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Expression and Characterization of a Serine Protease That Preferentially Cleaves Insulin-Like Growth Factor Binding Protein-5

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Abstract Insulin-like growth factor binding proteins (IGFBPs) play important roles in regulating the functions of insulin-like growth factors (IGFs). Because IGFBPs have very high affinity for IGF-I and IGF-II, they can regulate the amount of each growth factor that is able to bind to cell surface receptors, therefore, factors that alter IGFBP affinity have the capacity to regulate IGF actions. Protease activities that are present in cell culture systems and physiologic fluids have been shown to degrade IGFBP-5. Previously, a region of sequence in a serine protease was identified that was homologous with the N-terminal 90 amino acids of members of the IGFBP family and with members of the CCN family of proteins. In a prior study, the protease was expressed in human kidney cultured cells and the cell culture supernatants were shown to cleave IGFBP-5, however, it is unknown whether the purified protease would cleave IGFBP-5 and whether it would also cleave other specific forms of IGFBPs. In this study, we expressed this protease in an insect cell expression system, purified it to homogeneity and tested its capacity to cleave IGFBP-5. The expressed protease preferentially cleaved IGFBP-5, and it had minimal activity toward other forms of IGFBPs. The proteolytic activity of this IGFBPase is inhibited by serine protease inhibitors including PMSF and 3,4-dichloroisocoumarin, as well as by divalent metal ions such as, Zn and Cu. Mutation of the active site serine resulted in a major reduction in IGFBP-5 cleavage. The protease binds to heparin and its ability to degrade IGFBP-5 is blocked in the presence of heparin. Inhibition of the activity of the protease following its secretion by B104 cells resulted in inhibition of IGFBP-5 proteolysis and IGF-I stimulation of protein synthesis. Northern blotting revealed that the transcript was expressed in multiple human tissues, including placenta, uterus, prostate, testis, spinal cord, brain, liver, small intestine, thyroid, and spleen. The highest expression was in uterus and placenta, suggesting a possible role of sex steroids in regulating its expression. Understanding the mechanism of how cleavage of IGFBP-5 by this protease alters its activity will help to further our understanding of the biologic actions of the IGFs. J. Cell. Biochem. 94: 470–484, 2005.

Key words: proteolysis; insulin-like growth factor I

Insulin-like growth factor-I (IGF-I) and IGF-II are synthesized and secreted by a variety of cell types and act as autocrine or paracrine factors to regulate cell differentiation, proliferation, and anabolism in many cell types [Han et al., 1987]. IGF binding proteins (insulin-like growth factor binding proteins, IGFBPs) are ubiquitous components of the IGF system. Because their affinities for IGF-I and -II are greater than the IGF-I receptor, IGFBPs modulate IGF bioactivity by controlling IGF access to receptors and by exerting direct, non-IGF mediated effects [Shimasaki et al., 1991; Jones and Clemmons, 1995]. The IGFBP family consists of six distinct, high affinity members, termed IGFBP-1 to -6 [Jones and Clemmons, 1995]. In addition to these six proteins, a family of distantly related members exist termed the CCN proteins [Hwa et al., 1999]. These proteins have four modules, one of which has approximately 45% sequence homology with the amino terminal region of IGFBP-1 through -6, and conservation of 11 of the 12 cysteines in that region. In general, the members of the CCN family have low affinity for IGF-I (e.g., at least 1,000-fold lower than IGFBP-1–6).

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Metabolism of IGFBPs affects the interaction between the IGFs and the IGF-I receptor, thereby altering IGF actions [Chernausek et al., 1995; Conover et al., 1995a; Imai et al., 1997]. There are a number of reports describing identification of proteases for IGFBPs. Protease activity for IGFBP-3 has been demonstrated in human breast cancer (MCF-7) cell culture supernatants and in human serum [Hossenlopp et al., 1990; Giudice et al., 1992; Maile et al., 1999]. IGFBP-4 was found to be degraded in medium that had been conditioned by normal and transformed human fibroblasts [Chernausek et al., 1995; Conover et al., 1995a] bone cells and ovarian follicular cells [Chandrasekhar et al., 1995]. Human fibroblasts [Nam et al., 1996; Busby et al., 2000], osteoblasts [Kanzaki et al., 1994; Hakeda et al., 1996], chrondrocytes [Matsumoto et al., 1996; Olney et al., 1996], and smooth muscle cells [Imai et al., 1997; Overgaard et al., 2001] have been identified as sources of proteases that cleave IGFBP-5 [Imai et al., 1997]. Several specific proteases have been shown to cleave IGFBP-5 including complement C1s [Busby et al., 2000] and PAPPA-2 [Overgaard et al., 2001], Adam 12 [Loechel et al., 2000], Adam 9 [Mohan et al., 2002], plasmin [Campbell and Andress, 1997], kallikrein hK2 [Rehault et al., 2001], and thrombin [Zheng et al., 1998]. It has also been demonstrated that proteases are present in human serum during pregnancy that degrade IGFBP-3 and that the plasmin system is involved in dissociation of IGF-I from the IGFBP-3/IGF-I complex [Campbell et al., 1998; Shi et al., 2000]. A metalloprotease termed PPAP-A has been shown to cleave IGFBP-4 [Lawrence et al., 1999]. All these reports suggest that proteolysis of IGFBPs is an important regulatory process for controlling the availability of IGF-I and thereby regulating the IGF system.

Previously following screening expression libraries prepared from SV-40 transformed fibroblasts for genes that were preferentially expressed as compared to non-transformed fibroblasts [Zumbrunn and Trueb, 1996] detected and sequenced a cDNA encoding a protein whose sequence was similar to members of the CCN family. The protein contained an IGFBP-like domain and a separate serine protease domain. Since the protein contained IGF binding and protease domains, it was proposed to be an IGFBP protease. In a subsequent report, Hu et al. using differential display analysis of transcripts expressed preferentially in osteoarthritic cartilage detected the same cDNA transcript and pointed out its similarity to the HTRA protease family that had been previously identified in *Escherichia coli* [Gray et al., 2000]. These investigators expressed the protein in 293 cells and showed that it reacted with anti-HTRA antiserum and that it cleaved caseine. If serine 328 was changed to an alanine the expressed product did not cleave this substrate. They also showed that it formed a stable complex with α1 antitrypsin and that it could be detected in joint fluid and cartilage extracts [Hu et al., 1998]. In this paper, we report the properties of the purified protease that had been expressed in baculovirus transfected SF-9 cells and show that it preferentially cleaves IGFBP-5.

MATERIALS AND METHODS

Cloning of Full-Length IGFBPase cDNA

A strategy of combination of annealing, ligation, and polymerase chain reaction (PCR) was used to produce synthetic 5′ end of IGFBP protease (hereafter referred to as IGFBPase cDNA (Fig. 1). The 12 oligonucleotides used in the annealing reactions 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′; 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′; 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′; 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′; 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′; 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′; 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

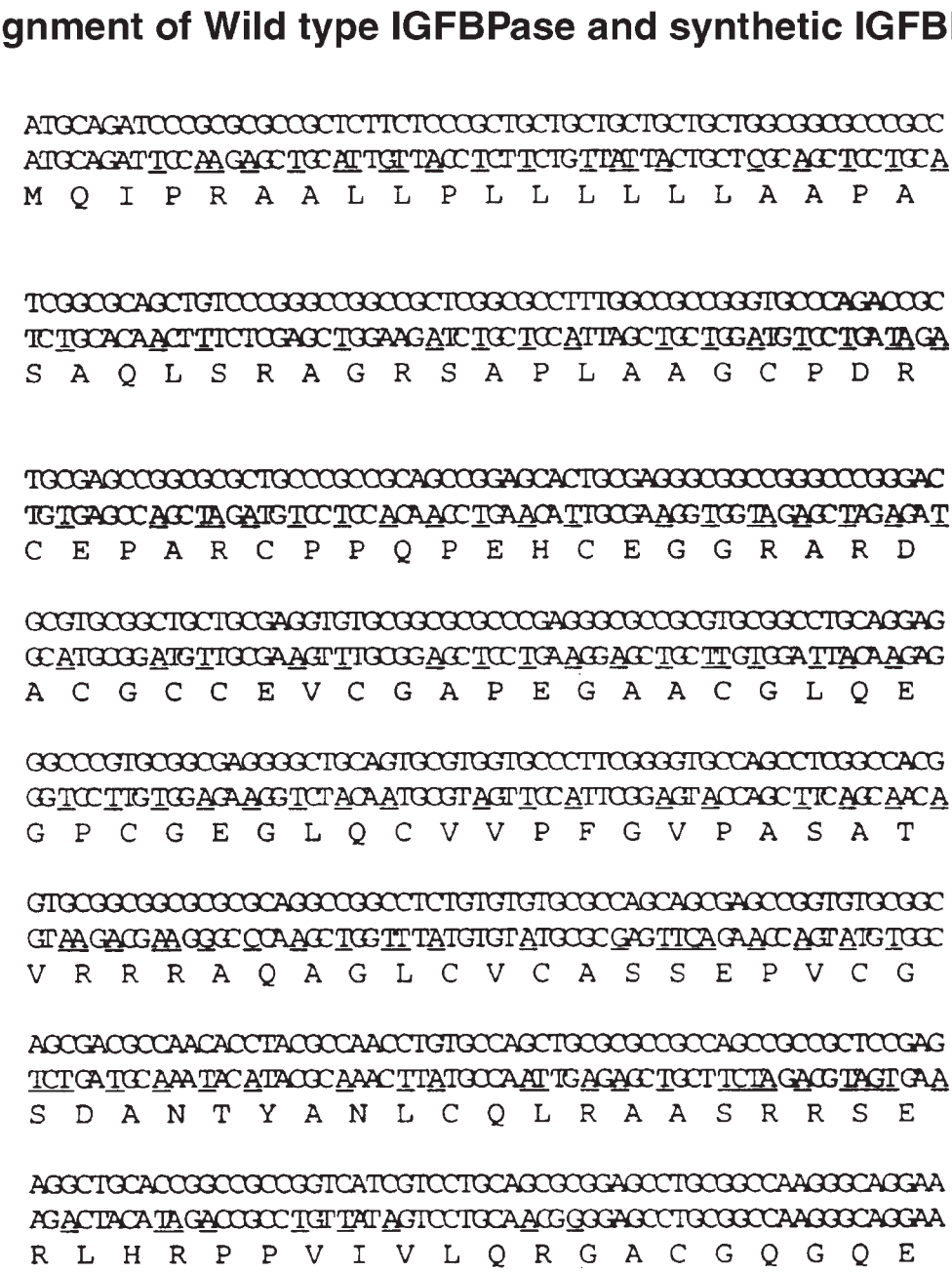

Fig. 1. The nucleotide and amino acid sequence of the insulin-like growth factor binding protein (IGFBP)-5 protease. The nucleotide sequence is shown in the two upper lines of each three-line group and the amino acid sequence is shown in the lower line. The bases that were substituted within the native sequence are underlined. The base substitutions were performed to reduce the G/C content in order to allow the successful synthesis of a PCR product.

TGA TGC AAA TAC ATA-3′; 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′; 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 GCA GGA A-3′; 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′; 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′; 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′. Complementary oligonucleotides of 50 pmoles each were mixed in 10 mM Tris-Cl, pH 7.4, 150 mM NaCl, followed by heating at 95°C for 3 min and slowly cooling to room temperature. All six annealed DNA fragments were mixed and ligated with 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′ and 5′-GTC TAA AGC TTG CGA AAC AAT TCG-3′ in a PCR reaction to amplify the fragment. 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 KCl, 1.75 mM $MgCl_2$, and 10 mM Tris-HCl, pH 8.4. PCR was carried out for 25 cycles with amplification as follows: 95°C denaturation for 1 min, 55°C annealing for 1 min, and then 72°C extension for 1 min. The PCR product was cloned into TA cloning vector (Invitrogen, Carlsbad, CA). The rationale for using several synthetic oligonucleotides was that the naturally occurring sequence had a very high GC content that did not allow efficient PCR.

The 3′ end 870 bp of IGFBPase was amplified in a PCR reaction using human placenta cDNA (Clontech, Inc., Palo Alto, CA) as template and 5′ end primer 5′-AAT TGT TTC GCA AGC TTC CGT TTT CTA AAC G-3′ and 3′ end primer 5′-AGT CTA GAA TTC CAT GAA GTC CAG CTC ATG CCT CTGCCT ATG G-3′. The fragment was amplified and cloned into the TA cloning vector using the same methods as described above.

The 5′ end fragment was excised from the TA cloning vector (Stragene, La Jolla, CA) with the restriction enzymes BamHI and HindIII and separated from vector by agarose gel electrophoresis (1% 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 of the TA cloning vector at Hind III site with rapid ligation reagents. The full-length IGFBPase cDNA was sequenced with ABI 377 sequencer (ABI Division, Perkin Elmer, Foster City, CA).

Expression of IGFBPase in Baculovirus-Insect Cell System and Purification of the Protein

The Bac-to-Bac Baculovirus Expression System (Gibco-BRL, Gaithersburg, MD) was used to express the protein. Briefly, the IGFBPase cDNA was amplified with a 5′ end primer 5′-CGTCGAGGATCCACT ATG CAG ATT CA AGA GCT G-3′and a 3′ end primer 5′-TACAGTGAATTCCTATGCTTTGTCATCGTCGTCCTTGTAATCTGGGTCAATTTCTTCGGGAATCAC-3′ in a PCR reaction. The 24 nucleotide (underlined) sequence contains the nucleotides that encode Flag peptide (Eastman Kodak, New Haven, CT). The PCR product was ligated into pFASTBac1 vector, which was then used to transform DH10Bac competent cells to generate recombinant bacmid. The bacmid DNA was transfected into sf9 insect cells to produce recombinants.

A high titer stock was used to infect sf 9 insect cells (multiplicity of infection >5) cultured in Sf-900 II SFM. The medium was collected 48 h after infection. Antiflag peptide antibody conjugated to agarose gel (4 ml) was exposed to 900 ml of the medium at 4°C for 2 h, and then the agarose-medium slury was poured into a 10 ml column. The gel was washed with 20 gel volumes of phosphate-bufferred-saline (PBS, pH 7.2), and bound IGFBPase was eluted with 4 gel volumes of Tris-glycine solution (pH 2.5). The eluate was immediately neutralized with 10× PBS and concentrated 20-fold with Microsep 10K (Filtron, Northborough, MA). The concentrated protein was dialyzed against PBS at 4°C overnight.

The amount of affinity-purified IGFBPase was estimated using BCA protein quantitation reagents (Pierce, Rockford, IL).

Cleavage of IGFBPs

The purified IGFBPase (200 nM) was incubated with 125 nM of IGFBP-5 in 20 μl of 25 mM HEPES (pH7.4), 25 mM CHES, 50 mM NaCl, and 0.025% Tween-20 (hereafter termed assay buffer) for 2 h 37°C. The reaction products were run on a 40%–20% gradient sodium dodecyl sulfate–polyacrylamide gel (SDS–PAGE) and subsequently transfered 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 h. The membrane was incubated with IGFBP-5 antibody (Austral

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

To determine if the IGFBPase cleaved other forms of IGFBPs, IGFBP-1 (Calbiochem, La Jolla, CA), IGFBP-2 (Austral Biologicals), IGFBP-3 (Upstate Biotechnology, Lake Placid, NY), IGFBP-4, IGFBP-5, or IGFBP-6 (Austral Biologicals), (125 nM) was added and the reaction carried out as described above. The products were analyzed by immunoblotting using specific antibodies (Austral Biologicals) for each form of IGFBP. The products of the reaction were immunoblotted using antibody dilutions that were recommended by the manufacturer, and the immune complexes were visualized as noted above.

Western Ligand Blotting

Each form of IGFBP (125 nM) was exposed to IGFBPase (200 nM) for 2 h at 37°C using the buffer described above. The reaction products were separated by SDS–PAGE as described above then transferred to a PVDF membrane. The membrane was blocked as described above and then exposed to 400,000 cpm/ml of ^{125}IGF-II (specific activity 150 μCi/μg) (Biosciences Corp., Piscataway, NJ). In additional experiments, an IGFBP-5 mutant (K138N, K139N IGFBP-5) that had been prepared and purified as described previously [Imai et al., 1997] was used as a substrate for the IGFBPase using the same concentration of reactants and incubation conditions as described above.

To analyze inhibitory effects of a spectrum of protease inhibitors on proteolytic activity of the IGFBPase, the protease inhibitors were incubated with IGFBPase in assay buffer at 37°C for 1 h, and then IGFBP-5 was added to the reactions, and the incubation was continued at 37°C for an additional 2 h. The concentrations of the protease inhibitors were as follows: aprotinin 1.5 μM; antipain, 370 μM; 3,4-DCI, 1 mM; E64, 140 μM; leupeptin, 5 μM; pepstatin A, 5 μM; PMSF, 5 mM; L-1-chloro-3-(4-tosylamido)-7-amino-2-heptanone-HCl (TLCK), 675 μM; L-1-chloro-3-(4-tosylamido)-4-phenyl-2-butanone (TPCK), 1.4 mM and EDTA, 10 mM.

To determine if heparin protected IGFBP-5 from being degraded by IGFBPase, IGFBP-5 was first incubated with heparin at concentrations of 100, 500, and 2,500 μg/ml in assay buffer at 37°C for 30 min. The IGFBPase was added, and the reaction was continued at 37°C for 2 h.

The volume for all above reactions was 20 μl. The IGFBPase used was 200 nM, and IGFBP-5 was used at 125 nM.

Heparin Binding of the IGFBPase

To determine if the IGFBPase would bind to heparin, IGFBPase (200 nM) was incubated in 30 μl of buffer containing 10 μl of heparin sepharose (2 mg/ml) (Pharmacia Biotech, Inc., Piscataway, NJ) and various doses of heparin (0, 2, 20, 200, 2,000 μg/ml) for 1 h at room temperature. The sepharose beads were washed with 1 ml of assay buffer for three times, and the bound IGFBPase was eluted with SDS–PAGE sample buffer and analyzed with immunoblotting as described above.

Northern Blotting

The multitissue blots containing total RNA that had been prepared from human tissue total RNA samples were purchased from Clontech, Inc. The membranes were incubated with Quick hybridization solution (Amersham) for 1 h at 42°C and then incubated in the same solution with a ^{32}P-labeled IGFBP protease cDNA probe for 3 h at 55°C. The probe was labeled with ^{32}P-dCTP by random priming [Fineberg and Vogelstein, 1983]. After hybridization, the membranes were washed with 1× SSC and 0.1% SDS at 55°C for three times, 15 min each. The membranes were then air dried and exposed to X-ray film overnight.

Development of IGFBPase Antiserum

A polyclonal antiserum that bound to the IGFBP-5 protease with high affinity was prepared in rabbits. The animals were immunized with 300 μg of the purified protein mixed with complete Freuds adjuvant. They were reimmunized after 6 weeks with 100 μg of the antigen and this was repeated every 4 weeks three times.

BRDU Incorporation Assay

To determine the effect of the protease on IGFBP-5 inhibition of cell growth MCF-7 breast carcinoma cells (ATCC, Rockville, MD) were plated at a density of 10,000 cells/cm^2 in 6-well

plates Falcon 3004 in 10% FBS. After allowing 4 h for attachment the medium was removed and replaced with serum-free medium. After 48 h IGF-I (100 ng/ml) or insulin (5 μg/ml) was added to triplicate cultures and the incubation continued for an additional 48 h in the presence of 5-bromodeoyxindine BRDU. Additional wells were exposed to IGFBP-5 (500 μg/ml) alone or in the presence of IGF-I or insulin or either of these reagents and 125 nM IGFBP-5 protease. After 48 h, the cultures were washed three times with PBS then lysed in 0.1 NaOH and BRDU incorporation into DNA determined by flourescence. The results are expressed as random fluorescence units (RFU).

Analysis of Protein Synthesis Response to IGF-I

To determine if proteolytic cleavage of IGFBP-5 altered the cellular protein synthesis response to IGF-I, a rat neuronal cell line (B104) (a gift from Dr. Steven Chernasek, University of Cinncinnatti) was grown to confluency in 6-well plates (Falcon 3084) as described previously [Chernausek et al., 1995]. One milliliter of serum free DMEM was added and the cells were allowed to condition the medium for 14 h. At that time IGF-I (50 ng/ml), IGFBP-5 (1.0 μg/ml), and a synthetic peptide containing the IGFBP-5 sequence between residues 129–141 (5 μg/ml) were added with 50 μCi/well of ^{35}S-methionine 1,150 μCi/μmol (ICN Biochemicals, Costa Mesa, CA). After 4 h, the amount of ^{35}S methionine incorporated into protein was determined as described previously [Imai et al., 1997]. To determine if IGFBP-5 was cleaved and if the synthetic peptide altered cleavage, the conditioned media samples were removed and 25 μl was analyzed by SDS–PAGE followed by immunoblotting for IGFBP-5 as described previously [Busby et al., 2000].

RESULTS

The full-length and truncated (AA 110–480) flag peptide-tagged forms IGFBPase that had been expressed in the baculovirus insect cell system were purified by affinity chromatography using a Flag antibody-agarose gel. The yields of the proteins were 1.5 and 1.3 mg/L. The purified products were 95%–98% pure as determined by staining with Commassie blue staining following SDS–PAGE (Fig. 2). The N-terminal amino acid sequence of the full-length expression product was SAPLAAG,

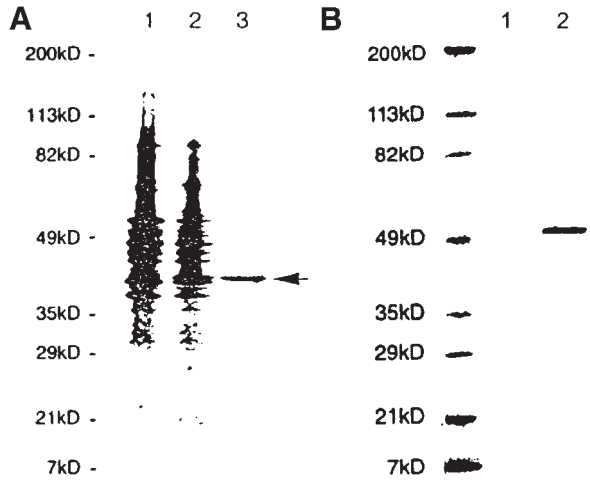

Fig. 2. Coumassie stained gel analysis of two purified forms of IGFBP-5 protease. The truncated (**panel A**, **lanes 1–3**) and full-length (**panel B**, **lane 2**) forms of IGFBPs were purified by heparin sepharose and flag immunoaffinity chromatography. Panel A: Lane 1, crude sf 9cell lysate; (lane 2) heparin sepharose purified SF-9 lysate; (lane 3) flag immunoaffinity purified truncated IGFBP-5 protease. The arrow denotes the band containing the IGFBP-5 protease. Panel B: Lane 1, blank; (lane 2) flag immunoaffinity purified full-length IGFBP-5 protease.

indicating that the first amino acid in mature protein expressed by insect cells is serine 30. This is similar to the *E. coli* expression product described previously [Hu et al., 1998]. The apparent molecular mass on reducing SDS–PAGE was 50 kDa (Fig. 2), which is in agreement with calculated molecular weight based on amino acid sequence derived from the cDNA of IGFBPase-flag (49,289 kDa). Human embryonic kidney cells (293) transfected with the same cDNA expressed a protein of same molecular weight (data not shown). The truncated product that had had the N-terminal sequence containing the IGFBP domain deleted had an Mr estimate of 35 kDa and the N terminal sequence was QAGLCVCASSEPVCG indicating that it contained the catalytic domain but not the IGF binding domain.

The purified full-length form of IGFBPase was used to determine which forms of IGFBPs would be cleaved. Each form of IGFBP was incubated with the IGFBPase and the products were analyzed by Western blotting. As shown in Figure 3, among the six forms of IGFBPs (IGFBP-1 to -6) that were tested, only IGFBP-5 was completely cleaved and two lower molecular weight fragments could be detected. One fragment had an Mr estimate of approximately 20 kDa, and the other was 17 kDa. Although the

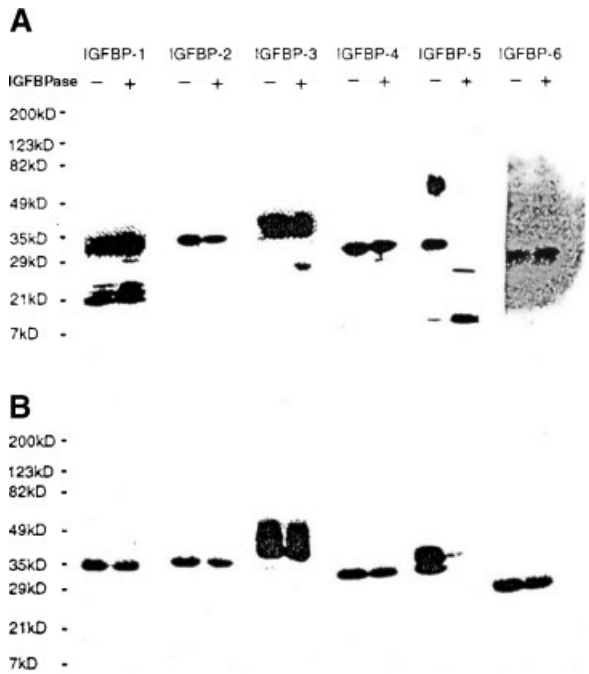

Fig. 3. Susceptibility of all six forms of IGFBPs to cleavage by the purified IGFBP-5 protease. **A**: All six forms of IGFBPs were exposed to 2 μg/ml of purified protease (baculovirus expressed immunoaffinity purified product) and the abundance of each intact form of IGFBP was determined by immunoblotting. Only IGFBP-5 was completely cleaved. **B**: To confirm that each form of IGFBP (except IGFBP-5) had remained intact their ability to bind to ^{125}I-IGF-II was determined by Western ligand blotting. All of the intact forms bound IGF-II and only IGFBP-5 was associated with a complete loss of binding after IGFBP-5 protease exposure.

majority of IGFBP-2 or IGFBP-3 was still intact, small fragments of 18 kDa from IGFBP-2 and a 28 kDa fragment from IGFBP-3 were seen after the samples were incubated for an extensive period with the IGFBPase, indicating that IGFBP-2 and -3 were slightly degraded (Fig. 3, panel A). The other forms of IGFBPs, IGFBP-1, -4, and -6, were not cleaved by the protease (Fig. 3A). To confirm that the forms that were visualized were functionally intact Western ligand blotting was performed (Fig. 3, panel B). The results show that each form of IGFBP had the expected binding capacity and that all forms except for IGFBP-5 remained intact following exposure to the protease. Incubation of ^{125}I-IGF-II with IGFBPase followed by SDS–PAGE with autoradiography showed no obvious degradation (data not shown). The above data indicate that the IGFBPase preferentially cleaves IGFBP-5.

In order to determine if the IGF binding motif of the IGFBPase was necessary for it to cleave IGFBP-5, the C-terminal fragment of the protein that contained the entire serine protease catalytic domain, but no IGF binding domain was utilized. The truncated IGFBPase completely cleaved IGFBP-5 and the degree of cleavage was comparable to that of the full-length protease protein (Fig. 4). This clearly indicates that the amino terminal IGF binding domain is not required for IGFBP-5 cleavage.

In order to determine the cleavage site on IGFBP-5, a mutant form of IGFBP-5, in which lysines 138 and 139 were mutated to asparagines that had been shown to be resistant to cleavage by a serine protease in pSMC media [Imai et al., 1997], was exposed to the IGFBPase. As shown in Figure 5, although the wild-type IGFBP-5 was degraded efficiently by the protease, the IGFBP-5 mutant remained intact. Resistance of the mutant IGFBP-5 to IGFBPase cleavage suggests that the mutated site may be a cleavage site. To confirm that this site was the site of cleavage, a synthetic peptide containing the IGFBP-5 region between residues 129 and 143 was tested for susceptibility to cleavage. Analysis of the cleavage products by mass spectroscopy showed that the peptide was cleaved, and the cleavage site determined from the mass estimates of the cleavage products was shown to be K^{138}, K^{139} (data not shown).

To assess the effect of protease inhibitors on the proteolytic activity of the IGFBPase, a number of protease inhibitors were incubated

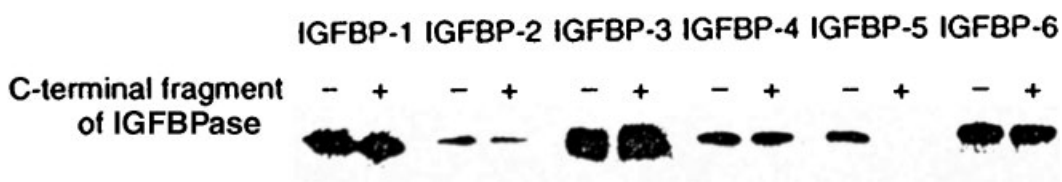

Fig. 4. Cleavage of IGFBP-5 by the C-terminal domain of the protease. Each form of IGFBP was exposed to the purified form of IGFBP-protease that had been expressed using a cDNA that did not encode the 100 N-terminal amino acids of the IGFBPase. Following a 3-h incubation, the products were analyzed by immunoblotting. Exposure of each form of IGFBP to this fragment showed that only IGFBP-5 was cleaved.

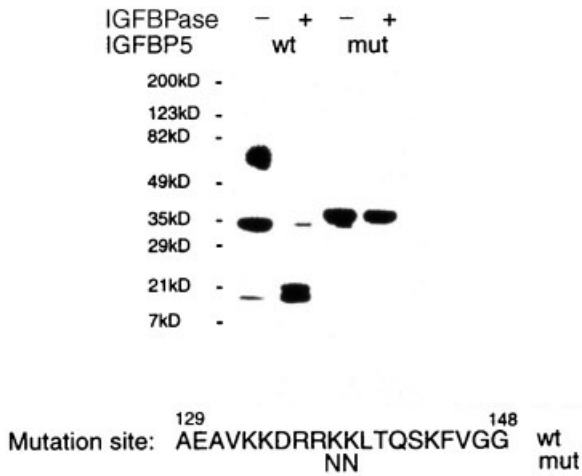

Fig. 5. Cleavage of native and mutant forms of IGFBP-5 by the purified protease. Wild-type IGFBP-5 and the IGFBP-5 mutant that had had lysines 138 and 139 converted to asparginines were incubated with the IGFBP-5 protease for 2 h at 37°C. The products of reaction were then analyzed by immunoblotting. As shown in the figure the wild-type protein is degraded whereas the mutant protein is resistant to proteolytic cleavage.

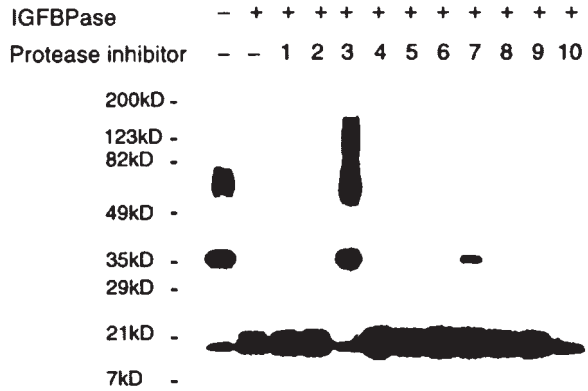

Fig. 6. Inhibition of IGFBP-5 proteolysis. The protease inhibitors listed were incubated with 2 μg/ml of the IGFBPase plus IGFBP-5. The inhibitors used were (**lane 1**) aprotinin (**lane 2**) antipain, (**lane 3**) 3, 4DCI, (**lane 4**) E64, (**lane 5**) leupeptin, (**lane 6**) pepstatin, (**lane 7**) PMSF, (**lane 8**) TLCK, (**lane 9**) TPCK, (**lane 10**) EDTA. As shown in the figure only 3,4 DCI and PMSF had any inhibitory activity and 3,4 DCI was the most potent. It is also notable that the protease activity is not divalent cation dependent as EDTA had no inhibitory activity.

individually with the protease for 1 h at 37°C, and then IGFBP-5 was added and the reaction continued as described in Materials and Methods. The results of immunoblotting showed that the serine protease inhibitor, aprotinin, the cysteine protease inhibitor, E64, the serine and cysteine protease inhibitors, antipain, leupeptin, TLCK, TPCK, the aspartic protease inhibitor, pepstatin A, and metalloprotease inhibitor EDTA had no effect on the proteolytic activity (Fig. 6). However, a serine protease inhibitor, 3,4-DCI strongly inhibited the proteolytic activity of the IGFBPase. Another serine and cysteine protease inhibitor, PMSF, also inhibited the proteolytic activity. These results indicate that IGFBPase is a serine protease, which is consistent with the fact that this protein contains a catalytic triad sequence that is typical of serine proteases. To further confirm this observation, we mutated the active site serine (S238) and tested the capacity of the purified mutant form to cleave IGFBP-5. As shown in Figure 7, the mutant had no activity after 2–4 h of incubation but there was a reduction in the intensity of the intact IGFBP-5 band after an overnight exposure.

To test whether the proteolytic activity of IGFBPase was metal ion dependent, degradation of IGFBP-5 by IGFBPase was carried out in the presence of different bivalent metal ions at concentration of 1 mM. Ca, Mg, and Mn showed no effect on the proteolytic activity, while Cu and Zn potently inhibited its activity (Fig. 8). The protease was fully active without adding metal ions to the reaction (Fig. 8, lane 2).

Heparin has been shown to inhibit IGFBP-5 proteolysis and to bind to IGFBP-5. In order to determine if heparin would alter IGFBP-5 cleavage by the IGFBPase, heparin was incubated with IGFBP-5 at 37°C for 30 min, and then the IGFBPase was added. Heparin inhibited the proteolytic activity of IGFBPase for IGFBP-5 in a concentration dependent manner (Fig. 9). The purified protease was also shown to bind to heparin sepharose (data not shown).

To determine if IGFBPase cleavage of IGFBP-5 resulted in an alteration in IGF-I action, MCF-7 breast cancer cells were exposed to increasing concentrations of IGF-I in the presence of a saturating concentration of IGFBP-5. As shown in Figure 10, BRDU incorporation was completely inhibited by a 5:1 molar excess of IGFBP-5 to IGF-I. However, if 300 nM IGFBPase was incubated with IGFBP-5 and IGF-I, the ability of IGFBP-5 to attenuate the IGF-I response was completely eliminated. Control cultures containing IGFBPase and IGF-I showed that it had no direct stimulatory effect. These findings indicate that using in vitro test culture conditions the IGFBPase cleaves IGFBP-5 allowing the IGF-I that is bound to IGFBP-5 to be released thus enabling it to stimulate DNA

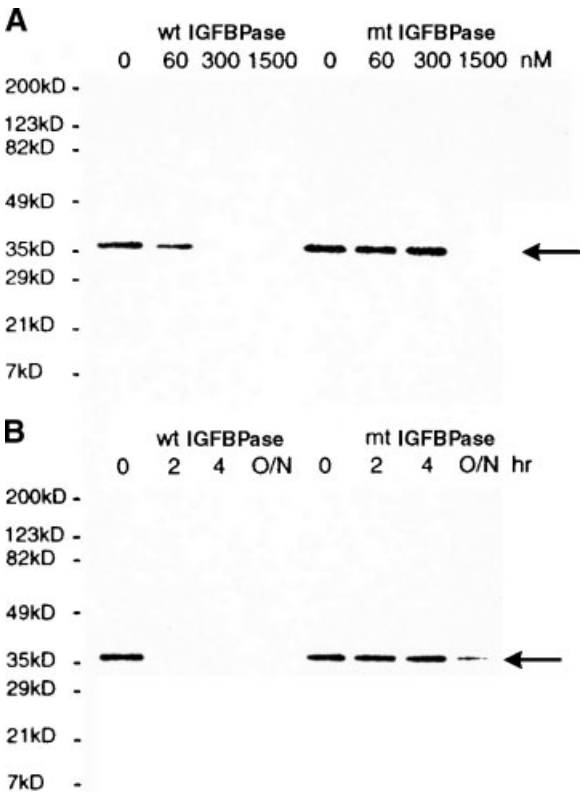

Fig. 7. Comparison of IGFBP-5 cleavage by wild-type and S238A mutant forms of IGFBPase. To further confirm that the protease was serine protease the active site serine 238 was mutated to alanine and the mutant protein was expressed in *Escherichia coli*. As shown in **panel A** following a 4 h-incubation increasing concentrations of the wild-type protease cleaved IGFBP-5 whereas increasing concentrations of the mutant up to 300 nM showed no cleavage. **Panel B**: Exposure to 300 nM the wild-type protease for 2 h resulted in complete cleavage whereas a 4 h exposure to the same concentration of mutant resulted in no cleavage.

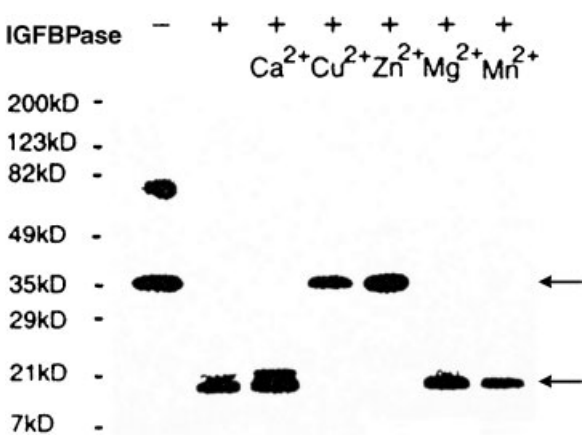

Fig. 8. Metal ion inhibition. To further analyze metal ion dependence the enzyme was incubated with Ca, Zn, Mg, and Cu. Both Zn and Cu inhibited proteolytic activity whereas Ca and Mg did not.

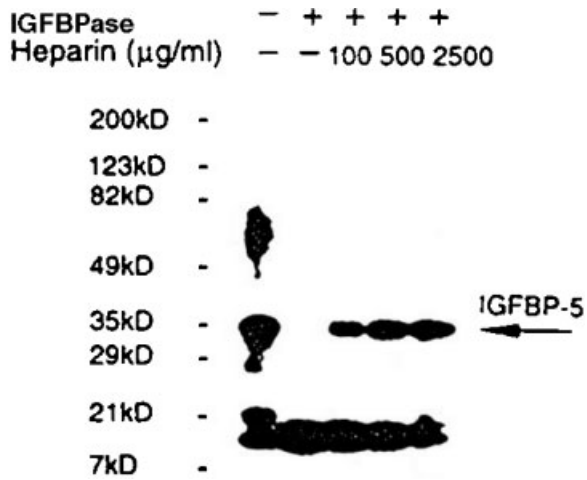

Fig. 9. Effect of heparin on IGFBP-5 proteolysis. Increasing concentrations of heparin were incubated with the IGFBP-5 protease and IGFBP-5. As shown in the figure, 100 μg/ml of heparin inhibited proteolysis and the degree of inhibition increased following exposure to increasing concentrations up to 2,500 μg/ml.

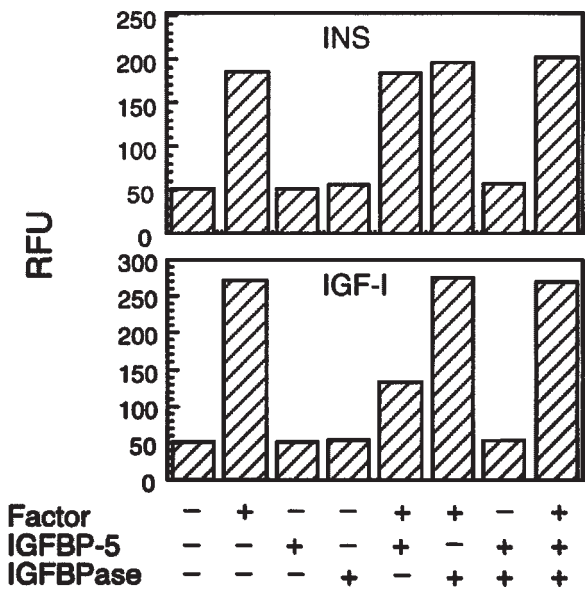

Fig. 10. Effect of IGFBPase on IGF-I mediated biologic actions. To determine the effect of the IGFBP-5 protease on IGF biological activity, a concentration known to cleave IGFBP-5 was co-incubated with IGFBP-5 and IGF-I (**bottom panel**) or insulin (**top panel**) and proliferation of MCF-7 cells was measured by BRDU incorporation. As shown in the figure, IGF-I (100 ng/ml) stimulated a 5.4-fold increase in cell number which was inhibited by coincubating with a molar excess of IGFBP-5 (e.g., 2.0 μg/ml). The addition of the IGFBP-5 protease (100 μM) alone caused no change in the cellular response to IGF-I, however, the addition of the protease with IGFBP-5 completely eliminated the inhibitory effect of IGFBP-5. When insulin was used as a control, there was no inhibition of insulin stimulation when IGFBP-5 was also added and no additional potentiation of its activity by IGFBP-5 protease addition.

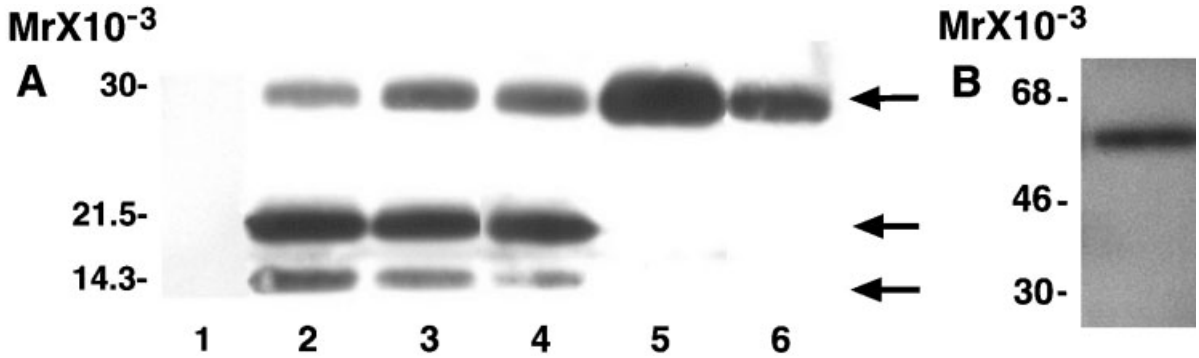

Fig. 11. Immunoblot of the IGFBP-5 in B104 cell conditioned medium. **A**: Conditioned medium was analyzed for the presence of intact IGFBP-5 following an 18-h incubation. Some cultures were exposed to IGFBP-5 or the synthetic IGFBP-5 peptide at the start of the incubation. Some cultures also received IGF-I for the last 4 h of the incubation. The treatments were as follows: (**lane 1**) IGF-I (50 ng/ml) + IGFBP-5 (0.5 μg/ml), (**lane 2**) IGFBP-5 (1.0 μg/ml), (**lane 3**) IGFBP-5 (1.0 μg/ml) + IGF-I (50 ng/ml), (**lane 4**) IGF-I (50 μg/ml) + IGFBP-5 (0.5 μg/ml), (**lane 5**) IGFBP-5 (1.0 μg/ml) + peptide B (5.0 μg/ml) + IGF-I (50 ng/ml), (**lane 6**) IGFBP-5 standard (no incubation). Intact IGFBP-5 and its major fragment are denoted by the arrows. **B**: The B104 cell condition medium obtained after an 18 h incubation was analyzed by immunoblotting using an antibody that is specific for the IGFBP-5 protease.

synthesis. These findings were extended to analyze the response of a cell type that serected the IGFBPase. When IGF-I was added to B104 cells (which have been shown to secrete the IGFBPase) it stimulated protein synthesis. Addition of IGFBP-5 for 14 h prior to the addition of IGF-I did not result in inhibition of the response to IGF-I. Analysis of the IGFBP-5 at the end of this incubation showed that it was extensively degraded (Fig. 11). Addition of the peptide that inhibited IGFBP-5 degradation (see Fig. 5) during the 14 h period prior to IGF-I addition resulted in restoration of the ability of IGFBP-5 to inhibit the cellular protein synthesis response to IGF-I (Table I). To confirm that the peptide had inhibited IGFBP-5 cleavage the conditioned medium obtained at the termination of the experiment was analyzed by immunoblotting. As shown in Figure 11, the addition of the synthetic peptide inhibited IGFBP-5 cleavage.

To determine the tissue distribution of the IGFBPase, a Northern blot containing total RNA from several human tissues was probed with an IGFBPase cDNA probe. As shown in Figure 12, uterus, and placenta contained the most abundant signal, although an easily detectible signal was present in heart, brain, thyroid, spinal cord, spleen, prostate, testis, small intestine, colon, and liver. Other tissues that were analyzed such as lung, skeletal muscle, kidney, pancreas, stomach, trachea, lymph node, adrenal, or thymus had minimal or no activity. This indicates that IGFBPase expression is selective and that female reproductive tract tissues contain the highest levels of expression.

DISCUSSION

Proteolytic activities that cleave IGFBP-5 have been described in the conditioned medium

TABLE I. Protein Synthesis Response of B104 Cells to IGF-I

Treatment	^{3}H-leucine incorporation into protein (CPM/well ± SD)
Serum free medium	5,984 ± 720
IGF-I (50 ng/ml)	8,796 ± 984*
IGFBP-5 (1.0 μg/ml)	5,772 ± 888
IGF-I (50 ng/ml) + IGFBP-5 (1.0 μg/ml)	8,101 ± 620*
IGF-I (50 ng/ml) + peptide B (5.0 μg/ml)	8,809 ± 933*
IGF-I (50 ng/ml) + IGFBP-5 (1.0 μg/ml) + peptide B(5.0 μg/ml)	6,303 ± 774**

IGFBP, insulin-like growth factor binding protein.
*$P < 0.01$ compared to the serum free medium.
**$P < 0.05$ compared to IGF-I + IGFBP-5.

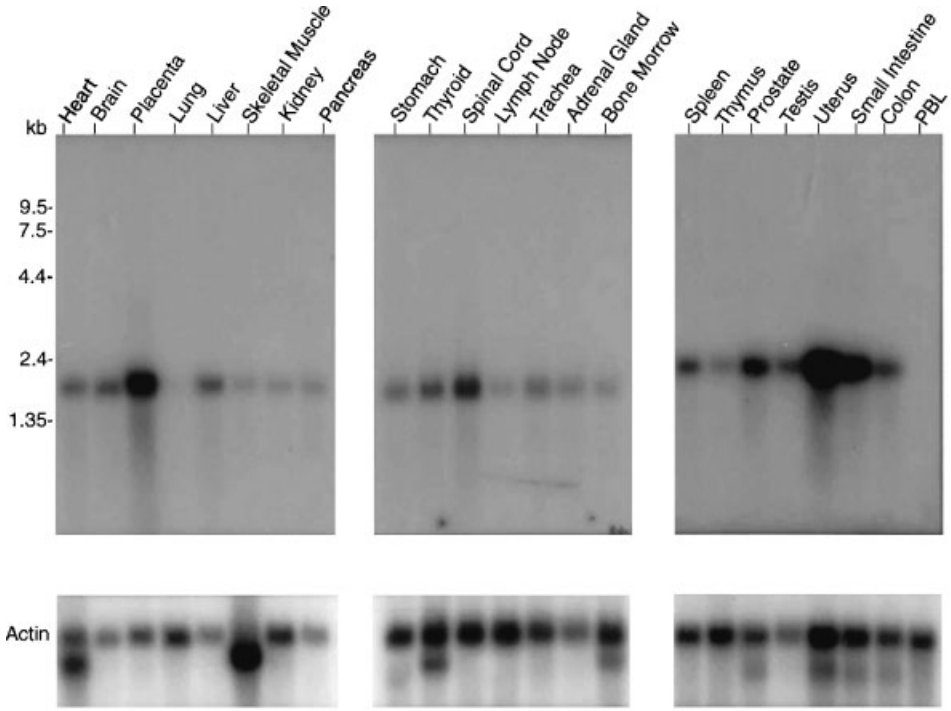

Fig. 12. IGFBP-5 protease mRNA abundance. To determine a relative abundance of the IGFBP-5 protease mRNA in various tissues, Northern blotting was performed. A single transcript was detected at 3.2 kB. Uterus, placenta, and small intestine were the most abundant human tissues. Easily detectible transcripts were present in spinal cord, prostate, testes, brain, thyroid, and colon. The transcript in adrenal gland, liver, heart, and spleen was less abundant. Lung, thymus, skeletal muscle, kidney, pancreas, lymph node, trachea, and bone marrow contained the lowest detectible level.

of several cell types and in physiologic fluids such as serum and joint fluid [Chernausek et al., 1995; Conover et al., 1995b; Imai et al., 1997]. Purification of several of these activities has led to identification of serine and metalloproteases that have proteolytic activity for IGFBP-5. Purification of human osteoblast condition medium led to the identification of Adam-9, as a IGFBP-5 protease [Mohan et al., 2002]. This protease was relatively specific for IGFBP-5 and did not cleave IGFBP-3, 4, and 6. Expression of the recombinant protein showed that it cleaved IGFBP-5 and that it also bound to alpha 2 macroglobulin but that alpha 2 macroglobulin did not inhibit its activity. Other ADAM family members, such as Adam-12s [Loechel et al., 2000] have been shown to cleave IGFBP-5. This protease had been shown previously to cleave IGFBP-3 [Shi et al., 2000]. Adam-12s activity for IGFBP-3 was completely removed by chelating Ca and readdition of Ca and/or Zn restored full activity. Similarly the activity of Adam-12s for IGFBP-5 could be inhibited by incubation with TIMP-3, a known metalloprotease inhibitor. Adam-12s was shown to have little activity against other forms of IGF binding proteins. The metalloprotease, PAPP-2A has also been shown to cleave IGFBP-5 [Overgaard et al., 2001]. Additionally, the matrix metalloproteases 2 and 9 have been shown to cleave IGFBP-5 although significantly higher concentrations of these proteases are required compared to Adam-12s and Adam-9 [Fowlkes et al., 1995].

Several serine proteases have been shown to be highly active in cleaving IGFBP-5. Analysis of the proteolytic activity in human fibroblast conditioned medium revealed that complement 1s was the active protease. Its activity could be inhibited by a variety of serine protease inhibitors and it was also shown to incorporate ^{3}H-diosopropylphosphoflordinate (DFP) [Busby et al., 2000]. This protease was specific for IGFBP-5 and it did not cleave other forms of IGFBPs. Human kallekein 2 and 3 have been shown to cleave IGFBP-5 but they also cleave other forms of IGFBPs. Hk2 cleaved IGFBP-2, 3, 4, and 5 whereas HK3 cleaved IGFBP-3 and 4 [Rehault et al., 2001]. Heparin was shown to enhance the capacity of HK3 to cleave IGFBP-3 and IGFBP-4 but whether it altered IGFBP-5 sensitivity to cleavage was not tested. Plasmin

has been shown to cleave IGFBP-5. Interestingly a fragment of IGFBP-5 containing the sequence of amino acids between 201 and 218 inhibited plasminogin activation thus resulting in decreased plasmin activity and decreased IGFBP-5 cleavage [Campbell and Andress, 1997]. Although each of these proteases has activity in cleaving IGFBP-5 whether the concentrations that are required for cleavage are present in interstitial fluids has not always been reported. Additionally, the relative amount of protease activity in a physiologic fluid is determined not only by the concentration of protease but also by the presence of protease inhibitors, however, most of these studies while documenting clearly that IGFBP-5 can be cleaved by these proteases have not analyzed the significance of IGFBP-5 cleavage in cell culture systems which are likely to contain protease inhibitors. In these studies, we have documented that the HTRA related protease that cleaves IGFBP-5 is secreted by a neuronal cell line and that inhibition of this enzymatic activity results in changes in the response of this cell type to IGF-I. This suggests that the protease activity is cleaving IGFBP-5 and that inhibition of IGFBP-5 cleavage results in a change in the cellular response to IGF-I.

An additional class of serine proteases that have been shown to cleave IGFBP-5 are the HTRA related family of proteases one member which is described in this manuscript. Originally the human homolog of HTRA-1 was discovered by a screening a subtraction library from SV40 transformed human fibroblasts [Zumbrunn and Trueb, 1996]. This study showed that SV40 transformation resulted in repression of this gene and cDNA sequencing revealed an 840amino acid protein whose N-terminus had a sequence that was similar to the N-terminal region of the IGF binding proteins and a C-terminal sequence that encoded a serine protease with high homology to the bacterial HTRA-1 protease. Based on these structural features, the authors proposed that this protease could potentially bind to the IGFs and that such binding could regulate the cleavage of IGFBPs. Subsequently Hu et al. [1998] cloned the identical protease from an expression library prepared from human osteoarthritic cartilage. They demonstrated that this transcript was increased sevenfold in osteoarthritic cartilage compared cartilage obtained from non-osteoarthritic controls. They expressed the protease and showed that serine 328 was required for biologic activity. They fully characterized the activity of this protease using a variety of substrates including caseine. The translated protein product had a molecular weight estimate of 50 kDa similar to the protein product identified in this report.

In these studies, we have expressed this serine protease in baculovirus and purified the recombinant product. The studies show that the protease cleaves IGFBP-5 at relatively low concentrations and this activity is relatively specific for IGFBP-5 having only minimal activity against two other forms of IGFBPs. Similar to the study those of Hu et al. [1998] mutation of the active site serine reduced the activity of this protease for IGFBP-5.

The protease has properties that are similar to a proteolytic activity that has been characterized in human fibroblast media in that it is a serine protease that cleaves IGFBP-5 at lysines 138–139. However, the protease in fibroblast media was shown to be glycosylated 92 kDa protein and to correspond to human C1s [Busby et al., 2000]. In contrast the protease described in this report is not glycosylated and has a molecular weight estimate of 50 kDa. Similarly immunoblotting of fibroblast media with the anti-HTRA protease antisera showed no detectible activity (data not shown) supporting the conclusion that human fibroblast media does not contain this activity.

This protease has an amino terminal sequence that is similar to the insulin like growth factor binding proteins and members of the CCN family of proteins. Our studies show that deletion of this sequence has no effect on proteolysis, that is, a purified fragment of the protease that does not contain the IGF binding domain had full proteolyic activity against IGFBP-5. Whether IGF binding would inhibit proteolytic activity was not tested, however, as has been shown for the other members of the CCN family, the affinity of proteins containing only this region of the complete IGF binding protein sequence for IGF-I or IGF-II is extremely low ($<10^{-6}$ M). This suggests that IGF-I binding to this protein does not occur at physiologically relevant IGF concentrations.

The catalytic domain of the protein contains remarkable similarity to the HTRA family of proteases. Hu et al. [1998] compared the sequences of HTRA proteases from a variety of species including human, calf, guinea pig, and

showed that they were remarkably similar to the same family of proteases expressed in *E. coli*. Gray et al. [2000] characterized a second member of this family HTRA2, and reported the sequence of a third family member HTRA-3. They compared the structures of both HTRA 2 and 3 to HTRA1 and showed the HTRA1 and 3 had more similarity. Both groups of investigators have pointed out that HTRA expression in bacteria is an important component of the response to stress and that these proteins are coinduced with heatshock proteins following oxidative stress or viral infection. HTRA expression is a required for bacteria to survive heatshock [Lipinska et al., 1990]. It has been postulated that this proteolytic activity is required to degrade misfolded proteins within the periplasmic space. Members of this family undergo autoactivation but the significance of autoactivation for IGFBP-5 cleavage has not been addressed. Members of this family also contained PDZ domains, which have been found to be important for protein/protein interactions. They have been shown to form protein complexes with cell surface proteins such as receptors or ion channels and may be important in targeting these proteins for degradation. The connection between a stress response regulated gene and IGF signaling is not immediately apparent. However, several studies have indicated that cellular responses to growth factors such as IGF-I may be modified by the generation of reactive oxygen species, an important component of the stress response. Therefore, a protease that regulates the presentation of IGF-I to receptors by controlling IGFBP-5 degradation in coordination with changes in signal transduction that occur in response to oxidative stress might have an important dual role in regulating IGF-I signaling.

Our results like those Hu et al. [1998], show that the mRNA encoding this protease is expressed in multiple human tissues. We have also found that the protease is expressed most abundantly in uterus and placenta. This raises the question whether sex steroids regulate the expression of the protease. The extent to which secretion of this protease and/or its activation contributes to alterations in IGF-I action that are modulated by IGFBP-5 remains to determined. The protease is a candidate for releasing IGFs from IGFBP-5 or liberating biologically active fragments of IGFBP-5 that could act directly on tissues [Standker et al., 1998; Miyakoshi et al., 2001; Song et al., 2001]. Investigators have shown that testes, bone, lung, uterus, placenta, and ovary are major sites of IGFBP-5 expression in human tissues [Schuller et al., 1994; Jones and Clemmons, 1995]. Similarly kidneys, spinal cord and skeletal muscle are important sites of its expression in mice. Importantly in these studies, all these tissues were shown to be sites of a high level of expression of the IGFBP-5 protease and this makes it possible that if sufficient substrate and protease were expressed together in these tissues this could modify the amount of IGF-I that is bound to IGFBP-5 and thereby alter IGF-I actions.

Heparin has been shown that inhibit several of the purified proteases, such as plasmin, that have been shown to degrade IGFBP-5. Heparin binding to IGFBP-5 has been shown to reduce affinity of IGFBP-5 for IGF-I. Therefore, it is possible that IGFBP-5 can undergo a conformational change in response to heparin binding that would alter the exposure of the proteolytic cleavage site thus altering its degradation. Our previous studies have shown that the region of IGFBP-5 that contains one of its heparin binding domains also contains the proteolytic cleavage site [Arai et al., 1996]. However, this does not exclude the possibility that heparin binding to IGFBP-5 also results in a conformational change that results in inhibition of its cleavage.

The mechanisms by which proteases alter IGF action have not been studied in detail. The references cited herein have described various purified proteases and determined their activation in cleaving IGFBP-5 and other forms of IGFBPs in vitro. However, most of the studies have not analyzed the effect of inhibiting a given protease on IGF-I responsiveness in a biologic test system. Our findings show that in MCF-7 cells the protease was able to liberate IGF-I enabling it to bind to receptors and activate mitogenesis. In contrast when the protease was not added IGFBP-5 fully inhibited IGF-I stimulation of mitogenesis. This observation suggests that that the protease could have this function in vivo although in vitro findings need to demonstrated in are in vivo model to determine the ultimate physiologic significance of this observation. We further utilized a rat neuronal cell line (B104 cells) that were known to release this protease and showed that when the activity of the protease was inhibited the cellular

protein synthesis response to IGF-I was also inhibited. Specifically addition of the peptide that was shown to modify IGFBP-5 cleavage by the IGFBPase resulted in an increase of intact IGFBP-5 and a decrease in the ability of IGF-I to stimulate protein synthesis. Therefore, our findings strongly suggest that at least in tissue culture manipulation of the levels of the activity of this protease can result in an alteration in IGF-I action.

In summary, we have expressed a serine protease that cleaves IGFBP-5 and is a member of the HTRA class of proteases. Expression of the purified protease showed that it had full activity for cleaving IGFBP-5. The protease is expressed in a wide variety of tissues and thus physiologic concentrations capable of modulating IGFBP-5 biologic actions may be present and thereby capable of regulating the availability of IGF-I to bind its receptor.

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