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Proteolytic Cleavage of Insulin-like Growth Factor Binding Protein 4 (IGFBP-4)

LOCALIZATION OF CLEAVAGE SITE TO NON-HOMOLOGOUS REGION OF NATIVE IGFBP-4*

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Insulin-like growth factor binding protein 4 (IGFBP-4) is a 24-kDa protein that binds insulin-like growth factor 1 (IGF-1) and IGF-2 with high affinity and inhibits IGF action *in vitro*. We recently described a protease produced by the B104 neuronal cell line that cleaves IGFBP-4, yielding an approximate 16-kDa immunoreactive protein that binds IGFs with reduced affinity. We analyzed fragments produced by exposing pure IGFBP-4 to the protease to determine potential cleavage sites. Electrospray mass spectrometry and amino acid sequencing indicated the 16-kDa fragment spanned the NH_2 terminus of native IGFBP-4 through Lys-120. There was evidence for an additional proteolytic fragment beginning at amino acid 132 and continuing to the COOH terminus. Proteolysis could be blocked by a synthetic peptide that spanned amino acids 117–126 but not by peptides that contained flanking sequences 111–120 or 125–135. Mutagenesis was used to alter the basic residue at position 120. The expressed mutant IGFBP-4 (K120A) was relatively resistant to cleavage, strongly suggesting that residues 120–121 represent the cleavage site. This region of IGFBP-4 is not homologous with other IGFBPs, explaining the apparent specificity of the protease for IGFBP-4. The 16-kDa IGFBP-4 fragment no longer inhibited IGF-1-stimulated thymidine uptake *in vitro*, suggesting that proteolytic processing of IGFBP-4 may have important functional consequences *in vivo*.

The insulin-like growth factors (IGFs)[1] are mitogens produced by many tissues and are postulated to act in a paracrine/autocrine manner (1). IGF action is thought to be greatly influenced by the IGF binding proteins (IGFBPs), a family of secreted proteins that bind the IGFs with high affinity (2). These proteins are expressed by a wide variety of tissues and may direct transport of the IGFs into organs. Also, *in vitro* experiments have shown that IGFBPs can either inhibit or enhance IGF action depending upon the experimental design and the form of IGFBP examined (3–7), suggesting that the IGFBPs modulate local IGF bioactivity *in vivo*. Six IGFBPs have been described (8). Although structurally related, they are the products of distinct genes that have differing tissue and developmental regulation of expression. Each IGFBP contains amino- and carboxyl-terminal regions that are homologous among the IGFBPs and rich in cystine or cysteine residues. In contrast, the middle third of the molecules is usually free of disulfides and has little shared sequence between the various IGFBPs.

All IGFBPs undergo some form of post-translational modification. IGFBP-3, -4, -5, and -6 manifest variable degrees of glycosylation (8). Phosphorylation of IGFBP-1 alters its affinity for IGFs and biologic actions (9). Recently, extracellular proteolytic processing has emerged as a frequent and potentially important mechanism governing the biologic properties of the IGFBPs (10–14). The responsible proteases have, in general, not been identified, although the IGFBP-3 protease produced by human fibroblasts in culture may be a member of the matrix metalloprotease family and the IGFBP-3 protease in seminal fluid appears to be prostate-specific antigen (15), a serine protease.

A protease(s) that cleaves IGFBP-4 is present in media of cultured fibroblasts (10, 11), decidual cells (16), osteoblasts (17–19), and smooth muscle cells (20). An unusual feature is that the IGFBP-4 ligand, IGF-1 or -2, greatly accelerates the rate of proteolysis when present in the reaction mixture (10, 11, 16). Although the exact mechanism has not yet been delineated, most data suggest that binding of an IGF by the IGFBP-4 makes the latter more susceptible to proteolytic cleavage, probably through a conformational change.

We recently described a glucocorticoid-inducible protease produced by the rat B104 neuronal cell line (21). This protease cleaves intact 24-kDa IGFBP-4 to a smaller, approximate 16-kDa species that binds IGFs with a reduced affinity. In order to better understand the nature of IGFBP-4 proteolysis, experiments were performed to identify proteolytic cleavage sites within the IGFBP-4 molecule and to determine the effect of mutagenesis on IGFBP-4 stability.

EXPERIMENTAL PROCEDURES

Reagents—The rat IGFBP-4 antibody raised against a synthetic 20-amino-acid peptide (residues 81–100) was described previously (22). An additional rat IGFBP-4 antibody raised against amino acids 111–120 was provided by N. Ling (Whittier Institute, La Jolla, CA), along with the corresponding synthetic peptide (no. 1). Peptides 2 and 3 (see Fig. 3) were synthesized by a commercial vender (Anaspec, San Jose, CA) and were judged to be greater than 90% pure. Rat IGFBP-4 was prepared from media conditioned by the B104 cell line as described previously (21). Restriction endonucleases and molecular biology reagents were purchased from New England Biolabs, Beverly, MA. IGF-1 was provided by Lilly.

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[1] The abbreviations used are: IGF, insulin-like growth factor; IGFBP, IGF binding protein; HPLC, high pressure liquid chromatography; PTH, phenylthiohydantoin; CHO, Chinese hamster ovary; ESMS, electrospray mass spectrometry.

Radioligand Blots and Immunoblots—Samples were subjected to 4%/12% discontinuous SDS-polyacrylamide gel electrophoresis and electroblotted to nitrocellulose membranes (Schleicher and Schuell). Radioligand blotting using ^{125}I-IGF-1 was performed by the method of Hossenlopp *et al.* (23). Immunoblotting was as described previously (22). Immunoreactive species were identified by either an avidin-biotin alkaline phosphatase conjugate system (Vectastain, Vector Labs, Inc., Burlingame, CA) or by chemiluminescent detection (Amersham Corp.).

Protease Preparation—Media containing the IGFBP-4 protease were prepared by dexamethasone treatment of a subline of B104 cells ($B104_{ns}$) that produced little IGFBP-4 (24). Cells were grown to confluency in fetal calf serum-containing media and then switched to serum-free media containing 100 nM dexamethasone (Sigma). After 2–3 days of exposure, harvested media (Dex-media) were centrifuged to remove cellular debris and saved at −70 °C. When required, conditioned media were concentrated using a microconcentrator (Centricon-10, Amicon, Beverly, MA).

Isolation of IGFBP-4 Fragments—Twenty-five μg of IGFBP-4 was exposed to the IGFBP-4 protease for 48 h. The pure peptide was incubated with 0.5 ml of Dex-media containing 0.1 volume of 0.5 M Tris buffer, pH 7.4. After 24 h at 37 °C, an additional 0.5 ml of media was added followed by a final 0.22 ml 12 h later. The reaction mixture was stored frozen and subsequently applied to an IGF-1 affinity column (binding capacity, 400–600 μg of IGFBP-4) equilibrated in 0.05 M NaH_2P0_4, pH 6.6, containing 2 mM EDTA and 0.1 M NaCl. After 16 h of recirculation, retained IGFBP-4 was eluted with 0.5 M acetic acid. Fractions containing the immunoreactive 16-kDa IGFBP-4 fragment (determined by immunoblotting) were loaded onto a reverse phase HPLC column (C4 Vydac, Hespena, CA) equilibrated in 0.04% trifluoroacetic acid and H_2O. Elution was accomplished with a 0–60% CH_3CN gradient over 1 h. Fractions were screened by immunoblotting, and those containing 16-kDa IGFBP-4 were subjected to further analysis.

Mass Spectrometry—Reverse-phase liquid chromatography fractions were evaporated to dryness and then dissolved in water:methanol: formic acid (50:50:1, v/v/v). Samples were introduced to a Sciex API III triple-quadrupole mass spectrometer (Sciex, Inc., Toronto, Canada) at 4 μl/min with a syringe pump. The device was equipped with an atmospheric pressure ion source used to sample positive ions produced from a pneumatically assisted electrospray interface. Sample ions generated by the electrospray process were mass-analyzed by scanning the first quadrupole in 0.1-atomic mass unit increments over approximately 10 s and passing mass-selected ions through the second and third quadrupoles, operated in the radiofrequency-only mode, to a channeltron detector. Mass calibration of the instrument was accomplished just prior to sample analysis by matching ions of polypropylene glycol to known reference masses stored in the mass calibration table of the mass spectrometer. Ten scans were averaged for each analysis. The average molecular weights of the sample peptides were calculated from the multiply protonated ions in the mass spectrum.

Amino Acid Sequence Analysis—Automated Edman degradation chemistry was used to determine the NH_2-terminal protein sequence. An Applied Biosystems, Inc. model 470A gas phase sequencer (Foster City, CA) was employed for the degradations using the standard sequencer cycle, 03RPTH. The respective PTH-derivatives were identified by reverse phase HPLC analysis in an on-line fashion employing an Applied Biosystems, Inc. model 120A PTH analyzer fitted with a Brownlee 2.1-mm (inner diameter) PTH-C18 column.

Mutagenesis—A full-length IGFBP-4 cDNA in pBluescript SK+ (pRBP4–503) served as a template for mutagenesis and was kindly provided by Dr. S. Shimasaki of the Whittier Institute (La Jolla, CA). In preparing the mutant IGFBP-4, the unique restriction sites *Msc*I (near the lysine 120 codon) and *Sph*I (approximately 200 base pairs 5′ to the lysine 120 codon) were exploited. An oligonucleotide primer pair was synthesized in which the upper primer contained the rat IGFBP-4 sequence that overlapped the *Sph*I site (5′ CTGTGGCTCAGGCATGCGCGGC 3′) whereas the lower primer contained the *Msc*I site as well as the introduced mutation (5′ ACTTTGGCCATATGCGCCTGCAGGC 3′). These primers were used to generate the 219-base pair DNA fragment by polymerase chain reaction using Vent DNA polymerase (New England Biolabs, Beverly, MA). The polymerase chain reaction was run for 23 cycles in buffers provided by the manufacturer using a 57 °C annealing temperature. The product was exposed to *Sph*I and *Msc*I and after isolation using agarose gel electrophoresis, ligated back into pRBP4–503 from which the matching sequence had been removed by enzymatic digestion. Integrity of the mutant was verified by DNA sequencing.

Mammalian Cell Expression and Isolation of Mutant IGFBP-4—The mutant IGFBP-4 cDNA was transferred to a pUC18-based mammalian expression vector that utilized a mouse metallothionein promoter (pNUT, provided by A. J. D'Ercole, University of North Carolina). Chinese hamster ovary cells (CHO K-1) were transfected by the following method. The cells were maintained in α-minimal essential medium containing 10% fetal calf serum supplemented with penicillin and streptomycin. Twenty-four hours before transfection the cells were seeded into 6-well tissue culture plates to approximately 15% confluency. DNA was introduced into the cells by a calcium phosphate precipitation procedure. The DNA/calcium precipitate was formed by mixing 0.5 ml of 0.25 M calcium chloride containing 10 μg of plasmid DNA in 0.5 ml of 2 × Hanks' basic salt solution. After incubation at room temperature for 15 min, 200 μl (2 μg of DNA) were applied to the cells in 2 ml of medium. Plates were incubated at 37 °C for 5 h after which calcium-containing media were removed and medium containing 10% glycerol was applied for 3 min. After extensive rinsing this medium was replaced, and the cells were incubated for 48 h. Treated cells were then trypsinized and plated 1:50 in 100-cm dishes with medium containing 1 μg/ml methotrexate. Fresh methotrexate was reapplied every 3–4 days for 10–12 days when colonies of stably transfected cells began to appear. Colonies were isolated by cloning rings, trypsinized, and transferred to individual wells of a 24-well plate. Medium was assayed for IGFBP-4 secretion by immunoblotting after the cells reached confluency. The methotrexate concentration was gradually increased to 50 μg/ml in the surviving clones.

Approximately 1 liter of conditioned medium was collected from CHO cells expressing the mutant IGFBP-4. The medium was clarified by centrifugation at 16,000 × *g* for 20 min, the pH was adjusted to 6.6 with Tris base, and then the medium was loaded at 1 ml/min at 4 °C onto a 4.4 × 3-cm phenyl-Sepharose column (Pharmacia Biotech Inc.) equilibrated with 0.05 M sodium phosphate buffer, pH 6.6, containing 0.1 M NaCl and 2 mM EDTA. After washing, a linear gradient of 0–20% CH_3CN in 0.05 M sodium phosphate, pH 6.6, 2 mM EDTA was used to elute bound proteins. The active fractions were identified by immunoblotting and partially lyophilized to remove the CH_3CN. The active pool was subjected to IGF-1 affinity column chromatography as described above. The partially lyophilized eluate was injected onto a Vydac C-4 reverse phase column (4.6 cm × 15 mm). An isocratic elution with 0.4% trifluoroacetic acid was conducted for 10 min followed by a 0–24% linear gradient of CH_3CN over 10 min. For the next 20 min the CH_3CN concentration was increased linearly from 24 to 34% and for the final 24 min from 34 to 100%. The mutant rat IGFBP-4 eluted as a single peak at 28–30% CH_3CN. The material was shown to be pure by both immunoblotting and ligand blotting.

Tritiated Thymidine Uptake—$B104_{ns}$ cells were used as targets for IGF action. Tritiated thymidine uptake in response to added IGF-1 was performed as described previously (24). Cells were exposed to IGF-1 and graded concentrations of intact IGFBP-4 or the 16-kDa fragment in serum-free media for 24 h prior to the addition of the [^{3}H]thymidine.

RESULTS

Characterization of Proteolytic Fragment of IGFBP-4—Proteolytic products of IGFBP-4 were initially examined using antibodies directed against specific IGFBP-4 peptide sequences. Antisera produced using amino acids 81–100 and 110–120 of the mature IGFBP-4 molecule both recognize a proteolytic fragment of approximately 16 kDa, indicating the 16-kDa species overlaps, at least in part, amino acids 80–120 (Fig. 1). Further characterization of the 16-kDa species was accomplished by subjecting purified 16-kDa IGFBP-4 (see "Experimental Procedures") to NH_2-terminal amino acid sequence analysis and electrospray mass spectrometry (ESMS). The amino acid sequence obtained was DEAIH(C)PP(C)S (where (C) indicates a possible cysteine), which corresponds to the published NH_2 terminus of native rat IGFBP-4. Following this, the exact molecular weight (M_r) of the isolated fragment was determined by ESMS. Analysis of the data indicated two species present in the isolate (Fig. 2); the observed M_r of one matches exactly that expected for an IGFBP-4 protein containing amino acids 1–120 while the other matched with amino acids 1–90. Because the 16-kDa IGFBP-4 seen on immunoblot reacted with the antibody raised against amino acids 110–120, it must represent the 1–120 species. The potential cleavage sites are indicated upon the schematic (Fig. 3).

Inhibition of Proteolysis by Synthetic Peptides and by Site-

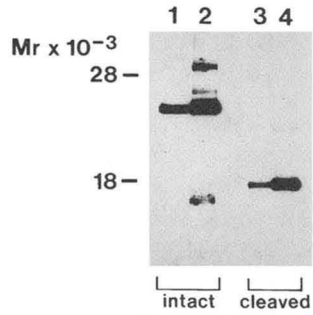


FIG. 1. **Recognition of proteolytic fragment of IGFBP-4 by antisera raised against differing synthetic peptides.** Intact (*lanes 1* and *2*) and proteolytically cleaved (*lanes 3* and *4*) IGFBP-4 were analyzed by immunoblot using antisera raised against amino acids 81–100 (*lanes 1* and *3*) and against amino acids 110–120 (*lanes 2* and *4*) of native IGFBP-4. Immunoblot was developed as described under "Experimental Procedures." Samples were run without prior disulfide bond reduction. Positions and molecular weight markers are shown to the *left*.

directed Mutagenesis—The data described suggest a putative cleavage site between Lys-120 and His-121. To assess the potential for cleavage at this site, the ability of synthetic peptides to inhibit the proteolysis of intact IGFBP-4 was examined. Peptides that either spanned or flanked the putative cleavage site (see Fig. 3) were incubated at high molar excess with pure native IGFBP-4 and Dex-media (Fig. 4). The synthetic peptide containing amino acids 117–126 inhibited proteolysis while the flanking peptides were ineffective.

Site-directed mutagenesis was used to alter the IGFBP-4 molecule, changing the basic lysine at position 120 to alanine. The mutant (K120A IGFBP-4) was expressed in CHO cells, isolated from conditioned media, and tested for sensitivity to proteolytic cleavage. Figs. 5 and 6 show the cleavage over time of the K120A IGFBP-4 compared with native IGFBP-4 in the presence and absence of IGF-1. Without added IGF-1, proteolytic degradation is minimal (Fig. 5). The addition of IGF-1 significantly enhanced cleavage of both mutant and native IGFBP-4. Although proteolytic cleavage of the mutant IGFBP-4 occurred eventually, the appearance of the 16-kDa species was significantly delayed compared with wild type IGFBP-4. The difference in apparent cleavage rates for native and mutant IGFBP-4 was more accurately quantified by incubating the purified proteins with the protease and assessing the decline in 24-kDa species over time through PhosphorImage analysis of a ligand blot (Fig. 6). This experiment confirmed that the proteolytic degradation of K120A IGFBP-4 is slower than that of the wild type protein. It also appears that 28-kDa (glycosylated) IGFBP-4 is relatively resistant to the protease.

Examination of the ligand blot data in Fig. 6 suggests greater resistance of the mutant to proteolytic cleavage than is apparent in Fig. 5. This likely reflects differences in time of exposure to the enzyme (15 *versus* 24 h) but also may reflect different levels of enzymatic activity found in differing batches of conditioned media. In multiple experiments, analyzed by both immunoblot and ligand blot, degradation of the K120A mutant IGFBP-4 was always attenuated relative to that of the wild type, but the amount of intact 24-kDa IGFBP-4 remaining at the end of each experiment varied. This is interpreted to mean the K120A IGFBP-4 is resistant, but not invulnerable, to cleavage by the B104 protease.

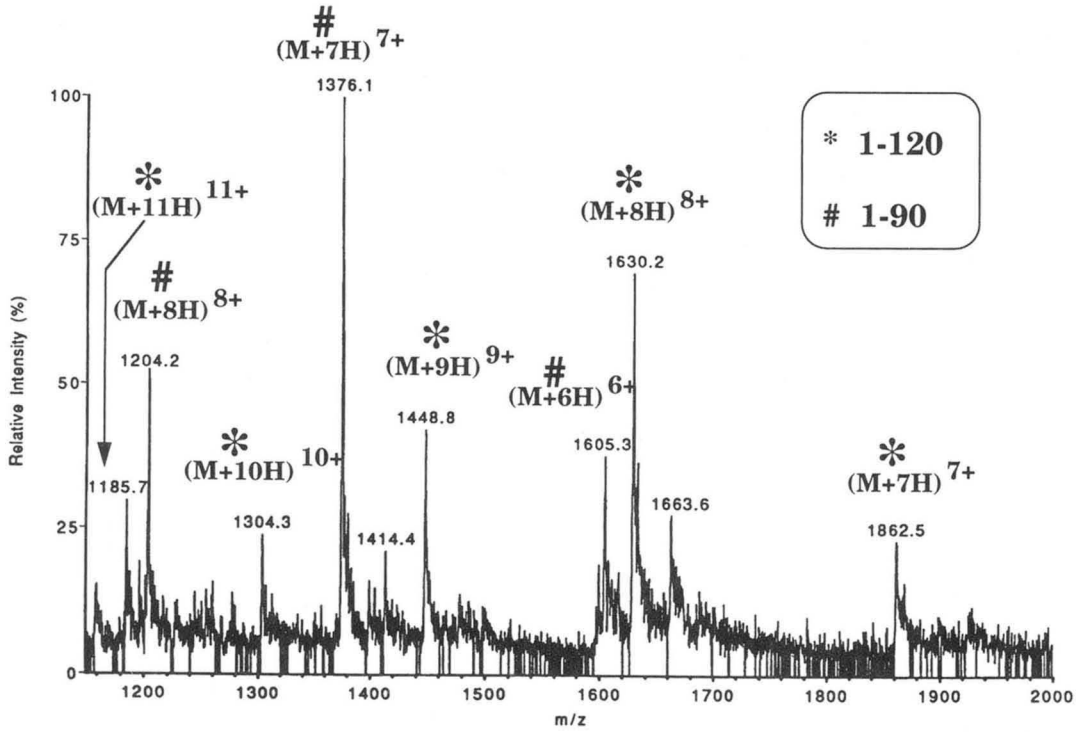
In light of the highly concordant immunologic amino acid sequence and ESMS data, substantial cleavage of the mutant IGFBP-4 was surprising due to the significant charge change expected by substituting alanine at position 120. In order to understand this better, the proteolytic fragments from the mutant were analyzed by amino acid sequencing and ESMS. The K120A IGFBP-4 was cleaved by exposure to Dex-media in the presence of 100 ng/ml IGF-1. The mixture was subjected to HPLC as described under "Experimental Procedures," and a fraction containing the 16-kDa species was mass-analyzed. Deconvolution of the mass spectra yielded observed M_r of 13,110.3 ± 0.8 and 11,322.2 ± 0.35 (mean ± S.D.). The M_r of the larger species matches the theoretical sequence from the NH_2 terminus through histidine 121 (expected M_r 13,112, accounting for the replacement by alanine at position 120). Scanning of the IGFBP-4 sequence for a string of amino acids matching the M_r of the smaller species yielded a sequence beginning at lysine 132 and continuing through the carboxyl terminus (expected M_r 11,324). In order to verify these data, proteins contained in a fraction adjacent to that mass analyzed (also containing the 16-kDa species by immunoblot) were subjected to NH_2-terminal sequence analysis. The data indicated three distinct IGFBP-4 sequences. One corresponded to the normal NH_2 terminus of intact IGFBP-4. The second began at lysine 132 (KVVGTPREEPRPVPQ), while the other began with valine 134 (VXTPREE*XXX*V*X*QGS, where *X* indicates inability to assign a residue for that position). The first two are thus compatible with ESMS data for K120A IGFBP-4 peptides of 1–121 and 132 through the COOH terminus. Concentration of the third species may have been too low for detection by ESMS.

The Effect of Proteolysis on IGFBP-4 Action—In a prior publication we showed that cleaved IGFBP-4 is capable of binding IGF-1 but with reduced affinity (21). In order to determine the effect of cleavage on biologic properties of IGFBP-4, the ability of intact IGFBP-4 to inhibit IGF-1-stimulated thymidine uptake was compared with that of cleaved IGFBP-4. The fragment was isolated by HPLC as above. Intact native 24-kDa IGFBP-4 produced the expected dose-dependent inhibition of IGF-1, using B104 cells as a target (Fig. 7). However, equivalent concentrations of cleaved IGFBP-4 were ineffective, indicating that proteolytic processing of IGFBP-4 changes its properties such that it no longer inhibits IGF-1 action.

DISCUSSION

Our data indicate that IGFBP-4 is proteolytically cleaved into two major fragments following exposure to a protease from B104 cells. One fragment begins at the NH_2 terminus and extends through amino acid 120. The other contains residue 132 through the COOH terminus. The B104 cell-derived protease appears to act at the bond between Lys-120 and His-121 (a trypsin-susceptible site) because proteolysis is blocked by an overlapping synthetic peptide and because mutation of lysine 120 attenuates the rate of cleavage. The residues surrounding this putative cleavage site (amino acids 116–121) are conserved between the human and rat but without a homologous sequence in the other IGFBPs (8). This is compatible with the reports of potentially related IGFBP-4 proteases from other species that appear to cleave IGFBP-4 selectively (10, 11, 16, 18, 19, 25).

An IGFBP-4 fragment spanning the NH_2 terminus through amino acid 90 was also detected by ESMS in one experiment. The origin and significance of this species is not clear. It might reflect further processing of the 1–120 species and/or be the result of the action of a distinct protease. Although we could not detect a 1–90 species by immunoblotting, in a prior publication we observed an appropriate sized fragment when radiolabeled IGFBP-4 was exposed to the protease (21). The relative amount of that species was very low, and therefore we believe the 1–90 IGFBP-4 is probably a minor component in the proteolytic processing, at least as applied to the B104 system. Fowlkes *et al.* (26) have recently examined the proteolysis of IGFBP-3 in human dermal fibroblast medium. They attributed the proteolytic activity to one or more matrix metalloproteinases that cleaved IGFBP-3 within its nonhomologous domain. Because



ESMS Compound M_r (± SD)	N	AA Sequence	Expected M_r
13,031.7 ± 1.5	5	1-120	13,032
9,625.6 ± 0.1	3	1-90	9,626.3

FIG. 2. **Mass spectrometric analysis of 16-kDa IGFBP-4.** The observed and expected M_r of two putative fragments and their potential amino acid sequences are given in the table at the *bottom*. *N* refers to the number of mass determinants for each. Charge states corresponding to the species of differing M_r are indicated.

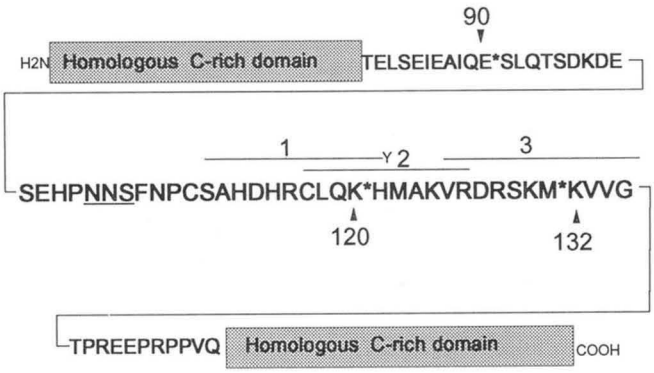


FIG. 3. **Schematic of rat IGFBP-4.** Regions of the amino acid homology among the IGF binding proteins are indicated as *shaded boxes*. The central, nonhomologous sequence is shown using the single alphabetic code. The specific sequences of synthetic peptides 1, 2, and 3 are indicated by the *labeled lines*. The *underlined* amino acids indicate the putative *N*-linked glycosylation site. Amino acid 120 is indicated. The *asterisk* shows potential cleavage sites based on ESMS data.

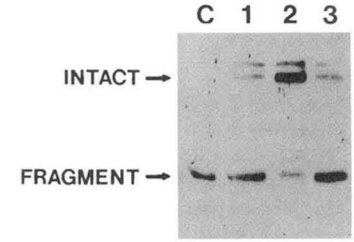


FIG. 4. **Inhibition of proteolysis by synthetic peptides.** Pure IGFBP-4 was incubated for 16 h at 37 °C with protease-containing media and 0.5 mg/ml synthetic peptide. *Lanes* labeled *1-3* contain peptides 1–3, respectively (see Fig. 3). *C* indicates control (no added peptide).

cleavage of IGFBP-3 does not occur in B104 conditioned media (21), the B104 protease is unlikely to be identical to those described by Fowlkes *et al.* (26). However, recognition of amino acid sequences within the nonhomologous regions of the IGFBPs by IGFBP proteases could be a general mechanism to explain specificity among the IGFBP proteases.

The single amino acid substitution we introduced was insufficient to abolish cleavage. It is possible that the mutation simply did not produce enough of a change to render the IGFBP-4 completely insensitive to cleavage at or around that site and that the His-121–Met-122 bond served as an alternate site. It is also possible that the primary cleavage site is not between amino acids 120 and 121 but a few residues toward the COOH terminus and that processing by a carboxypeptidase removes additional amino acids to produce the large NH_2-terminal fragment. Indeed, there are many basic amino acids between residues 120 and 132, indicating several potential cleavage sites. Although it is possible that initial cleavage occurs as distal as residues 132–134, that seems unlikely since the 125–135 synthetic peptide had no effect on proteolysis. A third possibility is that more than one IGFBP-4 protease in B104 cell media may produce the fragments. Because the major fragments observed by ESMS are not contiguous, proteolysis must occur at least at two points in the molecule.

The exact relationship between the protease found in the media of B104 cells and that described in other systems is not yet clear. The enhancing of proteolytic cleavage by IGF-1 is similar to that described for human and sheep fibroblasts (10, 11), human decidua (16), vascular smooth muscle cells (25), and

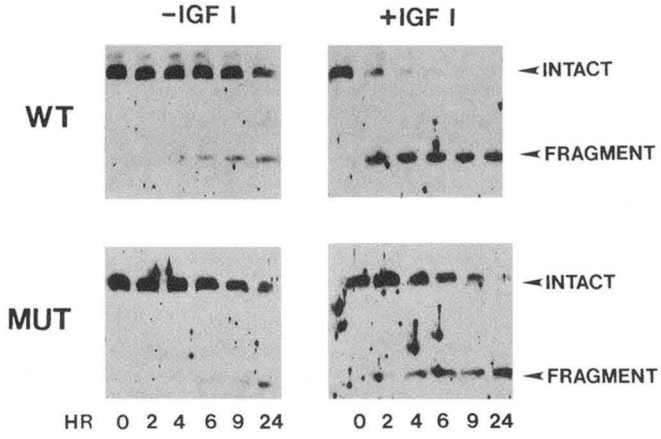


FIG. 5. **Immunoblot of native (*WT*) and mutant (*MUT*) IGFBP-4 following exposure to protease.** Pure peptides were incubated with protease containing media in the presence or absence of IGF-I as described under "Experimental Procedures" and analyzed on SDS-polyacrylamide gel electrophoresis followed by immunoblotting and chemiluminescent detection. In this experiment 100 ng of IGFBP-4 were incubated with 50 μl of media in a total reaction volume of 60 μl. *Bands* at the *top* of each gel represent intact IGFBP-4 while those near the *bottom* are the 16-kDa fragment. Time of exposure to the protease is indicated at the *bottom*.

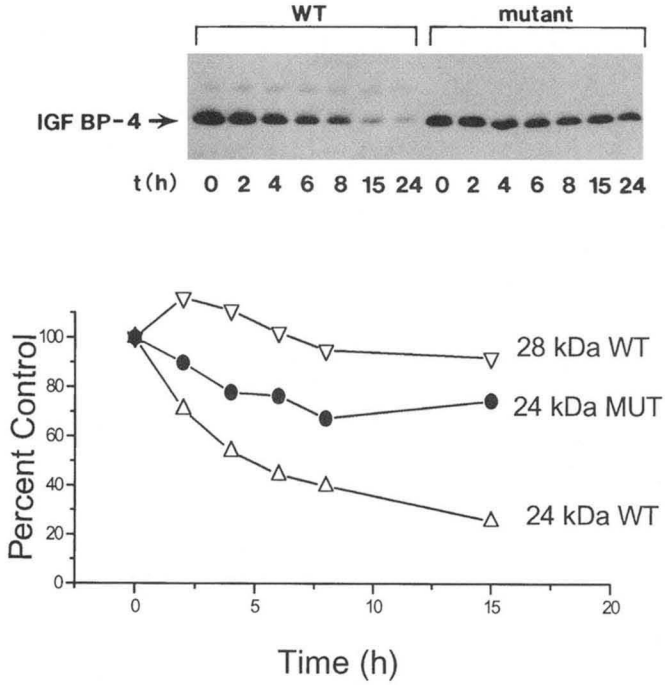


FIG. 6. *Top*, comparison of proteolytic degradation of native (*WT*) and mutant IGFBP-4 (*MUT*) assessed by ligand blot. Pure peptides were exposed to the protease-containing media and IGF-I and sampled at the times indicated. In this experiment 100 ng of IGFBP-4 were incubated with 50 μl of media on a total reaction volume of 60 μl. *Bottom*, PhosphorImage analysis of the ligand blot data. The experiment shown in the *top panel* was analyzed using the PhosphorImaging system with the numeric data plotted as percent of control (value at $t = 0$). The data for the *N*-glycosylated form of IGFBP-4 was derived by analysis of the less intense 28-kDa band observed just above the wild type IGFBP-4 band.

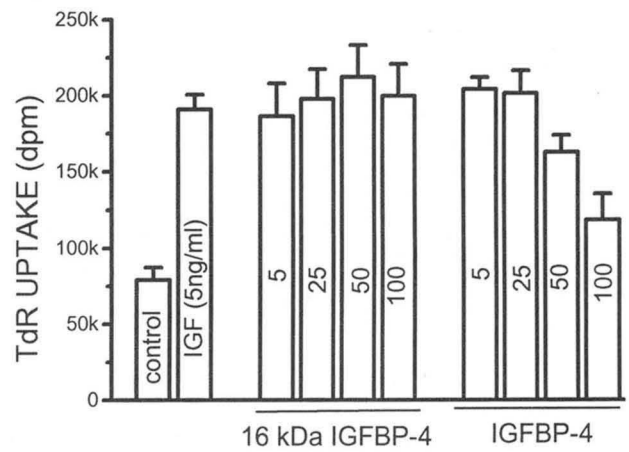


FIG. 7. **The effect of cleaved (16 kDa) IGFBP-4 and native IGFBP-4 on IGF-1-stimulated thymidine (*TdR UPTAKE*).** *Leftward bars* indicate thymidine uptake in the absence of added IGFBP-4 with and without (*control*) added IGF-1. Experiments with IGFBP-4 (as indicated) are in presence of 5 ng/ml IGF-1. *Numerals* within *bars* indicate concentration of added IGFBP-4 (ng/ml).

osteoblasts (18, 19). In our original description of the B104 cell protease, the addition of IGF-I was not required to demonstrate proteolysis (21), but we believe that this is because the B104 cells secrete small amounts of IGFs.[2] The response to various protease inhibitors has been similar, but not identical, among the proteases even though proteolytic fragments produced by each appear to be of similar sizes. The protease studied here may be the rat homologue of the IGFBP-4 protease found in other species, but there may be multiple IGFBP-4 proteases as well.

In a prior publication (21) we found that proteolytically cleaved IGFBP-4 could bind IGFs with an affinity that was reduced approximately 15-fold. Since the affinity of IGFBP-4 for IGF-I is approximately 1 log order higher than the affinity of the receptor for IGF-1 (27, 28), such a change might be predicted to abrogate the inhibitory properties of IGFBP-4, as was found in our experiments. This is in accord with prior reports showing that, in other systems, the growth inhibitory properties of IGFBP-4 are lost following exposure to the protease (10, 20).

Although the proteolytic processing of IGFBP-4 is likely to be an important mechanism regulating IGFBP-4 and IGF action, many questions remain. It is not known whether the 16-kDa IGFBP-4·IGF complex retains any activity and whether the complex can be bound by the IGF-I receptor as a functional unit. Whether any of the IGFBP-4 cleavage products have functions independent of the IGFs is also unknown. Nevertheless, a plausible scenario for the physiologic role of the IGFBP-4 protease might be as follows. The IGFBP-4 protease is induced or activated when IGF action is needed. The protease is specific for IGFBP-4 and preferentially attacks IGFBP-4 that is complexed with an IGF. Cleavage of IGFBP-4 releases sequestered IGFs and allows them to act on neighboring responsive cells, thereby providing a mechanism for directing the paracrine/autocrine actions of the IGFs.

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Note Added in Proof—Subsequent to the submission of this work Conover *et al.* (Conover, C. A., Durham, S. K., Zapf, J., Masiarz, F. R. & Kiefer, M. C. (1995) *J. Biol. Chem.* **270,** 4395–4400) described the cleavage of human IGFBP-4 by an IGFBP-4 protease. A fragment of IGFBP-4 was isolated that appeared to be homologous to the COOH-terminal fragment we identified. Mutations in the vicinity of the site equivalent to residues 132–134 of rat IGFBP-4 were associated with some reduction in protease sensitivity, suggesting that this region of IGFBP-4 might also be involved in the cleavage process.

[2] S. D. Chernausek, unpublished data.

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