either the candidate bioactive agent, or the competitor, or both, is added first to the ASP05 protein for a time sufficient to allow binding, if present. Incubations may be performed at any temperature which facilitates optimal activity, typically between 4 and 40°C. Incubation periods are selected for optimum activity, but may also be optimized to facilitate rapid high through put screening. Typically between 0.1 and 1 hour will

be sufficient. Excess reagent is generally removed or washed away. The second component is then added, and the presence or absence of the labeled component is followed, to indicate binding.

In a preferred embodiment, the competitor is added first, followed by the candidate bioactive agent. Displacement of the competitor is an indication that the candidate bioactive agent is binding to the ASP05 protein and thus is capable of binding to, and potentially modulating, the activity of the ASP05 protein. In this embodiment, either component can be labeled. Thus, for example, if the competitor is labeled, the presence of label in the wash solution indicates displacement by the agent. Alternatively, if the candidate bioactive agent is labeled, the presence of the label on the support indicates displacement.

In an alternative embodiment, the candidate bioactive agent is added first, with incubation and washing, followed by the competitor. The absence of binding by the competitor may indicate that the bioactive agent is bound to the ASP05 protein with a higher affinity. Thus, if the candidate bioactive agent is labeled, the presence of the label on the support, coupled with a lack of competitor binding, may indicate that the candidate agent is capable of binding to the ASP05 protein.

Alternatively, a preferred embodiment utilizes differential screening to identify drug candidates that bind to the native ASP05 protease, but cannot bind to modified ASP05 proteases. The structure of the ASP05 protease may be modeled, and used in rational drug design to synthesize agents that interact with that site. Drug candidates that modulate cleavage are also identified by screening drugs for the ability to either enhance or reduce specific cleavage by the ASP05 protease.

Positive controls and negative controls may be used in the assays. Preferably all control and test samples are performed in at least triplicate to obtain statistically significant results. Incubation of all samples is for a time sufficient for the binding of the agent to the ASP05 protease. Following incubation, all samples are washed free of non-specifically bound material and the amount of bound, generally labeled

agent determined. For example, where a radiolabel is employed, the samples may be counted in a scintillation counter to determine the amount of bound compound.

Screening for agents that modulate the activity of ASP05 may also be done. In a preferred embodiment, methods for screening for a bioactive agent capable of modulating the activity of an ASP05 protein comprise the steps of adding a candidate bioactive agent to a sample of ASP05, as above, and determining an alteration in the biological activity of the ASP05 protein. "Modulating the activity of ASP05" includes an increase in activity, a decrease in activity, or a change in the type or kind of activity present. Thus, in this embodiment, the candidate agent should both bind to ASP05 (although this may not be necessary), and alter its biological or biochemical activity, as defined herein. The methods include both *in vitro* screening methods, as are generally outlined above, and *in vivo* screening of cells for alterations in the presence, distribution, activity or amount of the ASP05 protein.

Thus, in this embodiment, the methods comprise combining an ASP05 protein and a candidate bioactive agent, and testing the biological activity of the ASP05 protein as is known in the art to evaluate the effect of the agent on the activity of ASP05. By "ASP05 activity" or grammatical equivalents herein is meant one of the putative identified functions of ASP05 including, but not limited to the ability of ASP05 to selectively cleave IGFBP, the ability of ASP05 to bind to IGFBP and the ability of ASP05 to alter IGF-mediated cell function.

In one embodiment, such modulation may result in response to the catalytic domain of ASP05 and may correspond to a decrease or an increase in selective cleavage. In a preferred embodiment, the activity of the catalytic domain of ASP05 is increased; in another preferred embodiment, the activity of the catalytic domain of ASP05 is decreased. Thus, bioactive agents that are antagonists (i.e. decrease the activity of the ASP05 protein) are preferred in some embodiments, and bioactive agents that are agonists (i.e., increase the activity of the ASP05 protein) may be preferred in other embodiments. For example, agents which bind to an ASP05 protein, could be

antagonists. In addition, agents which bind to an ASP05 protein, may increase specific cleavage by the ASP05 protein, and thus act as agonists.

In another embodiment, such modulation may result in response to the binding domain of ASP05 and may correspond to a decrease or an increase in binding. In a preferred embodiment, the activity of the binding domain of ASP05 is increased; in another preferred embodiment, the activity of the binding domain of ASP05 is decreased. Thus, bioactive agents that are antagonists (i.e. decrease the binding of an ASP05 protein) are preferred in some embodiments, and bioactive agents that are agonists (i.e., increase the binding of the ASP05 protein) may be preferred in other embodiments. For example, agents which decrease binding by an ASP05 protein, could be antagonists. In addition, agents may increase binding to the ASP05 protein, and thus act as agonists.

In a preferred embodiment, the invention provides methods for screening for bioactive agents capable of modulating the serine protease activity of an ASP05 protease. The methods comprise adding a candidate bioactive agent, as defined above, to a cell comprising an ASP05 protein or the ASP05 catalytic domain. Preferred cell types include, but are not limited to, SF9 and *E coli* cells and mammalian cells. The cells contain a recombinant nucleic acid that encodes an ASP05 protease; that is, the cells express ASP05. In a preferred embodiment, a library of candidate agents are tested in a plurality of assays with various IGFBPs. The effect of the candidate agent on selective cleavage, for example, is then evaluated. If ASP05 is acting, *i.e.*, there is no antagonistic agent present, IGFBP will not be selectively cleaved as detected by Western blot analysis with either antibody or ligand detection. However, if antagonistic agents are present, cleavage will not occur.

Detection of cleavage may be done as will be appreciated by those in the art. In one embodiment, purified ASP05 is incubated with various IGFBP, including, but not limited to IGFBP-1 through IGFBP-6. The reaction products to be analyzed are run on a SDS-PAGE gel and subsequently transferred to a polyvinylidene difluoride

(PVDF) membrane. In one further embodiment, the membrane is incubated with IGFBP-specific antibody, followed by incubation with a secondary antibody as exemplified by HRP-conjugated mouse IgG antibody, followed by subsequent visualization by chemiluminescence. In an alternate embodiment, detection is accomplished by incubation with ^{125}IGF-I (or ^{125}IGF-II) and subsequent autoradiography.

In addition, assays utilizing fluorescent sythetic peptides as the substrate may also be done as will be appreciated by those in the art, and include competitive-type assays.

A variety of other reagents may be included in the screening assays. These include reagents like salts, neutral proteins, *e.g.*, albumin, detergents, etc which may be used to facilitate optimal protein-protein binding and/or reduce non-specific or background interactions. Also reagents that otherwise improve the efficiency of the assay, such as protease inhibitors, nuclease inhibitors, anti-microbial agents, etc., may be used. The mixture of components may be added in any order that provides for the requisite binding.

In this way, bioactive agents are identified. Compounds with pharmacological activity are able to enhance or interfere with the activity of the ASP05 protein. The compounds having the desired pharmacological activity may be administered in a physiologically acceptable carrier to a host, as previously described. The agents may be administered in a variety of ways, orally, parenterally *e.g.*, subcutaneously, intraperitoneally, intravascularly, etc. Depending upon the manner of introduction, the compounds may be formulated in a variety of ways. The concentration of therapeutically active compound in the formulation may vary from about 0.1-100 wt.%.

The pharmaceutical compositions can be prepared in various forms, such as granules, tablets, pills, suppositories, capsules, suspensions, salves, lotions and the like. Pharmaceutical grade organic or inorganic carriers and/or diluents suitable for

oral and topical use can be used to make up compositions containing the therapeutically-active compounds. Diluents known to the art include aqueous media, vegetable and animal oils and fats. Stabilizing agents, wetting and emulsifying agents, salts for varying the osmotic pressure or buffers for securing an adequate pH value, and skin penetration enhancers can be used as auxiliary agents.

Without being bound by theory, it appears that ASP05 is an important enzyme involved in the regulation, differentiation, proliferation and function of many types of cells, including those derived from brain, blood, bone, liver, muscle, kidney. and the nervous system. Accordingly, disorders based on mutant or variant ASP05 forms or concentrations in various body fluids or tissues may be determined. In one embodiment, the invention provides methods for identifying cells containing variant forms or concentrations of ASP05 comprising determining the type and concetration of endogenous ASP05 in a body fluid and tissue. As will be appreciated by those in the art, this may be done using any number of techniques routinely used in the art. The method may include comparing the form or concentration of ASP05 to a known form or concentration of ASP05 *i.e.*, in a different biological sample.

In a preferred embodiment, the presence of a difference in the form or concentration of the ASP05 of the patient and the known form or concentration of ASP05 may be indicative of a disease state or a propensity for a disease state, as outlined herein.

The present discovery relating to the role of ASP05 in selective cleavage of IGFBP thus provides methods for screening for inhibitors of such cleavage.

In a preferred embodiment, the ASP05 proteins, and particularly ASP05 fragments, are useful in the study or treatment of conditions which are mediated by IGF, *i.e.*. to diagnose, treat or prevent IGF-mediated disorders. Thus, "IGF mediated disorders" or "disease state" include conditions involving disorders or cellular processes mediated by IGF, as well as conditions which have inappropriate IGF metabolism. Accordingly, IGF mediated disorders include, but are not limited to, proliferative

disorders such as cancer (various kinds) and the neointimal hyperplasia associated with restenosis, angiogenesis associated with tumor growth and neovascularization diseases of the eye such as, but not limited to, accelerated macular degeneration (AMD), diabetic retinopathy, retinopathy of prematurity (ROP), ischemic retinopathies, and neovascular glaucoma. Other IGF mediated disorders include, but are not limited to, bone metabolism disorders such as osteoporosis, and osteoarthritis, diseases of muscle, brain, ovary, uterus and placenta.

Thus, in one embodiment, methods of inhibiting IGFBP will involve selective antagonism of IGF function in a manner consistent with the use of such inhibitors for the treatment of human disease. In one embodiment, the methods comprise administering to a cell an anti-ASP05- antibody that reduces or eliminates the biological activity of the endogenous ASP05. Alternatively, the methods comprise administering to a cell or organism a recombinant nucleic acid encoding an ASP05. As will be appreciated by those in the art, this may be accomplished in any number of ways. In a preferred embodiment, the activity of ASP05 is decreased by decreasing the amount of ASP05 in the cell, for example by interfering with expression of the endogenous ASP05 or by administering a gene encoding a modified ASP05, using known gene-therapy techniques, for example. In a preferred embodiment, the gene therapy techniques include the incorporation of an exogeneous gene.

In one embodiment, the invention provides methods for diagnosing an IGF mediated disorder in an individual. The methods comprise measuring the activity and expression of ASP05 in a tissue from the individual or patient, which may include a measurement of the amount or specific activity of ASP05. This activity is quantified and compared to the activity of ASP05 from either an unaffected second individual or from an unaffected tissue from the first individual. When these activities are different, the first individual may be at risk for IGF mediated disorder. In this way, for example, monitoring of various disease conditions may be done, by monitoring the levels of ASP05. Similarly, ASP05 levels may correlate to the diseases and conditions enumerated above.

In one embodiment, the ASP05 proteins of the present invention may be used to generate polyclonal and monoclonal antibodies to the catalytic or binding domains of ASP05 proteins, which are useful as described herein. Similarly, the ASP05 proteins can be coupled, using standard technology, to affinity chromatography columns. These columns may then be used to purify ASP05 antibodies. In a preferred embodiment, the antibodies are generated to epitopes unique to the ASP05 protein; that is, the antibodies show little or no cross-reactivity to other proteins. These antibodies find use in a number of applications. For example, the ASP05 antibodies may be coupled to standard affinity chromatography columns and used to purify ASP05 proteins. The antibodies may also be used as blocking polypeptides, as outlined above, since they will specifically bind to the ASP05 protein.

In one embodiment, a therapeutically effective dose of an ASP05 or inhibitor thereof is administered to a patient. By "therapeutically effective dose" herein is meant a dose that produces the effects for which it is administered. The exact dose will depend on the purpose of the treatment, and will be ascertainable by one skilled in the art using known techniques. As is known in the art, adjustments for ASP05 degradation, systemic versus localized delivery, and rate of new protease synthesis, as well as the age, body weight, general health, sex, diet, time of administration, drug interaction and the severity of the condition may be necessary, and will be ascertainable with routine experimentation by those skilled in the art.

A "patient" for the purposes of the present invention includes both humans and other animals, particularly mammals, and organisms. Thus the methods are applicable to both human therapy and veterinary applications. In the preferred embodiment the patient is a mammal, and in the most preferred embodiment the patient is human.

The administration of the ASP05 or inhibitor thereof of the present invention can be done in a variety of ways, including, but not limited to, orally, subcutaneously, intravenously, intranasally, transdermally, intraperitoneally, intramuscularly,

intrapulmonary, vaginally, rectally, or intraocularly. In some instances, for example, in the treatment of wounds and inflammation, the ASP05 or inhibitor thereof may be directly applied as a solution or spray.

The pharmaceutical compositions of the present invention comprise an ASP05 or inhibitor thereof in a form suitable for administration to a patient. In the preferred embodiment, the pharmaceutical compositions are in a water soluble form, such as being present as pharmaceutically acceptable salts, which is meant to include both acid and base addition salts. "Pharmaceutically acceptable acid addition salt" refers to those salts that retain the biological effectiveness of the free bases and that are not biologically or otherwise undesirable, formed with inorganic acids such as hydrochloric acid, hydrobromic acid, sulfuric acid, nitric acid, phosphoric acid and the like, and organic acids such as acetic acid, propionic acid, glycolic acid, pyruvic acid, oxalic acid, maleic acid, malonic acid, succinic acid, fumaric acid, tartaric acid, citric acid, benzoic acid, cinnamic acid, mandelic acid, methanesulfonic acid, ethanesulfonic acid, p-toluenesulfonic acid, salicylic acid and the like.

"Pharmaceutically acceptable base addition salts" include those derived from inorganic bases such as sodium, potassium, lithium, ammonium, calcium, magnesium, iron, zinc, copper, manganese, aluminum salts and the like. Particularly preferred are the ammonium, potassium, sodium, calcium, and magnesium salts. Salts derived from pharmaceutically acceptable organic non-toxic bases include salts of primary, secondary, and tertiary amines, substituted amines including naturally occurring substituted amines, cyclic amines and basic ion exchange resins, such as isopropylamine, trimethylamine, diethylamine, triethylamine, tripropylamine, and ethanolamine.

The pharmaceutical compositions may also include one or more of the following: carrier proteins such as serum albumin; buffers; fillers such as microcrystalline cellulose, lactose, corn and other starches; binding agents; sweeteners and other flavoring agents; coloring agents; and polyethylene glycol. Additives are well known in the art, and are used in a variety of formulations.

The following examples serve to more fully describe the manner of using the above-described invention, as well as to set forth the best modes contemplated for carrying out various aspects of the invention. It is understood that these examples in no way serve to limit the true scope of this invention, but rather are presented for illustrative purposes. All references cited herein are expressly incorporated by reference in their entirety.

EXAMPLE I

Cloning of full-length ASP05 cDNA

A strategy combining annealing, ligation, and polymerase chain reaction (PCR) was used to produce a synthetic 5'-end of the ASP05 cDNA. The synthetic sequence comprised the first 156 nucleotides in the coding region but did not alter any of the encoded amino acids. The full length cDNA contains 1443 nucleotides (including stop codon). The complete native cDNA sequence, and the sequence used to expressed active protein is shown in Fig. 2. The twelve oligonucleotides used in the annealing were as follows:

oligo 1: 5'-ATG CAG ATT CCA AGA GCT GCA TTG TTA CCT CTT CTG TTA TTA CTG CTC GCA GCT CCT GCA TCT GCA CAA CTT TCT CGA GCT GGA A-3' (SEQ ID NO:4);

oligo 2: 5'-AGC AGA TCT TCC AGC TCG AGA AAG TTG TGC AGA TGC AGG AGC TGC GAG CAG TAA TAA CAG AAG AGG TAA CAA TGC AGC TCT TGG AAT CTG CAT-3' (SEQ ID NO:5);

oligo 3: 5'-GAT CTG CTC CAT TAG CTG CTG GAT GTC CTG ATA GAT GTG AGC CAG CTA GAT GTC CTC CAC AAC CTG AAC ATT GCG AAG GTG GTA GAG CTA GAG ATG CAT GCG G-3' (SEQ ID NO:6);

oligo 4: 5'-CGC AAC ATC CGC ATG CAT CTC TAG CTC TAC CAC CTT CGC AAT GTT CAG GTT GTG GAG GAC ATC TAG CTG GCT CAC ATC TAT CAG GAC ATC CAG CAG CTA ATG G-3' (SEQ ID NO:7);

oligo 5: 5'ATG TTG CGA AGT TTG CGG AGC TCC TGA AGG AGC TGC TTG TGG ATT ACA AGA GGG TCC TTG TGG AGA AGG TCT ACA ATG CGT AGT TCC ATT-3' (SEQ ID NO:8);

oligo 6: 5'-GGT ACT CCG AAT GGA ACT ACG CAT TGT AGA CCT TCT CCA CAA GGA CCC TCT TGT AAT CCA CAA GCA GCT CCT TCA GGA GCT CCG CAA ACT T-3' (SEQ ID NO:9);

oligo 7: 5'-CGG AGT ACC AGC TTC AGC AAC AGT AAG ACG AAG GGC CCA AGC TGG TTT ATG TGT ATG CGC GAG TTC AGA ACC AGT

ATG TGG CTC TGA TGC AAA TAC ATA-3' (SEQ ID NO:10);

oligo 8: 5'-AGT TTG CGT ATG TAT TTG CAT CAG AGC CAC ATA CTG GTT CTG AAC TCG CGC ATA CAC ATA AAC CAG CTT GGG CCC TTC GTC TTA CTG TTG CTG AAG CT-3' (SEQ ID NO:11);

oligo 9: 5'CGC AAA CTT ATG CCA ATT GAG AGC TGC TTC TAG ACG TAG TGA AAG ACT ACA TAG ACC GCC TGT TAT AGT CCT GCA ACG GGG AGC CTG CGG CCA AGG-3' (SEQ ID NO:12);

oligo 10: 5'-TCT TCC TGC CCT TGG CCG CAG GCT CCC CGT TGC AGG ACT ATA ACA GGC GGT CTA TGT AGT CTT TCA CTA CGT CTA GAA GCA GCT CTC AAT TGG CAT A-3' (SEQ ID NO:13);

oligo 11: 5'-GCA GGA AGA TCC CAA CAG TTT GCG CCA TAA ATA TAA CTT TAT CGC GGA CGT GGT GGA GAA GAT CGC CCC TGC CCT GGT TCA TAT CGA ATT GTT TCG CAA GCT T-3' (SEQ ID NO:14); and

oligo 12: 5'-AAG CTT GCG AAA CAA TTC GAT ATG AAC CAC GGC AGG GGC GAT CTT CTC CAC CAC GTC CGC GAT AAA GTT ATA TTT ATG GCG CAA ACT GTT GGG A-3' (SEQ ID NO:15).

Complimentary oligonucleotides (50 pmoles each) were mixed in 10 mM Tris-CI, pH 7.4, 150 mM NaCl, followed by heating at 95°C for 3 min and slowly cooling to room temperature. All 6 annealed DNA fragment pairs were then mixed and ligated with rapid DNA ligation reagents (Boehringer Mannheim Corp., Indianapolis, IN). This ligated cDNA was used as template, together with primers 5'-ACT ATG CAG ATT CCA AGA GCT G-3' (SEQ ID NO:16) and 5'-GTC TAA AGC TTG CGA AAC AAT TCG-3' (SEQ ID NO:17), in a PCR reaction to amplify the complete fragment. The PCR was performed in 100 μl of solution containing the cDNA fragment and primers (100 pmoles each), 0.2 mM of each dNTP, 0.1 U of Taq DNA polymerase (Perkin Elmer, Branchburg, NJ), 50 mM KCI, 1.75 mM

$MgCl_2$ and 10 mM Tris-HCI, pH 8.4. The PCR was carried out for 25 cycles with amplification profile as follows: 95°C denaturation for 1 minute, 55°C annealing for 1 minute and then 72 °C extension for 1 minute. The PCR product was cloned into TA cloning vector (Invitrogen, Carlsbad, CA)

The 3'-870 bp end of ASP05 was cloned by PCR using a human placenta cDNA (Clontech, Palo Alto, CA) as template and a 5' end primer of the sequence 5'-AAT TGT TTC GCA AGC TTC CGT TTT CTA AAC G-3' (SEQ ID NO:18) and a 3' end primer of the sequence 5'-AGT CTA GAA TTC CAT GAA GTC CAG CTC ATG CCT CTGCCT ATG G-3' (SEQ ID NO:19). This fragment was amplified and cloned in the same way as above.

The 5' end fragment was excised from the vector with restriction enzymes BamHI and HindIII and separated from vector with an 1% agarose gel. A DNA band with the expected size was extracted with Quick Gel Extraction Reagents (Qiagen, Inc., Chatsworth, CA). The purified DNA fragment was ligated to the 3' end fragment at Hind III site with rapid ligation reagents. The full-length ASP05 cDNA was completely sequenced in both directions using the ABI 377 DNA sequencer (ABI Division, Perkin Elmer, Foster City, CA).

EXAMPLE 2

Expression of ASP05 in baculovirus and purification of the protein.

Bac-to-Bac Baculovirus Expression System (GibcoBRL, Gaithersburg, MD) was used to express the protein. Briefly, the ASP05 cDNA tagged with flag epitope (Eastman Kodak, New Haven. CT) was ligated into pFASTBac1 vector, which was then transformed into DH1 OBac competent cells to generate recombinant bacmid. The bacmid DNA was transfected into sf9 insect cells to produce recombinant baculovirus. High titer stock was used to infect sf9 insect cells (multiplicity of infection>5) cultured in Sf-900 II SFM. The medium was collected 48 hours after infection. The expressed protein was purified by affinity chromatography using an agarose-linked antibody specific for the Flag epitope tag engineered into the C-terminal end of the expressed ASP05. Briefly, 4 ml of Flag agarose was incubated with 900 ml of the medium at 4°C for 2 hours, and then the agarose

medium slurry was poured into a 10 ml column. The gel was washed with 20 gel volumes of phosphate-buffered-saline (PBS, pH7.2), and bound ASP05 was eluted with 4 gel volumes of Tris-glycine solution (pH2.5). The eluate was immediately neutralized with 10x PBS and concentrated 20 fold with microsep 10K (Filtron, Northborough, MA). The concentrated protein was dialyzed against PBS at 4 °C overnight. The affinity-purified ASP05 was quantitated with BCA protein quantitation regents (Pierce, Rockford, IL).

EXAMPLE 3

Determination of N-terminal amino acid sequence of ASP05

Purified ASP05 (2 μg) was run onto a 4-20% sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) and subsequently transferred to a polyvinylidene difluoride (PVDF) membrane. The membrane was stained with Commassie Blue in 50% methanol for 5 minutes and destained with 10% acetic acid and 10% methanol. The protein band with correct molecular weight was cut and sent to Protein Structure Laboratories at University of California, Davis for sequence analysis.

The N-terminal sequence of insect cell expressed mature ASP05 is as follows: MQIPRAALLPLLLLLLAAPASAQLSRAGRSAPLAAGCPDRCEPARCPPQPEH CEGGSAPLAAG (SEQ ID NO:20).

EXAMPLE 4

Analysis of proteolytic activity of ASP05

Purified ASP05 (200nM) was incubated with 125nM of IGFBP-5 in 20 μl of 25 mM Hepes (pH7.4), 25 mM CHES, 50 mM NaCl and 0.025% Tween 20 (Assay buffer) at 37°C for 2 hours. The reaction products to be analyzed were run a 4-20% gradient sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) and subsequently transferred to a polyvinylidene difluoride (PVDF) membrane, which was then blocked with 1% bovine serum albumin (BSA) in 10 mM Tris-HCI, pH 8.0, 150 mM NaCl and 0.05% Tween 20 (TBST) for 1 hour. The membrane was then incubated with IGFBP-5 antibody (Autral Biologicals) for 1 hour. After washing with the TBST three times (15 minutes each) the membrane was incubated with

HRP-conjugated mouse IgG antibody for 1 hour. The PVDF membrane was washed again three times for 15 minutes each, and the proteins were visualized by ECL chemiluminescence (Amersham, Arlington Heights, IL). When testing if ASP05 cleaved IGFBP-1, IGFBP-2. IGFBP-3, IGFBP-4, or IGFBP-6, 125nM of each was added to the reaction in place of IGFBP-5 and the analysis carried out as described above. (See Figure 4A).

EXAMPLE 5

Ligand blot analysis of ASP05 cleavage activity of IGFBP's

Purified ASP05 (200nM) was incubated respectively with 125nM of IGFBP-1, IGFBP-2, IGFBP-3, IGFBP-4, IGFBP-5 and IGFBP-6 in 20ml of 25 mM Hepes (pH 7.4), 25 mM CHES, 50 mM NaCl and 0.025% Tween 20 (Assay buffer) at 37 °C for 2 hours. The reaction products were run onto a non-reducing 4-20% sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) and transferred to a nitrocellulose membrane. The membrane was incubated in 0.15 M NaCl, 0.01 M Tris-HCl, pH7.4 (TS) and 3% NP40 for 30 minutes at 4°C (Hereafter all steps were carried out at 4°C), then in TS, 1% BSA for 2 hours, followed by rinsing the membrane in TS plus Tween 20 for 10 minutes. The membrane was subsequently incubated with 400,000 cpm of ^{125}IGF-I (or ^{125}IGF-II) in 3 ml of TS, 1% BSA and 0.1% Tween 20 overnight. After washing twice in TS, 0.1% Tween20, 15 minutes each and once in TS for 15 minutes, the membrane was air-dried and exposed to X-ray film overnight. The film was developed with automatic film developer Konica QX-130A (Figure 4B).

EXAMPLE 6

Inhibitory effects of protease inhibitors on proteolytic activity of ASP05

To analyze inhibitory effects of protease inhibitors on proteolytic activity of ASP05, individual inhibitors were incubated respectively with ASP05 in assay buffer at 37 °C for 1 hour and then IGFBP-5 added as above. The reactions were further incubated at 37°C for 2 hours. The protease inhibitors analyzed (and the concentrations used) were as follows: aprotinin (1.5 μM); antipain (370 μM); 3,4-DCI (1 mM); E64 (140 μM); Leupeptin (5 μM); pepstatinA (5 μM); PMSF (5

mM); L-1-chloro-3-(4-tosylamido)-7-amino-2-heptanone-HCI (TLCK) (675 μM); L-1-chloro-3-(4-tosylamido)-4-phenyl-2-butanone (TPCK) (1.4 mM) and EDTA (10 mM). Results are shown in Figure 5 and indicate of the inhibitors tested, ASP05 is inhibited only by the serine protease inhibitors 3,4dichloroisocoumarin (lane 5) and PMSF (lane 9).

EXAMPLE 7

Assessment of autocleavage activity of ASP05

Purified ASP05 (200nM) was incubated in 20 μl of 25 mM Hepes (pH7.4), 25 mM CHES, 50 mM NaCl and 0.025% Tween 20 (Assay buffer) at 37°C for 15 hours. The reaction of 10 μl was run on a 4-20% sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) and subsequently transferred to a polyvinylidene difluoride (PVDF) membrane, which was then blocked with 1% bovine serum albumin (BSA) in 10 mM Tris-HCI, pH 8.0, 150 mM NaCl and 0.05% Tween 20 (TBST) for 1 hour. Next the membrane was incubated with ASP05 peptide antibody (Zymed, Inc.) for 1 hour. After washing with the TBST three times, 15 minutes each, the membrane was incubated with HRP conjugated rabbit IgG antibody for 1 hour. The PVDF membrane was washed again three times, 15 minutes each, and the proteins were visualized by ECL chemiluminescence (Amersham, Arlington Heights, IL) (Figure 3).

EXAMPLE 8

Construction of expression vector of C-terminal fragment of ASPO5

A polymerase chain reaction (PCR) was used to generate the cDNA encoding the C-terminal fragment of ASPO5. Two oligonucleotides used in the PCR were as follows: oligo 1: 5'-GAT CTA CGG ATC CCA AGC TGG TTT ATG TGT ATG C-3' (SEQ ID NO:21) and oligo 2: 5'-GAT CTA CCT GCA GTG GGT CAA TTT CGG CGG GAA TC-3' (SEQ ID NO:22). These two oligonucleotides were used as primers and full-length synthetic ASP05 cDNA was used as template in PCR reaction to amplify the fragment. The PCR was performed in 100μl of solution containing the template and primers (100 pmoles each), 0.2 mM of each dNTP, 0.1 U of Taq DNA polymerase (Perkin Elmer, Branchburg, NJ), 50 mM KCI, 1.75 mM

MgC12 and 10 mM Tris-HCI, pH 8.4. The PCR was carried out for 25 cycles with following amplification profile: 95 °C denaturation for 1 minute, 55 °C annealing for 1 minute and then 72 °C extension for 1 minute. The PCR product was extracted with phenol-chloroform and precipitated with ethanol. The DNA pellet was resuspended in 10 mM Tris-HCI, pH7.6, 1 mM EDTA (TE).

The amplified cDNA fragment was treated with restriction enzymes BamHI and PstI at 37 °C for 1 hour and purified with an 1% agarose gel. A DNA band with the expected size was excised from the gel and extracted with Quick Gel Extraction Reagents (Qiagen, Inc., Chatsworth, CA). The purified DNA fragment was ligated to pQE-30 vector (Qiagen, Inc., Chatsworth, CA) that was treated with BamHI and PstI and purified by way of an agarose gel in the same way as the amplified cDNA fragment. Restriction digestion was used to verify that the cDNA had inserted into the vector in the correct orientation.

The plasmid (0.2 μg) was mixed with 100 μl of SG13009 competent cells (Qiagen, Inc., Chatsworth, CA) and the mixture was kept on ice for 20 minutes, and then the mixture was heated at 42 °C for 90 minutes. After cooling on ice for 2 minutes, 500 μl of SOC medium containing 10 mM $MgCl_2$ were added to the mixture and the cells was shaked vigorously at 37 °C for 1 hour. The cells were spread on LB agar plate containing 100 mg/ml ampicillin and 10 mg/ml kanamycin and grown at 37 °C overnight.

EXAMPLE 9

Expression of the C-terminal fragment of ASPO5 in E.coli

A single transformed E. coli colony was picked from the plate and grown in LB broth containing 100 μg/ml of ampicillin and 10 mg/ml kanamycin at 37 °C overnight with vigorous shaking. The next morning the culture was diluted 1:50 with LB-broth and grown at 37 °C to ~0.5 of OD600. Isopropyl (3-Dthiogalacto-pyronoside (IPTG) was added to final concentration of 2 mM and the culture was continued to grow for another 3 hours. The cells were harvested by centrifugation and the cell pellet was resuspended in 200μl of deionized water. The cells were

lysed by adding 100µl of sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) sample buffer and heating at 95 °C for 2 minutes. The samples of 5µl each were separated on a reducing SDS-PAGE and the expressed C-terminal fragment of ASP05 was visualized by Commassie blue staining.

EXAMPLE 10

Scale-up expression and purification of C-terminal fragment of ASPO5

A transformed E. coli colony was grown in 3 ml LB containing 100 µg/ml Ampicillin and 10 mg/ml kanamycin at 37 °C overnight with vigorous shaking. Fifteen hours later the culture was transferred to 150 ml of prewarmed same medium and allowed to grow to OD600 of ~0.6. IPTG was added to final concentration of 2 mM and the culture was continued for another 5 hours. The cells were harvested by centrifugation and frozen at -80 °C. The next morning the cells were thawed in 20 ml of 6M guanidine-HCl, 0.1M Na-phosphate, and 0.01 M Tris-HCl, pH 8.0 (GuHCl). The cells were stirred at room temperature for 1 hour and spun at 10,000 g for 15 minutes at 4 °C. The supernatant was loaded onto a Ni-NTA column (Qiagen, Inc., Chatsworth, CA) pre-equilibrated with 10 column volumes of GuHCl. Subsequently the column was washed with 10 column volumes of GuHCl buffer and then 8 M urea, 0.1 m Na-phosphate, and 0.01 M Tris-HCl, pH 8.0, followed by 10 column volumes of 8 M urea, 0.1 m Na-phosphate, and 0.01 M Tris-HCl, pH 6.3. The bound protein was eluted with 5 column volumes of 8 M urea, 0.1 m Na-phosphate, and 0.01 M Tris-HCl, pH 4.5. The protein was dialyzed against 10 mM Mes buffer, pH5.5. After dialysis, the final concentration of urea was less than 10^{-7} M.

EXAMPLE 11

Analysis of proteolytic activity of C-terminal fragment of ASPO5

Purified C-terminal fragment of ASPO5 was incubated with 125nM of IGFBP-5 in 20 µl of 25 mM Hepes (pH7.4), 25 mM CHES, 50 mM NaCl and 0.025% Tween 20 (Assay buffer) at 37 °C for 2 hours. The reaction products were then run onto a 4-20% SDS-PAGE and subsequently transferred to a polyvinylidene difluoride (PVDF) membrane, which was then blocked with 1% bovine serum albumin (BSA) in 10 mM Tris-HCl, pH 8.0, 150 mM NaCl and 0.05% Tween 20 (TBST) for 1

hour. Next, the membrane was incubated with IGFBP-5 antibody (Autral Biologicals, San Ramon, CA) for 1 hour. After washing with the TBST three times, 15 minutes each, the membrane was incubated with HRP-conjugated mouse IgG antibody for 1 hour. The PVDF membrane was washed again three times, 15 minutes each, and the proteins were visualized by ECL chemiluminescence (Amersham, Arlington Heights, IL).

The fragment was tested for the ability to cleave other IGFBPs, IGFBP-1, IGFBP-2, IGFBP-3, IGFBP-4, or IGFBP-6. For these reactions, 125nM of each IGFBP was added respectively, instead of IGFBP-5 to the reaction as described above.

CLAIMS

1. A recombinant nucleic acid comprising a nucleic acid that will hybridize under high stringency conditions to the nucleotides 481 to 1113 of the nucleic acid sequence shown in Figure 1 (SEQ ID NO:1) or its complement and has at least a 10% decrease in GC content as compared to the nucleic acid sequence of the wild-type ASP05 nucleic acid of Figure 2 (SEQ ID NO:3).

2. The recombinant nucleic acid according to claim 1 that will hybridize under high stringency conditions to the nucleic acid sequence shown in Figure 1 (SEQ ID NO:3).

3. A recombinant nucleic acid comprising a nucleic acid sequence encoding amino acids 161 to 371 of the amino acid sequence of Figure 1 (SEQ ID NO:2), wherein said nucleic acid sequence has at least a 10% decrease in GC content as compared to the native nucleic acid sequence of Figure 3.

4. The recombinant nucleic acid according to claim 3 encoding the ASP05 protein of Figure 1 (SEQ ID NO:2).

5. The recombinant nucleic acid according to claim 1 comprising the nucleotide sequence of SEQ ID NO:1 or its complement.

6. The recombinant nucleic acid according to claim 1 comprising nucleotides 481 to 1113 of the nucleotide sequence of SEQ ID NO:1, or its complement.

7. The recombinant nucleic acid according to claim 1 consisting essentially of nucleic acid that will hybridize under high stringency conditions to the nucleotides 481 to 1113 of Figure 1 (SEQ ID NO:1).

8. The recombinant nucleic acid according to claim 1 consisting essentially of nucleic acid encoding amino acid residues 161 to 371 of the amino acid sequence of Figure 1 (SEQ ID NO:2).

9. A recombinant nucleic acid consisting essentially of nucleic acid encoding amino acid residues 30 to 104 of the amino acid sequence depicted in Figure 1 (SEQ ID NO:2).

10. An expression vector comprising the nucleic acid of claim 1.

11. An expression vector comprising the nucleic acid of claim 3.

12. A host cell comprising the nucleic acid of claim 1.

13. A host cell comprising the nucleic acid of claim 3.

14. A host cell comprising the expression vector of claim 10.

15. A host cell comprising the expression vector of claim 11.

16. A method of producing an ASP05 protein, comprising:
(a) culturing the host cell of Claim 12 under conditions suitable for expression of said ASP05 protein; and
(b) recovering said ASP05 protein from the cell culture.

17. An ezymatically active ASP05 protein comprising amino acids 161 to 371 of the amino acid sequence of Figure 1 (SEQ ID NO:2).

18. The enzymatically active ASP05 protein according to claim 17 comprising amino acids 1 to 481 of Figure 1 (SEQ ID NO:2).

19. A recombinant ASP05 protein encoded by a nucleic acid that will hybridize under high stringency conditions to the nucleotides 481 to 1113 of the nucleic acid sequence shown in Figure 1 (SEQ ID NO:1) or its complement and has at least a 10% decrease in GC content as compared to the nucleic acid sequence of the wild-type ASP05 nucleic acid of Figure 3 (SEQ ID NO:3).

20. The recombinant protein according to claim 19 encoded by a nucleic acid that will hybridize under high stringency conditions to the nucleic acid sequence shown in Figure 1 (SEQ ID NO:3).

21. The recombinant protein according to claim 19 comprising the amino acid sequence of SEQ ID NO:2.

22. The recombinant protein according to claim 19 consisting essentially of amino acids 161 to 371 of the amino acid sequence shown in n Figure 1 (SEQ ID NO:2).

23. A chimeric molecule comprising the catalytic domain of an ASP05 protein fused to a heterologous amino acid sequence.

24. An antibody which specifically binds to an ASP05 protein.

25. A method of selective cleavage of insulin-like growth factor binding protein (IGFBP) comprising, combining an IGFBP with an enzymatically active ASP05 protein comprising amino acids from about 161 to 371 of the amino acid sequence of Figure 1 (SEQ ID NO:2).

26. The method according to Claim 25 further comprising determining that said insulin-like growth factor binding protein has been cleaved.

27. A method of screening for a bioactive agent capable of modulating the activity of an ASP05 protein, said method comprising:
(a) combining a candidate bioactive agent and a protein comprising a catalytic domain of an ASP05 protein; and
(b) determining whether said agent modulates the activity of said ASP05 protein.

28. The method according to claim 27, wherein said ASP05 protein comprises the full length ASP05 protein.

29. A method of screening for a bioactive agent capable of modulating the activity of a serine protease capable of selective cleavage of IGFBP, said method comprising the steps of:
(a) adding a candidate bioactive agent to a cell comprising a recombinant nucleic acid encoding an ASP05 protein, wherein said ASP05 protein is expressed and
(b) determining whether said agent modulates the activity of said ASP05 protein relative to a cell lacking said expressed ASP05 protein.

30. The method according to claim 29 wherein a library of candidate bioactive agents are added to a plurality of cells.

31. A method of modulating cleavage of IGFBP in a cell comprising administering to said cell an exogenous compound that modulates the biological activity of an ASP05 protein.

32. The method of according to claim 31 wherein said modulation is a reduction or elimination of the biological activity of said ASP05 protein.

33. A method of diagnosing an IGF related condition in a first individual comprising:
a) measuring an activity of an ASP05 protein in a first tissue of said first

individual; and;

b) comparing said first ASP05 protein activity to an activity of an ASP05 protein in a second, unaffected individual or from a second, unaffected tissue in said first individual;

wherein the activity of said ASP05 protein from said first individual is modified in comparison to the activity of said ASP05 protein in said second individual or said second tissue, the first individual is at risk for an IGF related condition.

```
ATGCAGATTCCAAGAGCTGCATTGTTACCTCTTCTGTTATTACTGCTCGCAGCTCCTGCA   60
M  Q  I  P  R  A  A  L  L  P  L  L  L  L  L  L  A  A  P  A     20

TCTGCACAACTTTCTCGAGCTGGAAGATCTGCTCCATTAGCTGCTGGATGTCCTGATAGA  120
S  A  Q  L  S  R  A  G  R  S  A  P  L  A  A  G  C  P  D  R     40

TGTGAGCCAGCTAGATGTCCTCCACAACCTGAACATTGCGAAGGTGGTAGAGCTAGAGAT  180
C  E  P  A  R  C  P  P  Q  P  E  H  C  E  G  G  R  A  R  D     60

GCATGCGGATGTTGCGAAGTTTGCGGAGCTCCTGAAGGAGCTGCTTGTGGATTACAAGAG  240
A  C  G  C  C  E  V  C  G  A  P  E  G  A  A  C  G  L  Q  E     80

GGTCCTTGTGGAGAAGGTCTACAATGCGTAGTTCCATTCGGAGTACCAGCTTCAGCAACA  300
G  P  C  G  E  G  L  Q  C  V  V  P  F  G  V  P  A  S  A  T    100

GTAAGACGAAGGGCCAAGCTGGTTTATGTGTATGCGCGAGTTCAGAACCAGTATGTGGC  360
V  R  R  R  A  Q  A  G  L  C  V  C  A  S  S  E  P  V  C  G    120

TCTGATGCAAATACATACGCAAACTTATGCCAATTGAGAGCTGCTTCTAGACGTAGTGAA  420
S  D  A  N  T  Y  A  N  L  C  Q  L  R  A  A  S  R  R  S  E    140

AGACTACATAGACCGCCTGTTATAGTCCTGCAACGGGGAGCCTGCGGCCAAGGGCAGGAA  480
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GATCCCAACAGTTTGCGCCATAAATATAACTTTATCGCGGACGTGGTGGAGAAGATCGCC  540
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CCTGCCGTGGTTCATATCGAATTGTTTCGCAAGCTTCCGTTTTCTAAACGAGAGGTGCCC  600
P  A  V  V  H  I  E  L  F  R  K  L  P  F  S  K  R  E  V  P    200

GTGGCTAGTGGGTCTGGGTTTATTGTGTCGGAAGATGGACTGATCGTGACAAATGCCCAC  660
V  A  S  G  S  G  F  I  V  S  E  D  G  L  I  V  T  N  A  (H)  220

GTGGTGACCAACAAGCACCGGGTCAAAGTTGAGCTGAAGAACGGTGCCACTTACGAAGCC  720
V  V  T  N  K  H  R  V  K  V  E  L  K  N  G  A  T  Y  E  A    240

AAAATCAAGGATGTGGATGAGAAAGCAGACATCGCACTCATCAAAATTGACCACCAGGGC  780
K  I  K  D  V  D  E  K  A  (D)  I  A  L  I  K  I  D  H  Q  G    260

AAGCTGCCTGTCCTGCTGCTTGGCCGCTCCTCAGAGCTGCGGCCGGGAGAGTTCGTGGTC  840
K  L  P  V  L  L  L  G  R  S  S  E  L  R  P  G  E  F  V  V    280

GCCATCGGAAGCCCGTTTTCCCTTCAAAACACAGTCACCACCGGGATCGTGAGCACCACC  900
A  I  G  S  P  F  S  L  Q  N  T  V  T  T  G  I  V  S  T  T    300

CAGCGAGGCGGCAAAGAGCTGGGGCTCCGCAACTCAGACATGGACTACATCCAGACCGAC  960
Q  R  G  G  K  E  L  G  L  R  N  S  D  M  D  Y  I  Q  T  D    320

GCCATCATCAACTATGGAAACTCGGGAGGCCCGTTAGTAAACCTGGACGGTGAAGTGATT 1020
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```

FIGURE 1

FIGURE 1 (cont.)

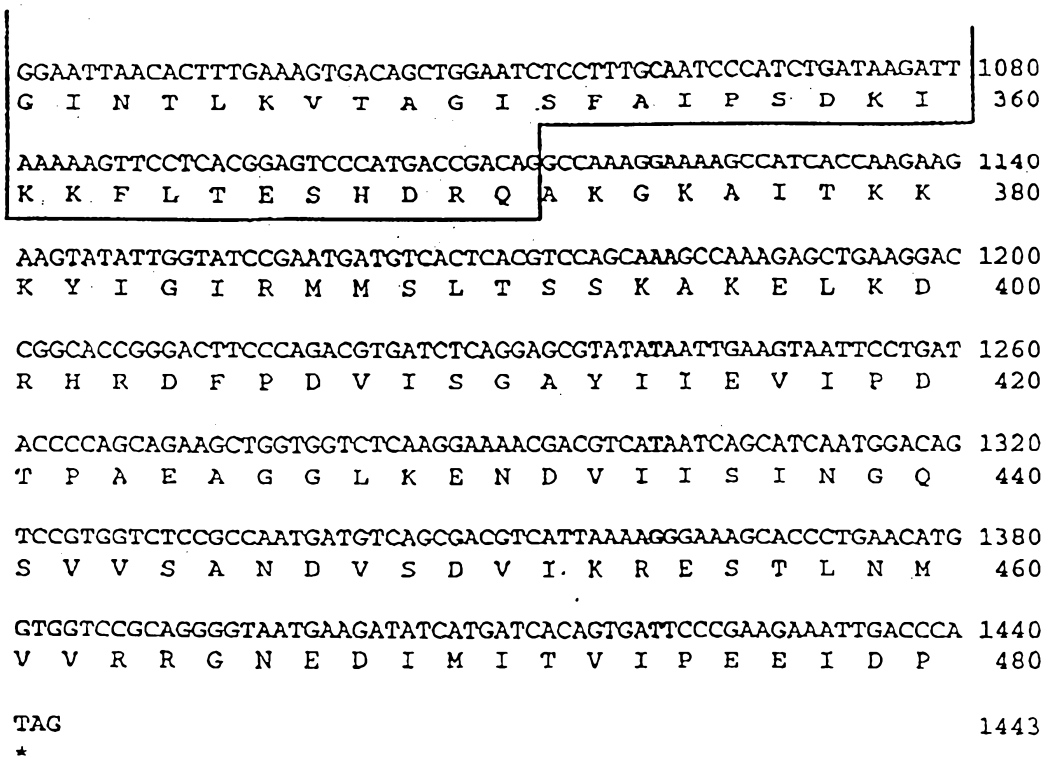
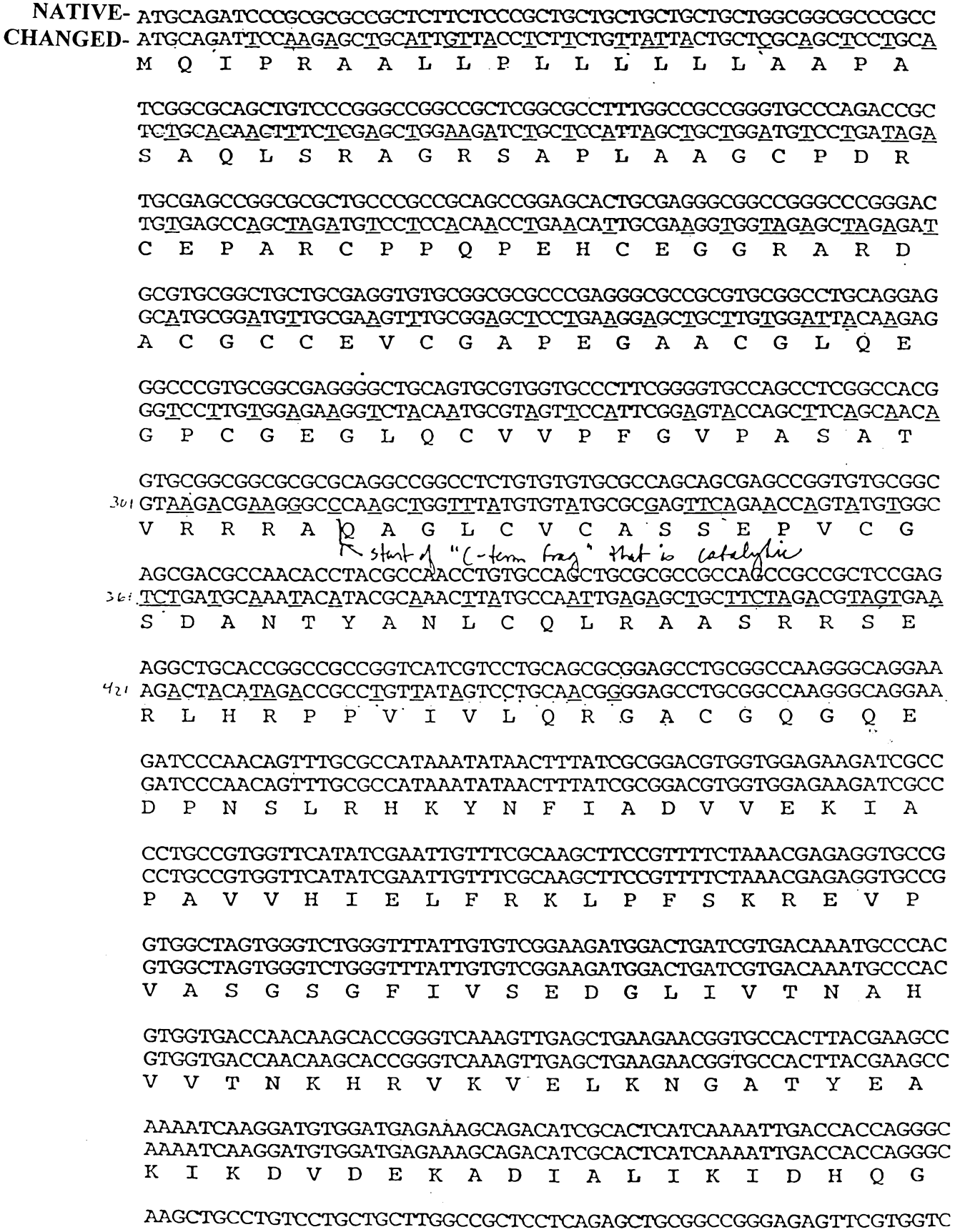


FIGURE 2A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CHANGED- ATGCAGATTCCAAGAGCTGCATTGTTACCTCTTCTGTTATTACTGCTCGCAGCTCCTGCA
M Q I P R A A L L P L L L L L L A A P A
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C E P A R C P P Q P E H C E G G R A R D
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A C G C C E V C G A P E G A A C G L Q E
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G P C G E G L Q C V V P F G V P A S A T
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301 GTAAGACGAAGGGCCCAAGCTGGTTTATGTGTATGCGCGAGTTCAGAACCAGTATGTGGC
V R R R A Q A G L C V C A S S E P V C G
start of "C-term frag" that is catalytic
AGCGACGCCAACACCTACGCCAACCTGTGCCAGCTGCGCGCCGCCAGCCGCCGCTCCGAG
361 TCTGATGCAAATACATACGCAAACTTATGCCAATTGAGAGCTGCTTCTAGACGTAGTGAA
S D A N T Y A N L C Q L R A A S R R S E
AGGCTGCACCGGCCGCCGGTCATCGTCCTGCAGCGCGGAGCCTGCGGCCAAGGGCAGGAA
421 AGACTACATAGACCGCCTGTTATAGTCCTGCAACGGGGAGCCTGCGGCCAAGGGCAGGAA
R L H R P P V I V L Q R G A C G Q G Q E
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D P N S L R H K Y N F I A D V V E K I A
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V A S G S G F I V S E D G L I V T N A H
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V V T N K H R V K V E L K N G A T Y E A
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AAAATCAAGGATGTGGATGAGAAAGCAGACATCGCACTCATCAAAATTGACCACCAGGGC
K I K D V D E K A D I A L I K I D H Q G
AAGCTGCCTGTCCTGCTGCTTGGCCGCTCCTCAGAGCTGCGGCCGGGAGAGTTCGTGGTC

FIGURE 2B

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A  I  I  N  Y  G  N  S  G  G  P  L  V  N  L  D  G  E  V  I

GGAATTAACACTTTGAAAGTGACAGCTGGAATCTCCTTTGCAATCCCATCTGATAAGATT
GGAATTAACACTTTGAAAGTGACAGCTGGAATCTCCTTTGCAATCCCATCTGATAAGATT
G  I  N  T  L  K  V  T  A  G  I  S  F  A  I  P  S  D  K  I

AAAAAGTTCCTCACGGAGTCCCATGACCGACAGGCCAAAGGAAAAGCCATCACCAAGAAG
AAAAAGTTCCTCACGGAGTCCCATGACCGACAGGCCAAAGGAAAAGCCATCACCAAGAAG
K  K  F  L  T  E  S  H  D  R  Q  A  K  G  K  A  I  T  K  K

AAGTATATTGGTATCCGAATGATGTCACTCACGTCCAGCAAAGCCAAAGAGCTGAAGGAC
AAGTATATTGGTATCCGAATGATGTCACTCACGTCCAGCAAAGCCAAAGAGCTGAAGGAC
K  Y  I  G  I  R  M  M  S  L  T  S  S  K  A  K  E  L  K  D

CGGCACCGGGACTTCCCAGACGTGATCTCAGGAGCGTATATAATTGAAGTAATTCCTGAT
CGGCACCGGGACTTCCCAGACGTGATCTCAGGAGCGTATATAATTGAAGTAATTCCTGAT
R  H  R  D  F  P  D  V  I  S  G  A  Y  I  I  E  V  I  P  D

ACCCCAGCAGAAGCTGGTGGTCTCAAGGAAAACGACGTCATAATCAGCATCAATGGACAG
ACCCCAGCAGAAGCTGGTGGTCTCAAGGAAAACGACGTCATAATCAGCATCAATGGACAG
T  P  A  E  A  G  G  L  K  E  N  D  V  I  I  S  I  N  G  Q

TCCGTGGTCTCCGCCAATGATGTCAGCGACGTCATTAAAAGGGAAAGCACCCTGAACATG
TCCGTGGTCTCCGCCAATGATGTCAGCGACGTCATTAAAAGGGAAAGCACCCTGAACATG
S  V  V  S  A  N  D  V  S  D  V  I  K  R  E  S  T  L  N  M

GTGGTCCGCAGGGGTAATGAAGATATCATGATCACAGTGATTCCCGAAGAAATTGACCCA
GTGGTCCGCAGGGGTAATGAAGATATCATGATCACAGTGATTCCCGAAGAAATTGACCCA
V  V  R  R  G  N  E  D  I  M  I  T  V  I  P  E  E  I  D  P

TAG
TAG
*
```

FIGURE 3

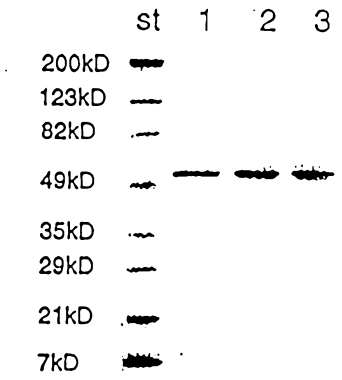

FIGURE 4A

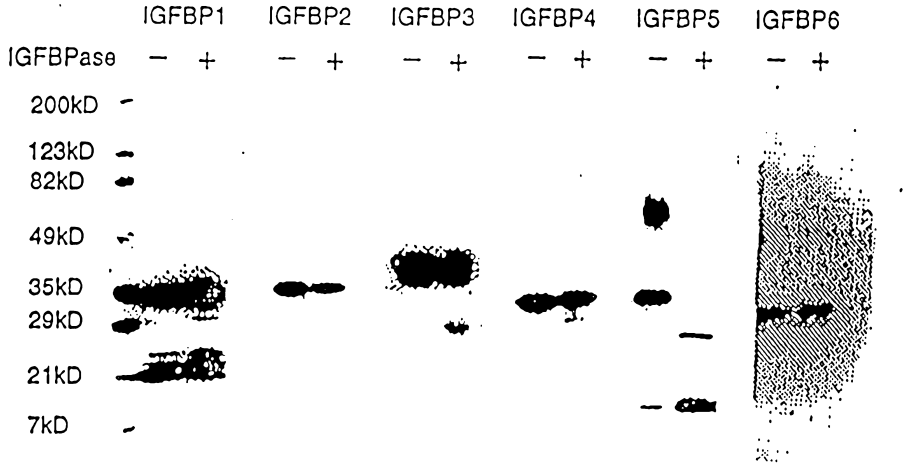

FIGURE 4B

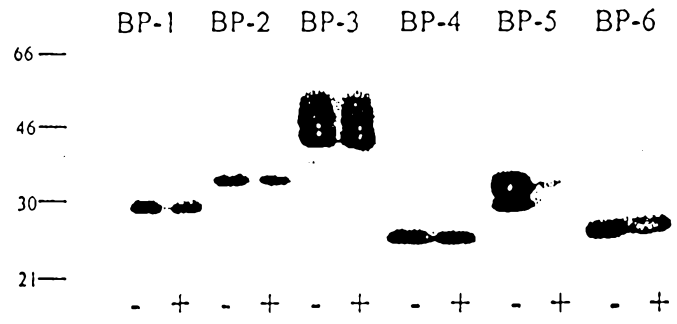

FIGURE 5

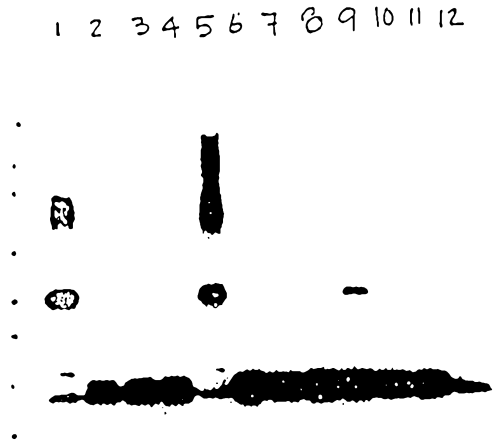

FIGURE 6

ATGAGAGGATCGCATCATCATCATCATCATGGATCCCAAGCTGGTTTATGTGTATGCGCG

AGTTCAGAACCAGTATGTGGCTCTGATGCAAATACATACGCAAACTTATGCCAATTGAGAG

CTGCTTCTAGACGTAGTGAAAGACTACATAGACCGCCTGTTATAGTCCTGCAACGGGGAGC

CTGCGGCCAAGGGCAGGAAGATCCCAACAGTTTGCGCCATAAATATAACTTTATCGCGGAC

GTGGTGGAGAAGATCGCCCCTGCCGTGGTTCATATCGAATTGTTTCGCAAGCTTCCGTTTT

CTAAACGAGAGGTGCCGGTGGCTAGTGGGTCTGGGTTTATTGTGTCGGAAGATGGACTGAT

CGTGACAAATGCCCACGTGGTGACCAACAAGCACCGGGTCAAAGTTGAGCTGAAGAACGGT

GCCACTTACGAAGCCAAAATCAAGGATGTGGATGAGAAAGCAGACATCGCACTCATCAAAA

TTGACCACCAGGGCAAGCTGCCTGTCCTGCTGCTTGGCCGCTCCTCAGAGCTGCGGCCGGG

AGAGTTCGTGGTCGCCATCGGAAGCCCGTTTTCCCTTCAAAACACAGTCACCACCGGGATC

GTGAGCACCACCCAGCGAGGCGGCAAAGAGCTGGGGCTCCGCAACTCAGACATGGACTACA

TCCAGACCGACGCCATCATCAACTATGGAAACTCGGGAGGCCCGTTAGTAAACCTGGACGG

TGAAGTGATTGGAATTAACACTTTGAAAGTGACAGCTGGAATCTCCTTTGCAATCCCATCT

GATAAGATTAAAAAGTTCCTCACGGAGTCCCATGACCGACAGGCCAAAGGAAAAGCCATCA

CCAAGAAGAAGTATATTGGTATCCGAATGATGTCACTCACGTCCAGCAAAGCCAAAGAGCT

GAAGGACCGGCACCGGGACTTCCCAGACGTGATCTCAGGAGCGTATATAATTGAAGTAATT

CCTGATACCCCAGCAGAAGCTGGTGGTCTCAAGGAAAACGACGTCATAATCAGCATCAATG

GACAGTCCGTGGTCTCCGCCAATGATGTCAGCGACGTCATTAAAAGGGAAAGCACCCTGAA

CATGGTGGTCCGCAGGGGTAATGAAGATATCATGATCACAGTGATTCCCGAAGAAATTGAC

CCACTGCAGCCAAGCTTAATTAGCTGA

FIGURE 7

MRGSHHHHHHGSQAGLCVCASSEPVCGSDANTYANLCQLR

AASRRSERLHRPPVIVLQRGACGQGQEDPNSLRHKYNFIA

DVVEKIAPAVVHIELFRKLPFSKREVPVASGSGFIVSEDG

LIVTNAHVVTNKHRVKVELKNGATYEAKIKDVDEKADIAL

IKIDHQGKLPVLLLGRSSELRPGEFVVAIGSPFSLQNTVT

TGIVSTTQRGGKELGLRNSDMDYIQTDAIINYGNSGGPLV

NLDGEVIGINTLKVTAGISFAIPSDKIKKFLTESHDRQAK

GKAITKKKYIGIRMMSLTSSKAKELKDRHRDFPDVISGAY

IIEVIPDTPAEAGGLKENDVIISINGQSVVSANDVSDVIK

RESTLNMVVRRGNEDIMITVIPEEIDPLQPSLIS*

FIGURE 8

Proteolytic activity that selectively cleaves IGFBP-5 resides in C-terminal half of ASPO5