Insulin-like Growth Factor (IGF)-binding Protein-3 (IGFBP-3) Functions as an IGF-reversible Inhibitor of IGFBP-4 Proteolysis*

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John L. Fowlkes, Delilia M. Serra, Carlyn K. Rosenberg, and Kathryn M. Thrailkill‡

From the Department of Pediatrics, Duke University Medical Center, Durham, North Carolina 27710

Previous studies have shown that insulin-like growth factor (IGF)-binding protein-4 (IGFBP-4) is degraded only in the presence of exogenous IGFs; however, we found that cation-dependent proteinase activity present in conditioned medium of MC3T3-E1 osteoblasts degrades 125I-recombinant human (rh)IGFBP-4 in the absence of IGFs. Addition of IGF-I, IGF-II, or insulin to conditioned medium had little affect on 125I-rhIGFBP-4 proteolysis, while extraction of IGFs resulted in only a ~10% reduction in proteinase activity. Since factors other than IGFs appeared to be involved in regulating IGFBP-4 proteolysis, we hypothesized that IGFBP-3, an IGFBP produced by many cell lines, but not MC3T3-E1 cells, might function as an inhibitor of IGFBP-4 proteolysis. Addition of rhIGFBP-3 to conditioned media inhibited 125I-rhIGFBP-4 proteolysis by 90% while IGF-I and IGF-II reversed the inhibitory effects of rhIGFBP-3 in a dose-dependent manner. 125I-rhIGFBP-4 proteolysis was not inhibited by N-terminal rhIGFBP-3 fragments that bind IGFs, but was inhibited by two synthetic peptides corresponding to sequences contained in the mid-region or C-terminal region of IGFBP-3. Both inhibitory peptides contain highly basic, putative heparin-binding domains and heparin partially reversed the inhibitory effects of rhIGFBP-3 on 125I-rhIGFBP-4 proteolysis. These data demonstrate that rhIGFBP-3 inhibits IGFBP-4-degrading proteinase activity and binding of IGFs or glycosaminoglycans to IGFBP-3 may induce conformational changes in the binding protein, causing disinhibition of the proteinase.

Insulin-like growth factor (IGF)1-binding proteins (IGFBPs) are a group of six homologous, yet distinct, proteins (IGFBPs 1–6) which bind both IGF-I and IGF-II with high affinity (for recent reviews, see Refs. 1–4). Among these IGFBPs, IGFBP-4 has been shown to function as an inhibitor of IGF bioactivity (1–4). For instance, IGFBP-4 was the only inhibitory IGFBP isolated from conditioned media from a colon adenocarcinoma cell line, although other IGFBPs were present in the conditioned medium (5). Furthermore, Malpe et al. (6) have demonstrated that treatment of human bone cells with antisense oligonucleotides directed against IGFBP-4 mRNA results in decreased production of IGFBP-4 and a striking increase in cellular proliferation, suggesting that IGFBP-4 plays a major role in regulating cellular proliferation. Since IGFBP-4 has been purified from a number of sources (1–4) and since mRNA for IGFBP-4 has been detected in all tissues studied by Shimasaki et al. (7), it is likely that IGFBP-4 may serve to restrain IGFBP-4 activity in many tissues.

Although IGFBP-4 functions as a potent inhibitor of IGF action, the factors controlling production, secretion, and turnover of IGFBP-4 have only recently been addressed. IGFBP-4 has been shown to decrease concentrations of IGFBP-4 in the conditioned media of human fibroblasts (8–10), human breast cancer cell lines (11), and human decidual cells (12, 13). However, IGF-I or IGF-II have little or no effect on IGFBP-4 mRNA in several cell lines (10, 14) and the effect of IGF-I on IGFBP-4 levels in conditioned media of human fibroblasts (8, 9) and human decidual cells (15) is not blocked by a monoclonal antibody to the type 1 IGF receptor. These observations suggest that IGFBP-4 might decrease IGFBP-4 concentrations via post-translational mechanisms.

Consistent with this hypothesis, recent studies from our laboratory have demonstrated that in human and sheep fibroblasts the addition of IGFs induces IGFBP-4 proteolysis by a proteinase(s) not yet identified (16). Since this original report, IGF-dependent IGFBP-4 proteolysis has been confirmed in human fibroblasts (17), and similar IGF-dependent IGFBP-4 proteolysis activity has been reported in human decidual cell cultures (15), vascular smooth muscle cells (18), and human bone cells (19). The action of the IGF-dependent IGFBP-4 proteinase(s) provides a novel mechanism through which IGFBPs can increase their own bioavailability and bioactivity.

In the current study, we use the murine MC3T3-E1 osteoblast cell line to explore the role of IGFBP-3 in the regulation and induction of IGFBP-4 proteolysis. Herein, we demonstrate that IGFBP-3 can function in a unique role as an inhibitor of IGFBP-4 proteolysis; however, its inhibitory effects can be reversed by the presence of IGFs.

EXPERIMENTAL PROCEDURES

Materials—Recombinant human (rh)IGFBP-3 produced in Escherichia coli (rhIGFBP-3) was kindly provided by Dr. Christopher Maack, Celtrix Pharmaceuticals, Santa Clara, CA (20). rhIGFBP-4 was purchased from Austral Biologicals, San Ramon, CA. rhIGF-I was kindly provided by Genentech Inc., South San Francisco, CA, and rhIGF-II was generously supplied by Lilly Research Laboratories, Indianapolis, IN. Recombinant human insulin was from Novo Nordisk Pharmacueticals Inc., Princeton, NJ. Polyclonal antisera to IGF-I was a gift from Dr. Lewis Underwood, University of North Carolina, Chapel Hill, NC, and a monoclonal antibody to rat IGF-II was purchased from Amano Pharmaceutical Co., Ltd., Nagoya, Japan. Reagents used for SDS-polyacrylamide gel electrophoresis (SDS-PAGE) were purchased from Bio-Rad. Low molecular mass heparin (6 KDA) from porcine intestinal mucosa and all proteinase inhibitors were purchased from Sigma, with the
exception of the proteinase inhibitors 3,4-dichlorosuccinamin (3,4-DCl) and l-trans-epoxysuccinyl-leucylamide-(4-guanidino)butane (E-64), which were purchased from Boehringer Mannheim. Na$_{131}$I and Hypafilm-ECL were obtained from Amersham Corp. Tissue culture plastic-ware was obtained from Corning Glass Works, Corning, NY. Growth media and cell culture reagents were obtained from Life Technologies, Inc.

MC3T3-E1 Cell Culture and Conditioned Media—Stock cultures of MC3T3-E1 osteoblasts were maintained in minimal essential medium containing 10% (v/v) fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 units/ml), as described previously (21). Stock cultures were subcultured every 3 days.

For the preosteoblastic differentiation, cells were plated at an initial density of 5 x 10$^4$ cells/well in 35-mm diameter multiwell plastic culture dishes; cells were then grown in a-minimal essential medium/10% fetal bovine serum supplemented with 10 mM β-glycerophosphate and 25 μg/ml ascorbic acid. Differentiating cultures were refed every 3 days throughout a 30-day culture period. Under these conditions, these cells display an orderly developmental pattern in culture; replication of preosteoblasts (days 1-10) is followed by growth arrest and the sequential expression of mature osteoblastic characteristics, including increased alkaline phosphatase activity (>day 10), matrix accumulation (days 14-21), osteocalcin expression (>day 21), and eventual mineralization (>day 25) (22). Every 2–3 days during the 30-day culture period, selected plates were washed and incubated for 48 h in serum-free medium, followed by the replacement of Eagle's minimal essential medium (MEM-10) + 0.1% bovine serum albumin. Cell-free conditioned media collected during these 48-h incubations were used in the subsequent experiments.

Preparation of IGF-extracted MC3T3-E1 Conditioned Media—Endogenous IGF-I and IGF-II present in MC3T3-E1 conditioned media were removed by immunoadsorption using anti-IGF-I and anti-IGF-II protein G-Sepharose. To prepare the immunoadfinity matrix, 38 μl of anti-IGF-I polyclonal antisera and 600 ng of an anti-IGF-II monoclonal antibody were incubated with 150 μl of protein G-Sepharose overnight at room temperature. The matrix was washed extensively with 20 mM Hepes, 1 mM NaCl, pH 7.4, to remove excess serum. Immunoadsorption was performed by incubating 400-μl samples of MC3T3-E1 conditioned media with 200 μl of the immunoadfinity matrix for 1–2 h at 4°C. The matrix was washed five times with 50 mM Tris-HCl, pH 7.4, and the supernatant was removed and used for 125I-rhIGFBP-4 degradation by MC3T3-E1 conditioned media as described above.

Degradation of 125I-rhIGFBP-4 by MC3T3-E1 CM—rhIGFBP-4 proteinase assays using cell-free conditioned media were performed as described previously, with minor modifications (16). rhIGFBP-4 was labeled with Na$_{131}$I using the chloramine-T method to a specific activity of ~50 μCi/μg of protein. To detect IGF-1 protease activity present in conditioned media, samples of cell-free conditioned medium (50 μl unless indicated otherwise) were incubated with 125I-rhIGFBP-4 (25,000 cpm; ~1 ng of rhIGFBP-4) at 37°C for 0–72 h. Protolytic degradation of 125I-rhIGFBP-4 was terminated by the addition of an equal volume of 2 X non-reducing sample buffer (23), followed by heating at 100°C for 3 min. Samples and prestained molecular weight markers were then electrophoresed through 15% SDS-polyacrylamide gels, dried under vacuum, and exposed to x-ray film to visualize intact and degraded 125I-rhIGFBP-4 fragments. In certain experiments, IGFBP-4 proteinase activity was characterized further in the presence of metal-dependent proteinase inhibitors: EDTA (10 mM) and 1,10-phenanthroline (1 mM); serine proteinase inhibitors: aprotinin (2.5 μg/ml), phenylmethanesulfonyl fluoride (10 μM), and 3,4-DCI (100 μM); or the cysteine proteinase inhibitor: E-64 (10 μM). In addition, various concentrations of rhIGF-1, rhIGF-II, intact rhIGFBP-4, and rhIGFBP-3 fragments produced by matrix metalloproteinase-3 (MMP-3) (as described below), N-terminal IGF-I, and IGF-II (monomeric fragments) that bind IGFs, synthetic peptides corresponding to sequences present in hIGFBP-3, and/or heparin were preincubated with MC3T3-E1 conditioned media for 3 h at 37°C prior to in vitro 125I-rhIGFBP-4 protease assay.

Preparation of IGF-1 Fragment Products—Preparation of 125I-rhIGFBP-4 was digested by 200 ng of MMP-3 (kindly provided by Dr. Hideaki Nagase, University of Kansas Medical Center, Kansas City, KS) in a total volume of 60 μl of 50 mM Tris, pH 7.5, 0.15 mM NaCl, 10 mM CaCl$_2$, 0.02% NaN$_3$, 0.05% Brij 35 (TNC buffer) for 8 h at 37°C (24). The digestion was stopped by the addition of EDTA (final concentration: 10 μM), and the digestion products were analyzed on 15% SDS gels under reducing conditions. Seven rhIGFBP-3 fragments, designated “a-f,” were characterized (see below). Treatment of the digest with ion-exchange chromatography (see Fig. 5). The remaining 125I-rhIGFBP-4 fragments produced by MMP-3 digestion, the digestion mixture was incubated with 1 ml of heparin-Sepharose (Sigma) overnight at 4°C. The matrix was washed with 50 μl Tris-HCl, pH 7.4, and heparin-bound fragments were eluted in 50 mM Tris-HCl, pH 7.4, containing 1 mM NaCl. All fractions were analyzed by SDS-PAGE and stained with Coomassie Blue. The wash fractions (i.e. 50 mM Tris-HCl, pH 7.4) contained only the smallest IGFBP-3 fragments (fragments e and f) produced by MMP-3, which correspond to the first 100–110 N-terminal amino acids of IGFBP-3. Fragments a-d bound to the heparin-Sepharose and, all four fragments were eluted in 50 mM Tris-HCl, pH 7.4, containing 1 mM NaCl. The inability of fragments a and f to bind heparin-Sepharose was anticipated, since their sequences do not contain either of the two putative heparin-binding sites present in IGFBP-3 (2, 25). In covalent cross-linking studies, fragments e and f bound specifically 125I-rhIGFBP-1 and 125I-rhIGF-II with IC$_{50}$ values of ~89.9 and ~3.03 milliunits/liter, respectively. The IC$_{50}$ values are consistent with a previous report demonstrating that an 88-amino acid N-terminal mutant of IGFBP-3 bound 125I-rhIGF-I (20). Fragments a-f together or fragments e and f together were tested for their abilities to alter 125I-rhIGFBP-4 degradation by MC3T3-E1 conditioned media as described above.

Preparation of Synthetic hIGFBP-3 Peptides—Peptides based on amino acid sequences contained in both the non-homologous mid-region of hIGFBP-3 (peptides I, II, and III) and in the highly conserved C terminus (peptide IV) (see Fig. 5) were produced by solid phase peptide synthesis using 9-fluorenylmethoxycarbonyl chemistry. The sequences are as follows: SRLRAYLPPAPAS (peptide I), KKAKDSSQRYKVEP (peptide II), DDTQDSSEKRETLEY (peptide III), and DKQGFYKKLPSK (peptide IV). All peptides were purified on a Vydac C-8 HPLC column using a Gilson automated HPLC system, and were shown to be >98% pure. Sequence verification was performed by electrospray mass spectrometry. All peptides were synthesized with an additional N-terminal cysteine for use in thiol-coupling reactions. The internal cysteine in peptide IV was acetylated because it is normally involved in a disulfide bond. All synthetic peptides were tested for their abilities to alter 125I-rhIGFBP-4 degradation by MC3T3-E1 conditioned media as described above.

Characterization of hIGFBP-4 Proteases in MC3T3-E1 Cultures—To determine if MC3T3-E1 osteoblasts produce IGFBP-4-degrading proteinases, conditioned media from different time points during differentiation were examined for their abilities to degrade 125I-rhIGFBP-4. When incubated with conditioned media from MC3T3-E1 osteoblasts, intact 125I-rhIGFBP-4 (28 kDa) was degraded into ~20- and ~14-KDa fragments (Fig. 1). By 24 h, >50% of the binding protein was degraded (p < 0.0001, n = 4). IGFBP-4 protease activity present in MC3T3-E1 conditioned media increased progressively as osteoblasts matured in culture; maximal proteinase activity was detected in conditioned media from osteoblasts displaying a differentiated phenotype. Therefore, all subsequent studies were performed with conditioned media from ~20 day cultures. To characterize further the IGFBP-4-degrading proteinase(s) present in MC3T3-E1 conditioned medium, samples of cell-free conditioned media were analyzed for their ability to degrade 125I-rhIGFBP-4 in the presence or absence of various protease inhibitors. As shown in Table I, the cation-dependent proteinase inhibitors (EDTA and 1,10-phenanthroline) significantly inhibited IGFBP-4-degrading proteinase activity. Serine (phenylmethanesulfonylfluoride, aprotinin, and 3,4-DCI) and
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TABLE I

| Proteinase inhibitor | Concentration | Inhibition (± S.E.) |
|----------------------|---------------|--------------------|
| Aprotinin             | 2.5 µg/ml     | 4.9 ± 3.1          |
| 3,4-DCI              | 100 µM        | 20.3 ± 10.4        |
| E-64                  | 10 µM         | 4.2 ± 2.3          |
| 1,10-Phenanthroline   | 1 mM          | 91.2 ± 7.4         |
| EDTA                  | 10 mM         | 93.2 ± 10.6        |
| PMFS                  | 10 mM         | 13.9 ± 2.1         |

*p < 0.001.  

**TABLE II**

| Treatment | Concentration | Proteolysis (% ± S.E.) |
|-----------|---------------|------------------------|
| Control   | 42.5 ± 2.0    |                        |
| IGF-I     | 10            | 45.3 ± 4.0             |
| IGF-II    | 10            | 50.7 ± 5.7             |
| Insulin   | 10            | 62.0 ± 4.3             |
|           | 500           | 43.1 ± 3.0             |
|           | 500           | 41.0 ± 2.4             |

*p = 0.01.

To identify the epitope(s) in the last ~150 amino acids of rhIGFBP-3 that were important for rhIGFBP-3 inhibitory effects on IGFBP-4 proteolysis in MC3T3-E1 osteoblasts, we performed studies using all cell lines, we and others have demonstrated that the addition of IGFBP-4 to conditioned media is essential for the induction of IGFBP-4 protease activity (reviewed in Ref. 1–4); in contrast, our current studies failed to demonstrate an absolute requirement for IGFBP-4.

Cysteine (E-64) proteinase inhibitors had little or no effect on degradation of 125I-rhIGFBP-4. These data suggested that the IGFBP-4-degrading enzyme in MC3T3-E1 conditioned media is a cation-dependent proteinase.

Effect of IGFBP-3 on 125I-rhIGFBP-4 Proteolysis—We examined the inhibitory effects of IGF-I, IGF-II, or insulin (26), while other cell lines exhibiting IGF-induced proteolysis do produce IGFBP-3 (15–19), we speculated that IGFBP-3 might function as an inhibitor of IGFBP-4 proteolysis. To determine whether exogenous IGFBP-3 inhibits the degradation of 125I-rhIGFBP-4 by MC3T3-E1 conditioned media, we found that the inhibitory domain(s) in IGFBP-3 resides in the non-homologous, mid-region of the molecule and/or in the C-terminal domain (Fig. 4) and that degradation of rhIGFBP-3 failed to destroy the inhibitory effects of rhIGFBP-3 on 125I-rhIGFBP-4 proteolysis.
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IGFBP-3 Peptides on 125I-rhIGFBP-4 Proteolysis—Effects of IGFBP-3 synthetic peptides on 125I-rhIGFBP-4 proteolysis by MC3T3-E1 cell-free conditioned media.

| Peptide no. | Inhibition (± S.E.) |
|-------------|---------------------|
| I           | 9.7 ± 1.7           |
| II          | 64.9 ± 4.8a         |
| III         | 0.00 ± 3.4          |
| IV          | 97.9 ± 6.1b         |

a p < 0.005.
b p < 0.0001.

In contrast, peptides I and III, used at the same concentrations, had no discernible inhibitory activities. Fig. 5 demonstrates that both peptides II and IV inhibited 125I-rhIGFBP-4 degradation in a dose-dependent manner (Fig. 5, panels A and B, respectively), and each produced a displacement curve parallel to the other peptide (Fig. 5). However, peptide IV (IC50 = 25 μM) was approximately 3-fold more potent than peptide II (IC50 = 74 μM) in inhibiting IGFBP-4-degrading protease activity. When used together, the peptides demonstrated no additive effect on inhibiting 125I-rhIGFBP-4 proteolysis (Fig. 5, panel C).

Effects of IGFs on the Inhibitory Activity of IGFBP-3 and IGFBP-3 Peptides on 125I-rhIGFBP-4 Proteolysis—Although exogenous IGFs had little or no effect on 125I-rhIGFBP-4 degradation, IGFs effectively reversed the inhibitory effects of rhIGFBP-3 (Fig. 6). When increasing concentrations IGF-I (Fig. 6, panel A) or IGF-II (Fig. 6, panel B) were added to MC3T3-E1 conditioned media containing 125I-rhIGFBP-4 and a maximal inhibitory dose of rhIGFBP-3 (2 μg/ml), both ligands effectively reversed the inhibitory effect of rhIGFBP-3 on 125I-rhIGFBP-4 proteolysis (Fig. 6). A 1:1 IGF-I:rhIGFBP-3 molar ratio produced a 50% reversal of IGFBP-3's inhibitory effect, while molar ratios of greater than 3:1 produced almost 100% reversal. IGF-I (IGF-I:IGFBP-3 IC50 = 29.1) was approximately 3 times less potent than IGF-II in reversing the inhibitory effects of rhIGFBP-3 on 125I-rhIGFBP-4 proteolysis (Fig. 6).

Because IGFs reversed the inhibitory effects of rhIGFBP-3 on 125I-rhIGFBP-4 proteolysis, IGF-I was examined for its ability to reverse the inhibitory effects of peptides II and IV on 125I-rhIGFBP-4 proteolysis by MC3T3-E1 conditioned media. As shown in Fig. 7, IGF-I had little or no effect on reversing the inhibitory effects of peptides II and IV on 125I-rhIGFBP-4 degradation. In covalent cross-linking studies, it was demonstrated that while 125I-IGF-I and 125I-IGF-II both bound to intact rhIGFBP-3 or IGFBP-3 fragments e and f, neither radioligand bound to peptides II and IV (data not shown). These data suggest that IGFBP-3 inhibits IGFBP-4 proteolysis via epitopes present within the amino acid sequences contained in peptides II and IV and that IGFs must be able to bind to IGFBP-3 in order to reverse the inhibitory effects of IGF on IGFBP-4 proteolysis.

Effect of Heparin on rhIGFBP-3 Inhibition of 125I-rhIGFBP-4 Proteolysis—As indicated in Fig. 5, peptides II and IV both contain putative heparin-binding domains (2, 25). Peptide II contains the sequence 149KKGHA153 which resembles a short heparin-binding domain (BBBXX; B = basic amino acid, and X = non-basic amino acid) and peptide IV contains the sequence 219YKKQCR226, which resembles a long heparin-binding motif (XBBBXXBXB) (25). This suggested that both of these highly basic, putative heparin-binding domains present in IGFBP-3 could inhibit IGFBP-4-degrading activity in MC3T3-E1.
conditioned media; therefore, we next examined the effect of heparin on modulating the inhibitory effect of rhIGFBP-3 on 

\[ {^{125I}}\text{rhIGFBP-4 proteolysis} \] on 

\[ {^{125I}}\text{rhIGFBP-4 proteolysis} \text{ by IGFBP-3 synthetic peptides.} \]

\[ {^{125I}}\text{rhIGFBP-4 was incubated with unconditioned media (lane 1) or MC3T3-E1 conditioned media (lanes 2-7) in the absence (lane 2) or presence (lanes 3-7) of increasing concentrations of peptide II (panel A and ), peptide IV (panel B and ), or both peptides II and IV (panel C and ) as described under "Experimental Procedures." The final concentration for each peptide is as follows: lane 3, 10 \( \mu \text{M} \); lane 4, 20 \( \mu \text{M} \); lane 5, 50 \( \mu \text{M} \); lane 6, 100 \( \mu \text{M} \); lane 7, 200 \( \mu \text{M} \). The percentage of proteolysis was calculated from densitometric data from two to four separate experiments as described under "Experimental Procedures."} \]

\[ {^{125I}}\text{rhIGFBP-4 proteolysis} \text{ by IGFBP-3 synthetic peptides.} \]

\[ {^{125I}}\text{rhIGFBP-4 was incubated with MC3T3-E1 conditioned media in the absence (lane 1) or presence (lanes 2-9) of various IGFBP-3 synthetic peptides (200 \( \mu \text{M} \)): peptide I, lanes 2 and 3; peptide II, lanes 4 and 5; peptide III, lanes 6 and 7; peptide IV, lanes 8 and 9. IGF-I (20 \( \mu \text{g/ml} \)) was added to lanes 3, 5, 7, and 9. Intact \[ {^{125I}}\text{rhIGFBP-4} \] and proteolytic fragments of \[ {^{125I}}\text{rhIGFBP-4} \] were separated by SDS-PAGE and detected by autoradiography. Molecular size markers are indicated on the left.} \]

\[ \text{DISCUSSION} \]

IGFBP-4 was first isolated from conditioned media from an osteosarcoma cell line and has now been identified in a variety of biological fluids and cellular conditioned media, where it...
functions as a potent inhibitor of IGF action (for recent reviews, see Refs. 1–4). Previously, we demonstrated that IGFs can induce the proteolytic degradation of IGFBP-4 into fragments that display little or no affinity for IGFs, providing a mechanism by which IGFs can directly increase their own bioavailability and/or bioactivity (16). Subsequently, Conover et al. (17) demonstrated that the degradation of IGFBP-4 increased IGF activity in human fibroblast cultures. How IGFs induce IGFBP-4 proteolysis remains unclear; we (16) and others (15) have hypothesized that IGFs induce IGFBP-4 degradation by binding to IGFBP-4, thus making it more susceptible to proteolysis, while others have proposed that IGFs directly activate an IGFBP-4 proteinase (17). Insights from the current studies would suggest that neither of these hypotheses is entirely correct, and that a more complex mechanism is involved in IGF-induced IGFBP-4 degradation.

Since other studies have now demonstrated IGF-inducible IGFBP-4-degrading proteinase activity in a number of cell lines (1–4), including bone cells (19), we chose to explore IGFBP-4 proteolysis in a murine bone cell line, MC3T3-E1 osteoblasts. Initial studies demonstrated that the IGFBP-4-degrading proteinase(s) present in MC3T3-E1 conditioned media cleaved the protein into two major fragments and that the proteinase was cation-dependent, making it similar to IGFBP-4-degrading proteinase activity reported in other cell lines (15–17). Nevertheless, one major difference existed between the IGFBP-4-degrading proteinase activity observed in MC3T3-E1 conditioned media compared with that produced by other cell lines; no exogenous IGFs were required to induce IGFBP-4 proteolysis (1–4, 15–19). In fact, addition of IGF-I to MC3T3-E1 conditioned media had no effect on IGFBP-4 degradation, while a maximal dose of IGF-II (500 ng/ml) only modestly increased the degradation of the binding protein. This is in marked contrast to our previous report using human and sheep fibroblast condition media, where we demonstrated that little or no proteolysis of IGFBP-4 occurred during a prolonged incubation (i.e. 72 h), yet with the addition of IGF-I or IGF-II, almost complete proteolysis of IGFBP-4 was achieved (16). Since MC3T3-E1 osteoblasts secrete both IGF-I and IGF-II (26, 27), it was possible that endogenous IGFs induced IGFBP-4 proteolysis in this cell line. In this regard, Durham et al. (28) have very recently demonstrated that in certain primary human bone cell lines, IGFBP-4 can be degraded without the addition of IGFs, while in other bone cell lines the addition of IGFs is necessary for IGFBP-4 proteolysis. They suggest that cell lines not requiring exogenous IGFs for IGFBP-4 proteolysis produced more IGFs than those cell lines requiring exogenous IGFs for IGFBP-4 proteolysis. Since immunoblotting of IGFs from conditioned media or the addition of IGF-binding, N-terminal fragments of IGFBP-3 to conditioned media had little or no effect on inhibiting IGFBP-4 degradation, endogenous IGFs do not appear to contribute significantly to the constitutive degradation of IGFBP-4 observed in MC3T3-E1 cells. Furthermore, since many cell lines that display IGF-induced IGFBP-4 proteolysis also produce some IGF-I and/or IGF-II, it seemed unlikely that the presence of IGFs in MC3T3-E1 conditioned media could account for the constitutive nature of the IGFBP-4-degrading proteinase activity.

We have demonstrated that MC3T3-E1 cells secrete immunoreactive IGF-I, -2, -4, and -5 (26), while IGFBP-3 is not produced by these cells (26) and the cells produce no IGFBP-3 mRNA (29). Based on the observation that MC3T3-E1 conditioned media contains little or no IGFBP-3, while other cell lines displaying IGF-dependent IGFBP-4 proteolysis do produce IGFBP-3 (15–19), we postulated that IGFBP-3 might function as an inhibitor of IGFBP-4 proteolysis. Indeed, the addition of rhIGFBP-3 to conditioned media significantly inhibited the constitutive degradation of IGFBP-4 by conditioned media from MC3T3-E1 cells in a dose-dependent manner. This demonstrated that exogenous IGFBP-3 is an IGFBP-4 proteinase inhibitor and also suggests that endogenous IGFBP-3 could function as a physiological inhibitor of IGFBP-4 proteolysis since it is present in a variety of biological fluids at concentrations 5–20 times greater (30) than the ICFs needed to inhibit IGFBP-4 degradation. IGFBP-3 may also be induced in certain cell systems and function as an IGFBP-4 proteinase inhibitor. For instance, Conover and colleagues (31) have demonstrated that treatment of human fibroblasts with phorbol esters induces an inhibitor(s) of IGFBP-4 proteolysis, while Albiston et al. (32) have shown that IGFBP-3 promoter activity is increased when transfected cells are treated with a phorbol ester. Therefore, it is possible that the phorbol ester-induced inhibitor of IGFBP-4 proteolysis is IGFBP-3.

Similar to the effects of intact IGFBP-3 and inhibitory IGFBP-3 fragments, two peptides inhibited IGFBP-4 proteolysis, while two other peptides did not. The feature common to both inhibitory peptides was that each contained one of the two highly basic, putative heparin-binding domains present in IGFBP-3 (2, 25). Further analysis revealed that peptide IV, which contains a long heparin-binding motif, was 3–4 times more potent in inhibiting IGFBP-4 proteolysis than was peptide II, which contains a short heparin-binding site, and there was no demonstrable synergy when both peptides were used together. This suggests that both peptides work through a similar inhibitory mechanism, although the specifics of the mechanism are currently unknown and are under investigation. Preliminary data from our laboratory demonstrate that other proteins, such as fibronectin and vitronectin, which are commonly found in cell cultures and which contain both long and short forms of heparin-binding motifs, do not inhibit IGFBP-4 proteolysis by MC3T3-E1 conditioned media.3 However, IGFBP-5, which contains almost the same stretch of basic residues that is present in IGFBP-3 and peptide IV (1), also inhibits IGFBP-4 proteolysis,3 suggesting that these basic domains are specific in their ability to inhibit IGFBP-4 proteolysis. These highly basic regions may interact with IGFBP-4 itself, protecting it from proteolysis; however, this mechanism seems doubtful since heterodimerization of IGFBP3s has not been reported to date. These regions may function as competitive substrates for the IGFBP-4 proteinase; however, this seems unlikely since IGFBP-4 itself contains no heparin-binding motifs (2) and since a recent study demonstrated that the cleavage site in human IGFBP-4 produced by the IGF-depend-
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In contrast to recent reports by Gockerman and Clemmons (36) and Arai et al. (37) demonstrating that heparin inhibits IGFBP-2 proteinase activity produced by porcine aortic smooth muscle cells and IGFBP-5 proteinase activity produced by human fibroblasts, respectively, our data suggest that heparin has little or no effect on IGFBP-4 degradation by MC3T3-E1 conditioned media. Nevertheless, heparin significantly reversed the inhibitory effects of IGFBP-3 and peptide IV on 125I-rhIGFBP-4 degradation by MC3T3-E1 conditioned media. The divergent effects of heparin on degradation of these various IGFBPs may simply reflect the differences in the proteinases that are involved. However, it is also possible that heparin alters the inhibition of these proteinases in divergent ways. For example, heparin has been shown to enhance inhibition of thrombin and factor Xa by antithrombin III (38), but decrease the rate of inhibition of neutrophil elastase by α2-proteinase inhibitor (39), although both proteinase inhibitors are members of the serpin family. The mechanism by which heparin interferes with IGFBP-3's ability to inhibit IGFBP-4 proteolysis is unclear; however, association of heparin with heparin-binding sites present in IGFBP-3 may make them less available to inhibit the proteinase. Because heparin has been shown to interfere with binding of IGFs to IGFBP-5 and IGFBP-3 (25), an alternative hypothesis would be that heparin causes dissociation of IGFs from endogenous IGFBPs, making IGFs available for binding to exogenous rhIGFBP-3, thereby partially mitigating the inhibitory effect of IGFBP-3 on IGFBP-4 proteolysis. Regardless of the mechanism, heparin, and possibly other glycosaminoglycans, may have a role similar to the IGFs in inducing IGFBP-4 proteolysis.

IGFBP-3 has been shown to both enhance and inhibit IGF activity in vitro (reviewed in Refs. 1–4). Our data would suggest that this dichotomy may be explained partially by the finding that IGFBP-3 inhibits IGFBP-4 proteolysis, thus decreasing IGF activity. However, when bound to IGFs, and possibly glycosaminoglycans, IGFBP-3 loses its capability to inhibit IGFBP-4 proteolysis, thus enhancing IGF activity by facilitating the degradation of IGFBP-4. Fig. 9 presents a summary of these studies and a hypothetical scheme of how IGFBP-3 might function in vivo to regulate IGFBP-4 proteolysis.

In the absence of IGFBP-3 (and possibly other IGFBPs containing heparin-binding motifs), IGFBP-4 is degraded constitutively (Fig. 9, A) into non-IGF binding fragments. In the presence of IGFBP-3, IGFBP-4 degradation is markedly attenuated (Fig. 9, B). However, the inhibitory effects of IGFBP-3 can be reversed via binding of IGFs to IGFBP-3 (Fig. 9, C). Similarly, if IGFBP-3 is bound to glycosaminoglycans or cell surfaces, heparin-binding domains present in the molecule may be less available for interaction with the proteinase (Fig. 9, D). This scenario may be altered in the event that IGFBP-3 is degraded by proteinases such as MMPs, since these proteinases can cleave the N-terminal IGF-binding domain from the heparin-
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binding domains present in the mid-region and C-terminal tail of the molecule (24). In this instance, fragments containing the heparin-binding motif(s) could inhibit proteinase activity; yet since they bind little or no IGFs, their inhibitory effects may not be reversible by IGFs, making them IGF-resistant IGFBP-4 proteinase inhibitors (Fig. 9, E). Together these data suggest a new role for IGFBP-3 as an IGFBP-4 proteinase inhibitor and they exemplify the intricate complexities involved in the IGF/IGFBP/IGFBP-proteinase system.

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John L. Fowlkes, Delila M. Serra, Carlyn K. Rosenberg and Kathryn M. Thrailkill

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