Proteolysis of insulin-like growth factor binding proteins (IGFBPs) by calpain

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Abstract

Calpains are non-lysosomal, Ca^{2+}-dependent cysteine proteases, which are ubiquitously distributed across cell types and vertebrate species. The rules that govern calpain specificity have not yet been determined. To elucidate the cleavage pattern of calpains, we carried out calpain-induced proteolytic studies on the insulin-like growth factor binding proteins IGFBP-4 and -5. Proteolysis of IGFBPs is well characterized in numerous reports. Our results show that calpain cleavage sites are in the non-conserved unstructured regions of the IGFBPs. Compilation of the calpain-induced proteolytic cleavage sites in several proteins reported in the literature, together with our present study, has not revealed clear preferences for amino acid sequences. We therefore conclude that calpains seem not to recognize amino acid sequences, but instead cleave with low sequence specificity at unstructured or solvent-exposed fragments that connect folded, stable domains of target proteins.

Keywords: calpain; Ca^{2+}-dependent cysteine protease; calpastatin; insulin-like growth factor binding protein.

Introduction

Calpains are a large family of Ca^{2+}-dependent cysteine proteases ubiquitously expressed in mammalian cells. The two major mammalian calpains are designated μ-calpain and m-calpain based on their *in vitro* activation, which requires micromolar and millimolar concentrations of Ca^{2+}, respectively (Goll et al., 2003). The two enzymes appear to be very similar in many biochemical properties and their structures also show a similar folding pattern (Pal et al., 2003) but significant differences are observed in the catalytic triad of residues of these two isoforms (Strobl et al., 2000). Calpains are heterodimers composed of a large catalytic subunit of 80 kDa (containing domains I–IV) and a small 28-kDa regulatory subunit (composed of domains V and VI). The small subunit is identical in both isoforms, but the large subunits are generated by two different genes (Dear and

Boehm, 2001; Sorimachi and Suzuki, 2001; Goll et al., 2003). Activation of the protease core of μ-calpain requires cooperative binding of two Ca^{2+} atoms at two non-EF-hand sites (Khorchid and Ikura, 2002; Moldoveanu et al., 2002, 2004). Substantial evidence indicates that calpains play a crucial role in a diverse spectrum of cellular responses, which include cell differentiation, apoptosis, cytoskeletal remodeling, signal transduction and, most recently identified, cell adhesion and motility (Croall and Demartino, 1991; Sato and Kawashima, 2001; Glading et al., 2002; Goll et al., 2003). Although definitive physiological roles are not yet established, dysregulated Ca^{2+} levels result in aberrant proteolysis by calpains, which contributes to tissue damage in heart and brain ischemia, cerebral infarction, acute neurological disorders such as stroke and, more recently identified, to Alzheimer's disease (Huang and Wang, 2001). Calpains have been also identified as a susceptible gene in type 2 diabetes (Kuwaki et al., 1989). Calpains are generally believed to exist and function in the cytoplasm, but a few studies have reported the existence of m-calpain in the extracellular spaces of various tissues (Glading et al., 2001; Nishihara et al., 2001). Immunolocalization studies have shown extracellular localization of calpains in diseased states (Kumamoto et al., 1992).

The rules that govern calpain specificity have not yet been determined. In this paper we attempt to characterize calpain preferred cleavage positions in the insulin-like growth factor binding proteins (IGFBPs). We have chosen to use these proteins for our calpain study for two reasons. First, proteolysis of IGFBPs is extensively characterized by numerous reports; this is because limited proteolysis of IGFBPs is the major mechanism for the release of IGFs from IGFBP/IGF complexes, generating IGFBP fragments with reduced affinity for IGFs (Binoux et al., 1999; Khandwala et al., 2000). This thereby increases the concentration of IGFs at the cell surface and allows IGFs to bind and activate the IGF receptor (Bunn and Fowlkes, 2003). Secondly, IGFBPs are structurally well characterized; they are composed of globular domains linked by large flexible fragments, thus providing model proteins that contain diverse structural elements (Kalus et al., 1998).

IGFBPs are also active in the cellular environment, as they may regulate cells by IGF-independent mechanisms (Oh and Rosenfeld, 1999; Baxter, 2000; Firth and Baxter, 2002). The effects of intracellular IGFBPs are complex, however, with both stimulatory and inhibitory actions. In addition, the regulation of intracellular IGFBPs by proteases influences the IGFBP extracellular concentration. Proteolytically cleaved fragments of the same IGFBP protein may display different cellular localizations. Several studies have identified IGFBP-3 in cell nuclei or directly demonstrated its nuclear transport by an importin β-mediated mechanism (Jaques et al., 1997; Wraight et al.,

1998). Competition studies suggested that IGFBP-5 uses the same nuclear transport system as IGFBP-3 (Schedlich et al., 1998). We have studied the calpain-induced proteolysis of two members of the IGFBP family (IGFBP-4 and IGFBP-5) in this paper. We expect, however, that calpain proteolysis will be very similar for other IGFPBPs because of the amino acid sequence and structural similarities of these proteins.

Results

The sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of the *in vitro* assay clearly showed that there were calpain cleavage sites in both IGFBP-4 and IGFBP-5 and both proteins were rapidly cleaved into smaller fragments (Figure 1A, B). For 1000 nM IGFBPs, as little as 20 nM μ-calpain was sufficient to induce cleavage and a further increase in the protease concentration up to 250 nM did not result in any change in the cleavage pattern. In addition, there was no change observed with increasing time: cleavage patterns obtained within the first 30 min were the same as those obtained after 4 h. This was expected, as calpain is an autolytic enzyme (Tompa and Friedrich, 2000; Li et al., 2002) and autolyses within the first 30 min, which results in protease activation, but an increased time interval does not result in any extra cleavage sites, which shows that calpain-mediated proteolysis is limited. The IGFBP protein stability assays showed that the proteins were stable and did not degrade at room temperature for up to 48 h, as shown in Figure 2. Figure 3A shows the Western blot analysis of IGFBP cleavage products after digestion with 20 nM μ-calpain. Figure 3B shows the results of the inhibitory assay of the endogenous protein inhibitor of calpains, calpastatin (Goll et al., 2003). Calpastatin efficiently blocked the cleavage of both IGFBP-4 and IGFBP-5, as no cleavage fragments of the proteins were observed. Domain I of calpastatin was chosen for the experiment as it inhibits μ- or m-calpain to a greater extent compared to other domains (Goll et al., 2003). This result clearly indicates that the cleavage pattern observed was entirely due to calpain and not to any other contaminating protease. In addition, no cleavage was observed in the absence of calcium, in agreement with the fact that calcium is necessary to induce the autocatalytic activity of calpain.

Characterization of the cleavage sites in IGFBPs was accomplished by subjecting proteolytic products to NH$_2$-terminal sequencing by Edman degradation. The amino acid sequences obtained for IGFBP-4 were: GSSHH, A$_3$IHC, M$_{73}$HGQG, A$_{87}$IQESL, A$_{123}$KIR, and G$_{139}$APR. Exact molecular weights of the isolated fragments were determined by matrix-assisted laser desorption/ionization-mass spectrometry (MALDI-MS). Similarly, for IGFBP-5, the amino acid sequences obtained from the N-terminal sequencing were: S$_3$FVH, C$_8$EPCD, Q$_{142}$SKFV, and A$_{153}$HPRI. The results of the fragments generated from polyvinylidene fluoride (PVDF) analysis and reverse-phase HPLC were mapped onto the IGFBP-4 and IGFBP-5 sequences and all the theoretically possible cleavage fragments for both IGFBPs were simulated.
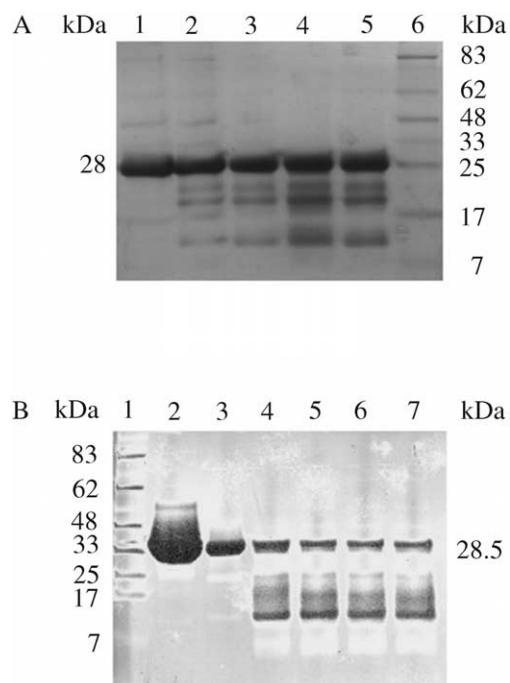

Figure 1 SDS-PAGE analysis of fragments generated by calpain cleavage.
(A) SDS-PAGE analysis of IGFBP-4 proteolysis by μ-calpain. Lane 1 contains aliquots of the undigested protein as the control. Lanes 2–5 contain aliquots of the digestion reaction of IGFBP-4 cleavage at different calpain concentrations (20, 50, 100 and 250 nM) in the calpain assay buffer. Lane 6 shows the prestained molecular mass protein marker (kDa).
(B) SDS-PAGE analysis of IGFBP-5 digested by μ-calpain. Lane 1 shows the prestained marker. Lane 2 contains the undigested IGFBP-5 protein. Lanes 3–7 show IGFBP-5 cleavage at different time intervals of 0 min, 30 min, 1 h, 2 h, and 4 h, respectively, at a constant calpain concentration of 20 nM in the calpain reaction buffer. IGFBP-4 is a 28-kDa protein and IGFBP-5 is 28.5 kDa in molecular mass.

Mass spectrometry showed that fragments of the following sizes were generated for IGFBP-4: 14.75, 13.10, 11.01, 10.90, 4.10, and 2.45 kDa (Figure 4A). For IGFBP-5, fragments of 15.69, 16.81, 12.70, and 11.60 kDa were obtained (Figure 4B). These mass spectrometry results, together with the results from N-terminal sequencing, identified cleavage sites at amino-acid positions 2, 86, 122, and 138 in IGFBP-4 (Figure 5). Hence, in IGFBP-4 calpain appears to act on the bond between Glu 2 and Ala 3, generating a fragment of approximately 2.45 kDa,
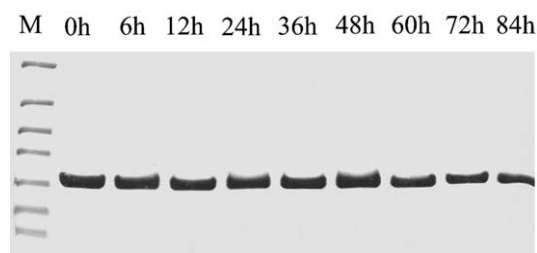

Figure 2 IGFBP stability gel showing the protein at different time intervals.
Lane M shows the prestained molecular mass protein marker (kDa) and the remaining lanes contain aliquots of the protein in the calpain assay buffer at different time intervals. The protein remains stable even after 3 days.
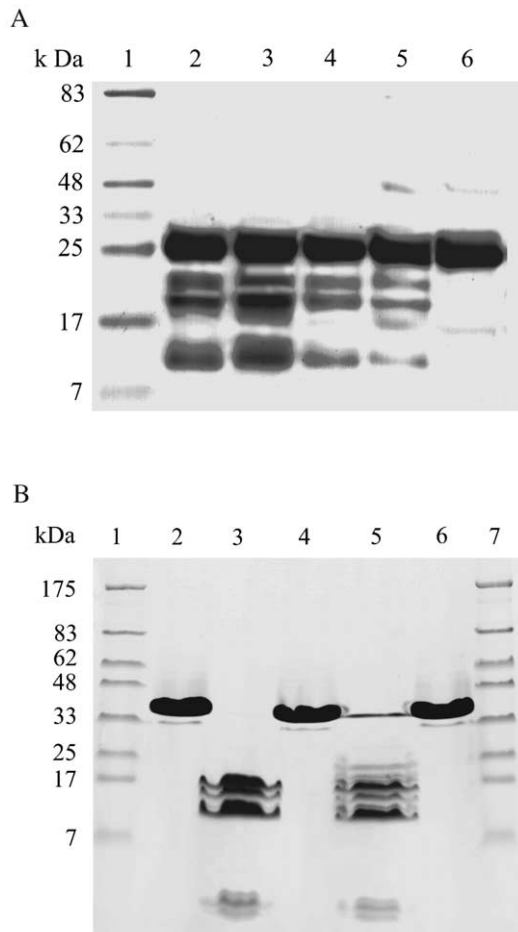
Figure 3 Western blot analysis and calpastatin inhibitory assay.
(A) Western blot of IGFBP fragments generated by μ-calpain protease cleavage. IGFBP digested with calpain was resolved by SDS-PAGE and transferred onto a nitrocellulose membrane. Cleaved fragments of IGFBP were detected by immunoassaying with IGFBP polyclonal antibodies. Lane 1 shows the prestained marker. Lanes 2–5 highlight the cleavage products of IGFBP. Lane 6 consists of the undigested IGFBP protein as control.
(B) Calpastatin inhibitory and Ca^{2+}-dependent protease assay. Lanes 1 and 7 show the molecular weight protein marker. Lane 2 shows IGFBP protein incubated with the protease, μ-calpain and calpastatin in the absence of Ca^{2+}, where no cleavage occurs. Lanes 3 and 5 contain aliquots of IGFBP-4 and IGFBP-5, respectively, digested with 20 nM μ-calpain in the absence of calpastatin and supplemented with 1 mM Ca^{2+}. Lanes 4 and 6 show the inhibition of the IGFBP-4 and -5 cleavage by μ-calpain in the presence of calpastatin at molar concentrations of 1:1 with calpain.

which corresponds to the 20 His/T7-tag, residues along with the first three residues of the amino terminus of the protein. The other three major cleavage sites were found in the weakly conserved regions of the protein at Glu 86 and Ala 87, generating fragments of approximately 11.01 and 16.0 kDa. There was another small fragment of 4.10 kDa observed in mass spectrometry instead of the large 16.0-kDa fragment. This discrepancy arose because this larger fragment was further cleaved at another major internal cleavage site, Phe 122 and Ala 123, which generated fragments of 4.10 and 13.10 kDa. The last major site was the bond between Asp 138 and Gly 139, which resulted in a fragment of 10.90 kDa. The NH_2-terminal sequencing of IGFBP-4 yielded another

weak cleavage site at the bond between Leu 72 and Met 73, which corresponds to the N-terminal region of the protein, but mass spectroscopy did not show any such fragment with the corresponding molecular weight. Hence, this site was not considered as a major cleavage site.

In IGFBP-5, we identified cleavage sites at residues 2, 141, and 152, as shown in Figures 4B and 5. Three major cleavage sites were observed: the first site is between the residues Gly 2 and Ser 3 located at the very beginning of the protein at the amino-terminus. NH_2-terminal sequencing of IGFBP-5 also showed another very close site between His 7 and Cys 8, but this was probably due to degradation of the N-terminus following initial cleavage, as no corresponding fragment was evident in the
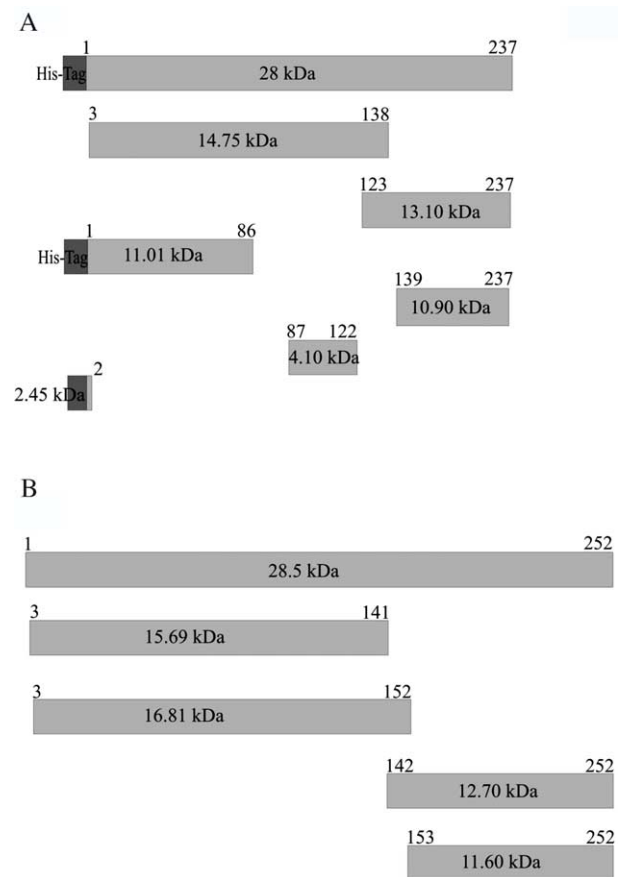

Figure 4 Schematic representation of the fragments generated by calpain cleavage.
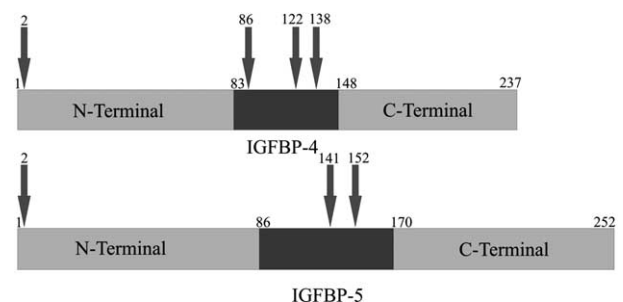Fragments for IGFBP-4 (A) and IGFBP-5 (B).

Figure 5 Summary of the location of the principal calpain cleavage sites in IGFBP-4 and IGFBP-5.
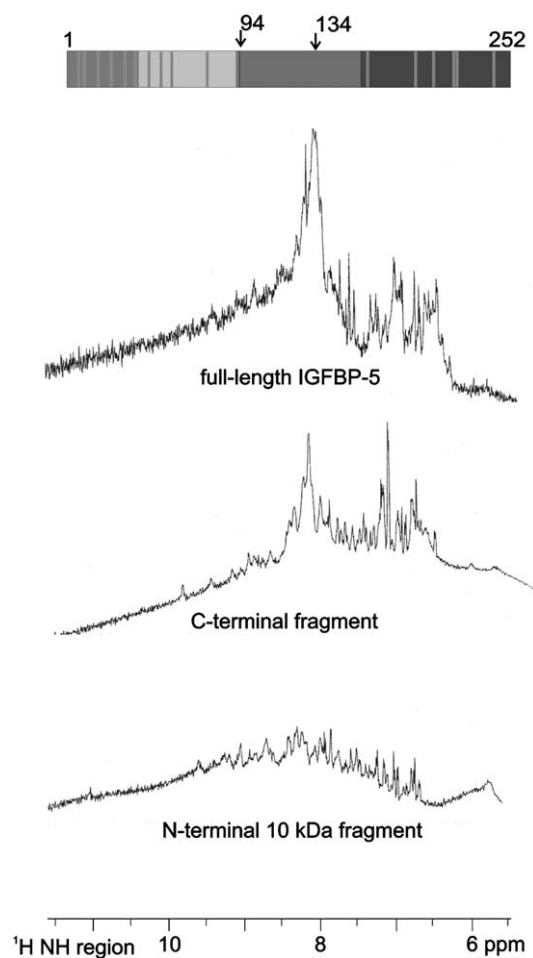
Figure 6   The amide region of one-dimensional NMR spectra of the recombinant human IGFBP-5.

The upper, middle and lower panels show the full-length protein, a C-terminal fragment of 112 (residues 135–246) and an N-terminal fragment of 94 residues, respectively. The fragments were created by limited proteolysis with endoproteinase Asp-N. A schematic domain structure of IGFBP-5 consists of the N-terminal domain (light gray), a central domain (gray) and the C-domain (dark gray). Sticks mark cysteines.

mass spectra. The other two cleavage sites are located in the weakly conserved domain of the protein, as in IGFBP-4, but not at the same residues. The first, located between Thr 141 and Gln 142, results in fragments of 15.69 and 12.70 kDa, and the second, between Thr 152 and Ala 153, generated fragments of 16.81 and 11.61 kDa.

Figure 6 shows one-dimensional ^1H NMR spectra of IGFBP-5. Inspection of such spectra yields semi-quantitative information on the extent of folding in proteins or their domains (Rehm et al., 2002). The upper panel shows the spectrum of the full-length IGFBP-5; this spectrum exhibits a large peak at 8.3 ppm and some signals downfield (i.e., at higher ppm values) close to the noise level. The appearance of intensities at chemical shifts near ∼8.3 ppm is an indicator of a disordered protein, as this is a region characteristic of backbone amides in a random coil configuration (Weber et al., 2000; Rehm et al., 2002). On the other hand, large signal dispersion beyond 8.3 ppm proves a protein to be folded. Due to the different chemical environment and thus the varying

shielding effects, the resonances of single protons will be distributed over a wide range of frequencies. A typical intensity pattern of a folded protein is shown in the lower panel, which shows the spectrum of the N-terminal fragment of IGFBP-5. The spectrum of the C-terminal fragment indicates that there are also some unstructured regions located in the C-terminal fragment (Figure 6, middle). Based on these spectra, we can conclude that the central variable domain of IGFBP-5 is in a random coil conformation. Nearly identical estimates were obtained from NMR for IGFBP-3 and IGFBP-4 (data not shown).

Discussion

The IGFBP family comprises six proteins (IGFBP-1–6) that bind to IGFs with high affinity, and a group of IGFBP-related proteins (IGFBP-rP 1–9), which show lower affinity for IGFs (Khandwala et al., 2000; Clemmons, 2001). IGFBPs-1–6 share a high degree of similarity in their primary protein structure (identities approx. 30–40%), with highest conservation at the N- and C-terminal regions (Figures 6 and 7), which participate in binding to IGFs (Baxter, 2000; Payet et al., 2003; Shand et al., 2003). The structure of the N-terminal domain of IGFBP-5 discloses a rigid, globular fold that consists of a centrally located three-stranded antiparallel β-sheet (Zeslawski et al., 2001). This is in contrast to the central, weakly conserved domain in IGFBPs, for which the NMR spectra presented here indicate a highly flexible, disordered conformation (Figure 6). This domain is also the site of proteolytic activity in IGFBPs (Figure 5). Our data indicate that there are four major calpain cleavage sites in IGFBP-4 and three in IGFBP-5 (Figure 5) all located in this region of the protein, except for the first cleavage sites in the N-terminal domain of both the proteins. Comparison of the cleavage pattern of IGFBP-5 with endoproteinase Asp-N reveals that Asp-N cleaves in a generally similar manner to calpain (i.e., in the central domain of the protein); however, the specific bonds are different (Kalus et al., 1998).

Figure 7 and Table 1 show locations of the cleavage sites on IGFBPs found in the present study and those compiled from the literature for other proteases. All IGFBPs, except IGFBP-6, are highly sensitive to proteolysis (the resistance of IGFBP-6 may be due to the absence of hydrophobic residues, such as tyrosine, tryptophan and phenylalanine, a very small variable domain and a low number of basic amino acid residues). Generally, for all IGFBPs two–three major proteolytic cleavage sites always occur at the beginning and towards the end of the variable domain (Figure 7). Calpain follows this rule too. Although the N- and C-terminal domains of IGFBPs are resistant to proteolytic attack, degradation of the C-terminal regions has been observed for IGFBP-3 (for plasmin, PSA, cathepsin D, and thrombin) and IGFBP-5 (thrombin) in in vitro studies. Figure 7 also includes our in vitro proteolysis of the recombinant human IGFBP-5 (residues 158–247) with thrombin. This fragment shows two extra cleavage sites compared to proteolysis of a full-length IGFPB-5 at physiological concentrations of thrombin (Zheng et al., 1998). A corresponding C-terminal region of IGFBP-4 (residues

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Location of protease cleavage sites in IGFBPs

hIGFBP-1 C V Q E S D A S A P H A A E A G S P E S P E S T E I T E E E L L D M F 113
hIGFBP-2 C E K R R D A E Y G A S P E Q V A D N G D D H S E G G L V E N H V D S 129
hIGFBP-3 C V N A S A V S R L R A Y L L P A P P A P G N A S E S E E D R S A G S 121
hIGFBP-4 C M E L A E I E A I Q E S L Q P S D K D E G D H P N N S F S P C S A H 113
hIGFBP-5 C L N E K S Y R E Q V K I E R D S R E H E E P T T S E M A E E T Y S P 114
hIGFBP-6 C L P A R A P A V A E E N P K E S K P Q A G T A R P Q D V N R R D Q Q 114

hIGFBP-1 H L M A P S E E D H S I L W D A I S T Y D G S K A L H V T N I K K W K 148
hIGFBP-2 T M N M L G G G G S A G R K P L K S G M K E L A V F R E K V T E Q H R 164
hIGFBP-3 V E S P S V S S T H R V S D P K F H P L H S K I I I I K K G H A K D 156
hIGFBP-4 D R R C L Q K H F A K I R D R S T S G G K M K V N G A P R E D A R P V 148
hIGFBP-5 K I F R P K H T R I S E L K A E A V K D R R K K L T Q S K F V G G A 149
hIGFBP-6 N R P G T S T T P S Q P N S A G V Q D T E M                         136

hIGFBP-1                                               E P C R E I L Y 156
hIGFBP-2 Q M G K G G K H H L G L E E P K K L R P P P A R   T P C Q Q E L D 196
hIGFBP-3 Q R Y K V D Y E S Q S T D T Q N F S S E S K R E T E Y G P C R R E M E 191
hIGFBP-4 P Q                                             G S C Q S E L H 158
hIGFBP-5 E N T A H P R I I S A P E M R Q E S E Q           G P C R R H M E 177
hIGFBP-6                                                 G P C R R H L D 144

hIGFBP-1 R V V E S L A K A Q E T S - - G E - E - I S K F Y L P N C N K N G F Y 187
hIGFBP-2 Q V L E R I S T M R L P D E R G P L E H L Y S L H I P N C D K H G L Y 231
hIGFBP-3 D T L N H L K F L N V L S V R G - - - - V - - - H I P N C D K K G F Y 219
hIGFBP-4 R A L E R L A A S Q - - S - R T H - E D L Y I I P I P N C D R N G N F 189
hIGFBP-5 A S L Q E L K A S E M R V P R A - - - - V Y - - - L P N C D R K G F Y 205
hIGFBP-6 S V L Q Q L Q T E V Y - - - R G - A Q T L Y - - - V P N C D H R G F Y 172
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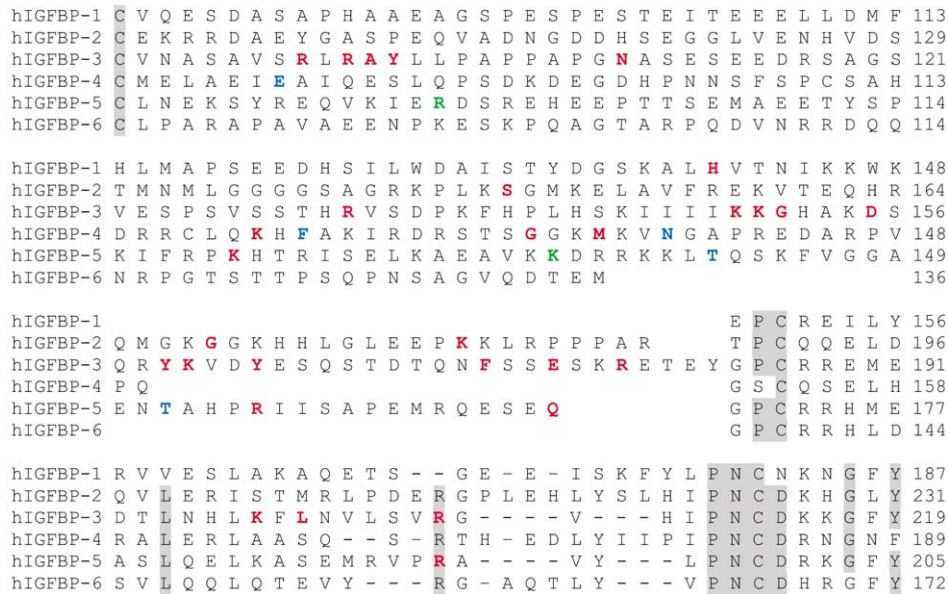
Figure 7 Sequence alignment of all the IGFBP non-conserved regions.
Grey, conserved amino acids; red, protease cleavage sites reported in the literature to date (see Table 1); blue, amino acids after which calpain cleaves; green, amino acids after which endoproteinase Asp-N cleaves.

171–252) was completely resistant to thrombin proteolysis in our experiments (data not shown).

Since IGFBP-3 and IGFBP-5 show structural similarities and both are synthesized by cell types derived from the mesenchyma, calpain is expected to cleave IGFBP-3 in the same fashion as it cleaves IGFBP-5. There is evidence that a calpain-like cysteine protease is a key regulatory component required for passage through the G_1 phase of the cell cycle in eukaryotes (Mellgren, 1997). As IGFBP-3 can traverse into the nucleus, calpain might also regulate the IGF-independent functions of IGFBP-3 (Baxter, 2000).

There are a limited number of reports on the subsite specificity of calpains and these have been reviewed by Croall and Demartino (1991) and more recently by Goll et al. (2003). In general, the literature data indicate that the calpains cleave target proteins at a limited number of sites and produce large polypeptide fragments rather than small peptides (Sasaki et al., 1984). Early studies suggested that the calpains preferentially cleaved peptide bonds with a Leu or a Val residue in the P_2 position. More complete data, however, indicated that subsite specificity of the calpains is controlled by the conformation of the polypeptide chain and not by amino acid

Table 1 Proteolytic cleavage sites in IGFBPs.

	Protease (source)	Cleavage sites (P1 residue)	Reference
IGFBP-1	Stromelysin-3	140	Manes et al., 1997
IGFBP-2	Fetal rat liver cell line	147	Wang et al., 1988
	Human milk	169, 181	Ho and Baxter, 1997
IGFBP-3	Plasmin	95, 97, 149, 150, 151 155, 160, 198, 220	Lalou et al., 1997 Booth et al., 1996
	PSA	97, 132, 159, 173, 179	Fielder et al., 1994
	Human serum	97, 148, 149, 150, 154	Booth et al., 1996
	MMP-1	99	Fowlkes et al., 1994
	MMP-2 (human fibroblasts)	99	Fowlkes et al., 1994
	MMP-3	99, 109, 176	Fowlkes et al., 1994
	Cathepsin D	98, 159, 163, 200	Claussen et al., 1997
	Thrombin	97, 206	Booth et al., 1996
	MCF-7 cell line	97	Salahifar et al., 2000
IGFBP-4	Rat neuronal cells	90, 120, 132	Chernausek et al., 1995
	Human fibroblasts	135	Conover et al., 1995
	Cathepsin D	135	Claussen et al., 1997
	Calpain (human erythrocytes)	3, 86, 122, 138	This work
IGFBP-5	Human osteoblasts	169	Andress, 1995
	Porcine aortic smooth muscle cells	137, 138	Imai et al., 1997
	Thrombin	120, 156, 192	Zwad et al., 2002
	PAPP-A	143, 144	Laursen et al., 2001
	Calpain (human erythrocytes)	2, 140, 151	This work
	Asp-N	94, 134	Kalus et al., 1998

Table 2 Calpain cleavage sequences.

Protein	Cleavage	
	Site	Sequence
IGFBP-4 (this work)	2	GDEAIH
	86	EIEAIQ
	122	KHFAKI
	138	KVNGAP
IGFBP-5 (this work)	2	SLGSFV
	141	KLTQSK
	152	ENTAHP
Crystallin (Shih et al., 1998)		
βB1	12	ATAAVN
	15	GPPPGP
βA3	11	ELESLP
	17	TTKMAQ
βB2	8	QTQAGK
βB3	5	EQHSTP
	10	PEQAAA
Calpain (autolysis;	9	KLAKDR
Brown and Crawford, 1993)	19	GLGSHE

Cleavage occurs after the third residue of the cleavage sequence.

sequence (Fischer et al., 1986; Harris et al., 1988; Croall et al., 1996; Stabach et al., 1997). For example, 9 of the 11 calpain cleavages in the α-tropomyosin polypeptide are in the COOH-terminal half of the molecule (Goll et al., 2003) where the COOH-terminal half of the helix is significantly less stable than the NH_2-terminal half, suggesting that subsite specificity of the calpains depends on the conformation of the polypeptide, with a more open structure favoring cleavage.

Our present study has not revealed clear preferences for amino acid sequences, although in many cases a hydrophobic residue is present in the P_2 position (Table 2 and Figure 7). For completeness, we also include in Table 2 two reports not covered by the reviews (Croall and Demartino, 1991; Goll et al., 2003). These data pertain to the m-calpain cleavage of N-terminal propeptides of β-crystallin, which are highly mobile and in random coil conformations (PDB: 1E7N; Clout et al., 2000).

In conclusion, we propose that calpains seem not to recognize amino acid sequences in target proteins, but cleave with low sequence specificity at unstructured or solvent-exposed fragments that connect folded, stable domains of target proteins, suggesting that calpains may modulate the functions of substrate proteins by cutting their interdomain regions.

Materials and methods

Chemicals, plasmid, expression host strain and growth media

Unless otherwise specified, all reagents were obtained from Sigma (Taufkirchen, Germany). Luria broth, agar, and antibiotics were purchased from Sigma-Aldrich. BL21 (DE 3) *E. coli* protein expression strains bought from Invitrogen (Karlsruhe, Germany) are designed for high-level protein expression using T7 RNA polymerase-based expression systems. pET 28a expression vector, based on the T7 promoter-driven system, was obtained from Novagen (Darmstadt, Germany). The restriction enzymes *Nde*I and *Bam*HI were from New England Biolabs (Frankfurt,

Germany). Pfu polymerase and DNA ligase were purchased from Stratagene (La Jolla, USA). Antibodies and isopropyl β-D-thio-galactoside (IPTG) were obtained from Santa Cruz Biotechnology (Santa Cruz, USA) and Peq Labs (Erlangen, Germany), respectively. Plasmid miniprep kit and Ni-NTA resin were bought from Qiagen (Hilden, Germany). The nitrocellulose membranes, polyvinylidene fluoride (PVDF) membranes and the ion exchange and size exclusion chromatography columns were purchased from Amersham Pharmacia Biotech (Freiburg, Germany). Bradford reagent was from Bio-Rad (Munich, Germany). The ultrafiltration membranes used were from Amicon (Schwalbach, Germany).

Cloning and protein expression

Human recombinant IGFBP-5 protein was a generous gift from Roche Diagnostics (Penzberg, Germany). The IGFBP-4 cDNA was also obtained from Roche Diagnostics in BP4-2/pFDX500 vector. To purify the protein as a His-tag fusion protein, the IGFBP-4 cDNA (full-length residues 1–237) was subcloned into pET-28 (a) vector (Qiagen) with restriction sites at *Nde*I and *Bam*HI in frame to a His-tag. Standard procedures were adopted for the construction of the vector pET-28 (a) with the desired insert (full-length IGFBP-4) which was expressed as a fusion protein in *E. coli* strain BL21 (DE 3) under kanamycin selection. The encoded amino acid sequences were *gsshhhhhhssglvprgs*MDEAIH…LADSFRE [the protein of interest is shown in capital letters and the amino acids of the His-tag and thrombin cleavage site (underlined) are in italics]. Expression was carried out in shaking cultures at 300 rpm and 37°C. The cultures were induced with 1 mM IPTG at a cell density of OD_{600}=0.85. Cells were harvested by centrifugation after 3 h of induction.

Protein purification

The cell pellets were resuspended in lysis buffer and kept for 1 h shaking. To obtain complete lysis, the cells were sonicated using a macro tip, with which pulsed, high-frequency sound waves are used to agitate and lyse cells. To prevent excessive heating, ultrasonic treatment was applied in multiple short bursts to the sample immersed in an ice bath. The sonication was carried out for 2 min at a duty cycle of 50% and output control 7. The sonication/lysozyme treatment was carried out by first treating the cells with the lysozyme and then sonicating the extract. All of the extracts were clarified by centrifugation for 60 min at 60 000 *g* at 4°C. The supernatants were recovered and then analyzed for total protein by the Bradford protein assay. The supernatant was loaded onto a Ni-NTA column and protein elution was carried out using a pH gradient method (Qiagen). The fractions containing IGFBP-4 were pooled and β-mercaptoethanol was added at a concentration of 10 mM. The final volume was concentrated to approximately 2 ml by ultrafiltration and then dialyzed against 6 M guanidinium chloride. The protein solution was then diluted into refolding buffer (Kalus et al., 1998). After 24 h of refolding at 4°C, the protein solution was again concentrated by ultrafiltration and dialyzed against phosphate buffer and loaded onto an anion exchange column (Mono S) in which the proteins were bound to the column material in 25 mM phosphate buffer, 25 mM NaCl, pH 7.2, and bound proteins were fractionated by a linear gradient of 25–700 mM NaCl over 20 column volumes. The fractions containing the protein were pooled and size exclusion chromatography was carried out on a Superdex 75 column.

μ-Calpain storage conditions

μ-Calpain was isolated from human erythrocytes as described previously (Gabrijelcic-Geiger et al., 2001). The purified enzyme

was dialyzed against 10 mM Tris, 150 mM NaCl, 1 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride (calpain storage buffer) and stored at 4°C until use. On the day of use, a small aliquot was taken and reactivated with 14 mM β-mercaptoethanol prior to the proteolysis assays.

IGFBP-4 and IGFBP-5 limited proteolysis assays

Purified IGFBP-4 and IGFBP-5 were incubated separately with calpain at a molar ratio of 1:50 in calpain assay buffer (25 mM Tris, 150 mM NaCl, pH 7.3) supplemented with CaCl$_2$ and kept for different time intervals at room temperature. Addition of 10 mM EDTA stopped the reactions. The fragments were separated by SDS-PAGE and visualized by Coomassie Blue staining.

Calpastatin inhibitory and protein stability assays

Domain 1 of calpastatin was added in equimolar ratio to calpain. Purified IGFBP-4 and IGFBP-5 were incubated separately with calpain and calpastatin at the molar ratio of 1:50:1 (protein/calpain/calpastatin) in calpain assay buffer supplemented with CaCl$_2$ to a final concentration of 1 mM and kept for different time intervals at room temperature. Addition of 10 mM EDTA and SDS-PAGE loading buffer stopped the reaction. Subsequently, the protein fragments were separated by SDS-PAGE and visualized by Coomassie blue staining. To ensure that the respective proteins were not degrading autocatalytically and that the degradation was due to calpain cleavage, we carried out a stability test by keeping the proteins at room temperature in calpain assay buffer without adding calpain, and taking aliquots for SDS-PAGE at different time intervals for up to 3 days; no degradation was observed, even after 3 days.

Electrophoresis and Western blot analysis

Aliquots of the protein samples treated with calpain were resolved on 12% SDS-PAGE, which was carried out using the Laemmli procedure. Western blot analysis was then carried out using primary antibodies against the protein. Alkaline phosphatase-conjugated secondary antibodies were then used against the primary antibody and the membrane was further developed by incubation with 5-bromo-4-chloro-3-indolyl phosphate (BCIP)/nitroblue tetrazolium (NBT) alkaline phosphatase substrate for 15–30 min.

N-Terminal sequencing of the protein fragments using PVDF membranes, reversed-phase HPLC and mass spectrometry

The reaction mixtures once resolved on SDS-PAGE were used to electroblot the protein fragments onto a PVDF membrane using the standard procedure described by Sambrook et al. (1989) so that the NH$_2$-terminal amino acids of the intact and cleaved products could be sequenced. The electroblot was carried out using semi-dry electroblotting apparatus at constant power supply of 110 mV for 1 h. The protein fragment-blotted PVDF membrane was stained with Coomassie blue dye. Coomassie-stained bands were cut from the membrane and used for N-terminal sequencing by Edman degradation. The results obtained from the sequence analysis were then mapped on the IGFBP sequences (Swiss-Prot, primary accession number P22692 for IGFBP-4 and P24593 for IGFBP-5). To further confirm the results of N-terminal sequencing, the reaction mixtures were purified by reverse-phase HPLC, sequenced and analyzed by mass spectrometry using MALDI-MS.

NMR spectroscopy

Standard 1D proton spectra were acquired on a Bruker DRX 600 spectrometer at 300 K. The protein samples were typically 0.3–0.6 mM in phosphate-buffered saline (PBS).

Acknowledgments

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