Substrate specificity of the metalloproteinase pregnancy-associated plasma protein-A (PAPP-A) assessed by mutagenesis and analysis of synthetic peptides: substrate residues distant from the scissile bond are critical for proteolysis

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Human pregnancy-associated plasma protein-A (PAPP-A) cleaves insulin-like growth factor (IGF) binding protein-4 (IGFBP-4), causing a dramatic reduction in its affinity for IGF-I and -II. Through this mechanism, PAPP-A is a regulator of IGF bioactivity in several systems, including the human ovary and the cardiovascular system. PAPP-A belongs to the metzincin superfamily of zinc metalloproteinases, and is the founding member of a fifth metzincin family, the pappalysins. Herein, we first determined that PAPP-A cleaves IGFBP-4 at a single site (Met-135/Lys-136), and we analysed the influence of ionic strength, pH and zinc ion concentration on the cleavage reaction. Secondly, we sought to delineate the role of substrate residues in PAPP-A-mediated cleavage by the construction and analysis of 30 IGFBP-4 mutants in which various residues were replaced by alanine, by the analysis of eight mutants of IGFBP-5 (found recently to be a second PAPP-A substrate), and by cleavage

analysis of synthetic peptides derived from IGFBP-4. Our data reveal a complex mode of substrate recognition and/or binding, pointing at important roles for several basic residues located up to 16 residues N-terminal to the scissile bond. An unexpected parallel can be drawn with an intracellular enzyme, the mitochondrial processing peptidase, that may help us to understand properties of the pappalysins. Further, proteinase-resistant variants of IGFBP-4 and -5, presented here, will be useful tools for the study of proteolysis in cell-based systems, and our finding that a synthetic peptide can be cleaved by PAPP-A provides the basis for development of quantitative assays for the investigation of PAPP-A enzyme kinetics.

Key words: insulin-like growth factor binding protein, metzincin, mitochondrial processing peptidase, proteolysis.

INTRODUCTION

The biological activities of insulin-like growth factor-I (IGF-I) and IGF-II are tightly regulated by their binding to one of six IGF binding proteins, IGFBP-1 to IGFBP-6. The binding capabilities of the IGFBPs are regulated, in turn, by specific cleavage of these proteins, which results in decreased affinity for the IGFs [1,2]. Pregnancy-associated plasma protein-A (PAPP-A) has been identified as a proteinase acting on IGFBP-4 [3], and recently IGFBP-5 was shown to be a second PAPP-A substrate [4]. Interestingly, efficient proteolysis of IGFBP-4 requires the presence of IGF, whereas the cleavage of IGFBP-5 by PAPP-A is slightly inhibited by IGF [4].

PAPP-A and other components of the IGF system have been studied in several biological systems, where PAPP-A appears to function as a local regulator of IGF bioavailability. Secretion of PAPP-A from human fibroblasts [3], osteoblasts [3,5] and vascular smooth muscle cells [6,7], as well as the presence of PAPP-A in the ovaries of humans [8] and several other animal species [9], has been established. Because it has been demonstrated that the proteolytic activity against IGFBP-4 can be inhibited with polyclonal antibodies against PAPP-A, PAPP-A is most probably the only IGFBP-4 proteinase present in these systems. During human pregnancy, PAPP-A is expressed abundantly in

the placenta [10]. As judged by reverse transcription–PCR analyses, no tissue has been found that does not express detectable levels of PAPP-A mRNA [11].

The plasma of pregnant women contains a higher concentration of PAPP-A (approx. 50 mg/l in the third trimester) than found elsewhere [12], but in this fluid the vast majority of PAPP-A ($\approx 99\%$) exists as a disulphide-bound 2:2 complex of 500 kDa with the 206-residue pro-form of eosinophil major basic protein (proMBP) [13]. In the PAPP-A/proMBP complex, proMBP functions as a proteinase inhibitor of PAPP-A, and the measurable PAPP-A activity in pregnancy serum is caused by the minor fraction of uncomplexed PAPP-A [14].

PAPP-A was recently recognized as a member of the metzincin superfamily of metalloproteinases [15]. Metzincin families [16] include the astacins, the adamalysins, the serralysins, the matrix metalloproteinases and the pappalysins, of which PAPP-A is a founding member [15]. PAPP-A2, a novel proteinase 45% identical to PAPP-A, which cleaves IGFBP-5, but not IGFBP-4, is the second member of the pappalysin family [17].

The active site of all metzincins is based on the elongated zinc-binding motif (HEXXHXXGXXH). Further, a structurally conserved methionine residue, located in the so-called Met-turn, is an invariable feature of all known proteinases of this super-family [16]. In addition to the common proteolytic domain, most

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Abbreviations used: IGF, insulin-like growth factor; IGFBP, IGF binding protein; MPP, mitochondrial processing peptidase; PAPP-A, pregnancy-associated plasma protein-A; proMBP, pro-form of eosinophil major basic protein; RP-HPLC, reverse-phase HPLC.
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metzincins contain several other domains [16]. The PAPP-A polypeptide chain has 1547 residues [18], of which about 310, located in the N-terminal half of the sequence, comprise the proteolytic domain [15]. Except for the conserved zinc-binding motif, the proteolytic domains of PAPP-A and PAPP-A2 do not show sequence homology with other proteinases. Members of the metzincin families are found among both prokaryotes and eukaryotes, and their natural substrates cover a wide spectrum of proteins [16]. The substrate specificity within families is defined in part by preferences at amino acid positions in the vicinity of the scissile bond, but no simple cleavage site motifs can be defined [16,19].

In an effort to understand the roles of PAPP-A in complex biological systems such as the ovary and the cardiovascular system, a fundamental understanding of the biochemistry of PAPP-A is important. We have therefore analysed the PAPP-A cleavage of IGFBP-4 and -5 mutants, and of synthetic peptides derived from IGFBP-4. Surprisingly, amino acids distant from the scissile bond are critical for cleavage by PAPP-A. An unexpected, interesting parallel can be drawn with an intracellular enzyme, mitochondrial processing peptidase (MPP).

EXPERIMENTAL

Determination of cleavage site

For cleavage site determination, 10 μg of purified, recombinant IGFBP-4 was digested in the presence of equimolar concentrations of added IGF-II using purified PAPP-A {0.1 μg immobilized to Protein G–agarose (Life Technologies), as previously described [4]}. The reaction mixture was spotted directly on to a glass filter for Edman degradation [20].

Analysis of conditions for cleavage of IGFBP-4 and -5 by PAPP-A

Purified recombinant IGFBP-4 (3 μg) [15] was ^{125}I-labelled [21] and cleavage analysis in the presence of added IGF-II (Bachem) was performed as previously described [4], but with variations as specified below. PAPP-A in standard serum-containing medium was used [15]. The extent of cleavage was visualized by SDS/PAGE followed by autoradiography using a PhosphorImager (Molecular Dynamics). To establish conditions for cleavage of IGFBP-4 by recombinant PAPP-A, three basic parameters were varied, with a constant time of incubation (2 h). Analysis of dependence on salt was carried out in 0–200 mM NaCl, 1 mM CaCl$_2$ and 50 mM Tris, pH 7.5. Analysis of dependence on pH was carried out in 100 mM NaCl and 1 mM CaCl$_2$; the buffer used was 50 mM Mes or 50 mM Tris, adjusted to pH values between 6 and 9, as specified. Analysis of dependence on zinc ion concentration was carried out in 100 mM NaCl/1 mM CaCl$_2$/50 mM Tris, pH 7.5, and 0–50 μM added ZnCl$_2$. Analyses of conditions for PAPP-A proteolysis of IGFBP-5 [4] were carried out similarly.

Plasmid constructs

Human cDNA encoding IGFBP-4 was cloned into the *Xho*I/*Kpn*I sites of pcDNA3.1/*Myc*-His(−)A (Invitrogen) as recently described [15]. The construct encoded the 258-residue proIGFBP-4, followed immediately by residues GTKLGP, the *myc* epitope (EQKLISEEDL), residues NSAVD, and H$_6$ (amino acids are given as the one-letter code), increasing the molecular mass by 3.1 kDa, which causes the PAPP-A cleavage products to co-migrate in SDS/PAGE [15]. Mutagenesis of this vector was carried out by overlap extension PCR [22]. In brief, outer primers were 5′-GAGGAGCTGGTGCGAGAGCC-3′ (5′ end)

and 5′-AAGGACAGTGGGAGTGGCACC-3′ (3′ end), corresponding to residues 24–30 of IGFBP-4 (note that human IGFBP-4 and IGFBP-5 are numbered with the N-termini of the mature polypeptides, Asp and Leu respectively, as residue 1), and nucleotides 1204–1184 of pcDNA3.1/*Myc*-His(−)A respectively. Internal primers with an overlap of approx. 22 bp were used to generate mutated fragments that were digested with *Bst*EII/*Acc*65I, and swapped into the wild-type construct.

Human cDNA encoding IGFBP-5 was cloned into the *Xho*I/*Hin*dIII sites of pcDNA3.1/*Myc*-His(−)A as recently described [17]. The construct encoded the 272-residue proIGFBP-5, followed immediately by residues KLGP, the *myc* epitope (EQKLI-SEEDL), residues NSAVD and H$_6$, increasing the molecular mass by 2.9 kDa. Mutagenesis of this vector was carried out by overlap extension PCR. Outer primers were 5′-CACTGCTTAC-TGGCTTATCGA-3′ (5′ end) and 5′-AAGGACAGTGGGAG-TGGCACC-3′ (3′ end), corresponding to nucleotides 839–859 and 1204–1184 respectively of pcDNA3.1/*Myc*-His(−)A. Internal primers with an overlap of approx. 22 bp were used to generate mutated fragments that were digested with *Xho*I/*Hin*dIII and swapped into the wild-type construct.

All PCRs were carried out with *Pfu* DNA polymerase (Stratagene), and all constructs were verified by sequence analysis. Plasmid DNA for transfection was prepared using a QIAprep Spin Kit (Qiagen).

Tissue culture, transfection and analysis of recombinant protein

Human embryonic kidney 293T cells (293tsA1609neo) were maintained in high-glucose Dulbecco's modified Eagle's medium supplemented with 10 % (v/v) fetal bovine serum, 2 mM glutamine, non-essential amino acids and gentamicin (Life Technologies). Cells were plated on to 6 cm tissue culture dishes, and were transfected 18 h later by calcium phosphate co-precipitation using 10 μg of plasmid DNA [14]. After a further 48 h, the supernatants were harvested and replaced by serum-free medium for another 48 h. To verify that the mutated proteins were expressed as efficiently as the wild-type protein, their levels were compared by SDS/PAGE and Coomassie Blue staining of ethanol-precipitated serum-free medium (100 μl). Further, the functionality of the expressed binding proteins was confirmed by ligand blotting. In brief, proteins separated by SDS/PAGE were blotted on to a PVDF membrane (Immobilon-P; Millipore) and processed further by a standard procedure [23] using iodinated IGF-I [4].

Cleavage analysis of mutated IGFBPs

Cleavage of recombinant IGFBP-4 and mutated variants of this protein was analysed by Western blotting. Briefly, approx. 40 ng (final concentration 60 nM) of wild-type or mutated IGFBP-4, as contained in 4 μl of cell culture medium, was incubated with 2 ng of recombinant human PAPP-A (0.25 nM) [15] in the presence or absence of 75 nM IGF-II (Bachem). The total reaction volume was 20 μl. For estimation of the effects of amino acid substitutions, conditions were chosen that resulted in approx. 50 % degradation of wild-type proteins (approx. 4 h at 37 °C in 100 mM NaCl/1 mM CaCl$_2$/50 mM Tris, pH 7.5). The reaction mixtures (10 μl) were separated on reducing SDS/PAGE (16 % gel), blotted on to an Immobilon-P PVDF membrane, and the C-terminal cleavage products were detected with a monoclonal anti-c-myc antibody (clone 9E10; A.T.C.C.) using peroxidase-conjugated secondary antibodies (P260; DAKO). Blots were allowed to dry, and then incubated with primary antibody diluted in 10 mM sodium phosphate, 150 mM NaCl and 0.01 % Tween 20, pH 7.2 (PBST), containing 2 % (w/v) skimmed milk
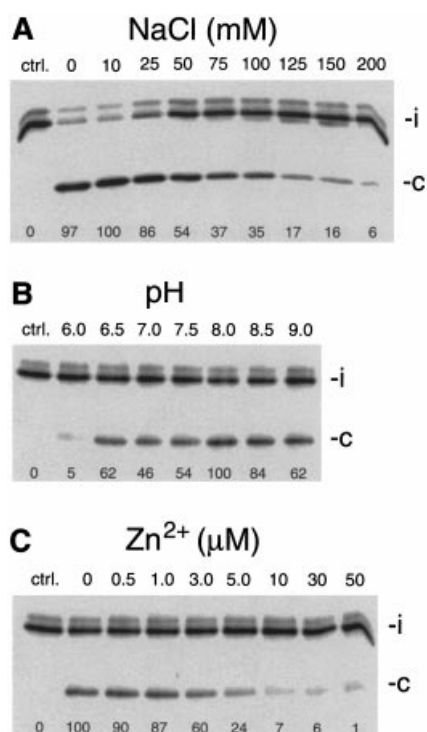
Figure 1   Cleavage of IGFBP-4 by recombinant PAPP-A

(**A**) Dependence on salt concentration. Radiolabelled IGFBP-4 was incubated with recombinant PAPP-A in 1 mM CaCl₂, 50 mM Tris, pH 7.5, and 0–200 mM NaCl as specified above each lane. Neither salt nor PAPP-A was added to the reaction loaded in the first lane (ctrl.). Bands corresponding to intact (i) and cleaved (c) IGFBP-4 are indicated. The N- and C-terminal cleavage products co-migrate because of the C-terminal tag, as detailed in the Experimental section. Values at the bottom of each lane specify percentage cleavage; the lane with the highest amount of IGFBP-4 cleavage product was set to 100%. (**B**) Dependence on pH. The same experiment was carried out, varying the pH of the reactions (at 100 mM NaCl). (**C**) Dependence on zinc ion concentration. Essentially the same experiment was carried out, with added zinc chloride.

powder for 1 h at 37 °C, according to the protocol of the manufacturer (Millipore). Blots were washed in PBST, incubated further with secondary antibody diluted in PBST containing 2 % (w/v) skimmed milk powder, and then washed again with PBST. The blots were developed using enhanced chemiluminescence (ECL®; Amersham), and the degree of cleavage, compared with that of wild-type protein, was measured by densitometry. Cleavage of wild-type and mutant IGFBP-5 was analysed by the same procedure.

Cleavage analysis of synthetic peptides

Peptides were prepared on an Applied Biosystems 433A peptide synthesizer, and chromatographed by reverse-phase HPLC (RP-HPLC) on a 4 mm × 250 mm column packed with Nucleosil C18 300–305 (Macherey-Nagel). Linear gradients were formed from 0.1 % (v/v) trifluoroacetic acid (solvent A) and 0.075 % (v/v) trifluoroacetic acid in 90 % (v/v) acetonitrile (solvent B), increasing the amount of solvent B to 50 % over 30 min at a flow rate of 1 ml/min; the eluates were monitored at 215 nm. The identities of purified peptides were verified by MS on a Voyager DE-PRO from Applied Biosystems by Profundis Biotech ApS (Science Park Aarhus, Denmark), and the amounts of individual peptides were determined by amino acid analysis [24] following lyophilization and redissolving in 10 mM HCl (at approx.
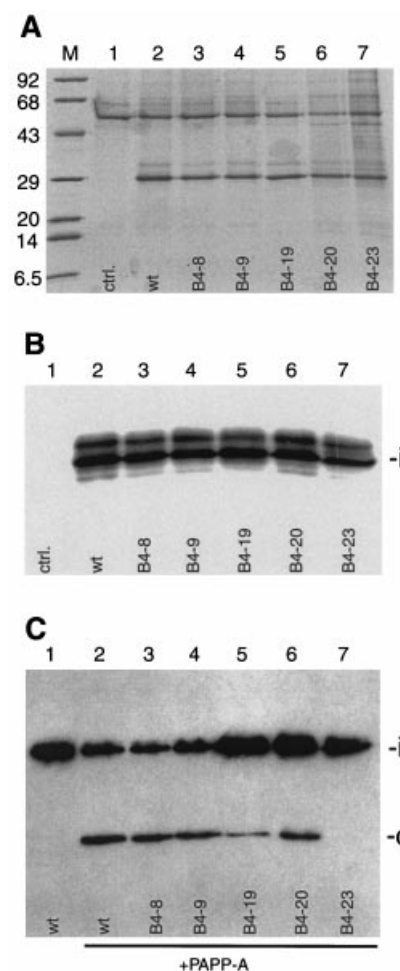

Figure 2   Expression and analysis of wild-type and mutated IGFBP-4

Wild-type and mutated IGFBP-4 were expressed in mammalian cells and analysed, as shown here with selected examples. (**A**) Non-reducing SDS/PAGE of precipitated proteins contained in serum-free medium (100 µl) from transiently transfected cells (Coomassie Blue stained gel). Molecular mass markers of the sizes (in kDa) indicated were loaded in the left lane (M). Lane 1 contains medium from cells transfected with empty vector (ctrl.). Lane 2 contains medium from cells transfected with wild-type (wt) IGFBP-4 cDNA. Lanes 3–7 contain medium from cells transfected with mutated IGFBP-4 cDNAs (B4-8, B4-9, B4-20, B4-21 and B4-24 respectively; see Figure 3A), as indicated at the bottom of the lanes. The apparent molecular mass of both wild-type IGFBP-4 and all mutated variants is close to 29 kDa, including the C-terminal tag. (**B**) Ligand blotting using iodinated IGF-I to confirm its binding to wild-type as well as mutated IGFBP-4 proteins. The position of intact IGFBP-4 is indicated (i). Samples loaded are as in (**A**). (**C**) Cleavage of wild-type and mutated IGFBP-4 proteins by PAPP-A (lanes 2–7) in the presence of IGF-II. Intact (i) and cleaved (c) IGFBP-4 were visualized by immunoblotting using anti-c-myc antibody, which recognizes the C-terminal tag. Samples loaded are as in (**A**) and (**B**), except for lane 1, which contains wild-type IGFBP-4. Sample identities are indicated.

50 µM). The peptides were added to immobilized PAPP-A contained in 100 mM NaCl/1 mM CaCl₂/50 mM Tris (pH 7.5)/0.01 % sodium azide, or to agarose beads processed similarly with mock medium. The final concentration of peptide was 5 µM, i.e. 250-fold higher than the concentration of PAPP-A (20 nM). IGF-II (5 µM) was added in some experiments. The reaction mixtures (100 µl) were incubated at 37 °C for 20 h, and then analysed using the same RP-HPLC system. Eluted peptides were identified by amino acid analysis and/or MS, and the degree of cleavage was determined by measuring peak heights.

RESULTS

PAPP-A cleaves IGFBP-4 at a single site

To determine unequivocally the site(s) at which PAPP-A cleaves IGFBP-4, purified recombinant proteins (300 pmol of IGFBP-4) were incubated in the presence of added IGF-II. Complete digestion was verified by SDS/PAGE, and Edman degradation was performed directly on the reaction mixture. This procedure ensured detection of a small peptide fragment that might have been released following a second, hypothetical cleavage of the IGFBP-4 polypeptide chain. Such a peptide would escape detection if the material was first separated by SDS/PAGE and blotted on to a membrane. Two IGFBP-4 sequences were observed: Asp[1]-Glu-Ala-Ile-His and Lys[136]-Val-Asn-Gly-Ala.

Thus PAPP-A cleaves IGFBP-4 at a single site, between Met-135 and Lys-136.

Establishment of conditions for the cleavage reaction using recombinant PAPP-A

Before further analysis, we characterized the cleavage reaction with regard to salt dependence, pH dependence and the influence of zinc ions. Radiolabelled IGFBP-4 was digested by PAPP-A in the presence of IGF-II, and cleavage was evaluated by SDS/PAGE followed by autoradiography (Figure 1). Surprisingly, at physiological ionic strength (50 mM Tris/HCl and 100 mM NaCl), the amount of cleavage product generated was only 35 % of that following cleavage in 50 mM Tris without added NaCl,

Figure 3   Cleavage analysis of mutant IGFBP-4 and IGFBP-5 proteins

Cleavage by PAPP-A of mutant IGFBP-4 in the presence of IGF-II (**A**) and mutant IGFBP-5 (without added IGF) (**B**) was analysed. The substituted residues of the individual mutants (B4-1 to B4-30 and B5-1 to B5-8) are shown. The ability of the mutants to be cleaved by PAPP-A was compared with the PAPP-A cleavage of the wild-type proteins under conditions that resulted in 50 % cleavage of the latter. Band intensities were measured after Western blotting: + + + + +, no effect compared with wild type [or slightly increased proteolysis (B5-4)]; + + + +, cleavage reduced to 99–81 %; + + +, cleavage reduced to 80–51 %; + +, cleavage reduced to 50–21 %; +, cleavage reduced to 20–1.0 %; 0, no detectable cleavage under standard assay conditions. The scoring was based on at least three independent experiments. Residue positions −16, −10, −8, −1 and +1 are indicated, and experimentally determined cleavage sites are indicated with arrows.

and addition of 200 mM NaCl dramatically inhibited proteolysis (Figure 1A). Maximum cleavage was observed at pH 8, and proteolysis was effectively abrogated at pH 6 (Figure 1B). Interestingly, although the metalloproteinase PAPP-A is a zinc-dependent enzyme, an inhibitory effect of very low concentrations of zinc ions ($< 0.5\ \mu$M) was apparent, and 50 μM Zn^{2+} almost completely blocked proteolysis (Figure 1C). Based on this, we carried out all subsequent incubations under carefully controlled conditions near physiological ionic strength and pH, in 100 mM NaCl, 1 mM $CaCl_2$ and 50 mM Tris, pH 7.5. No addition of zinc was made. The same set of experiments was carried out with IGFBP-5, with similar results (not shown).

Expression and analysis of mutated variants of IGFBP-4

By successive rounds of mutagenesis, a total of 30 IGFBP-4 mutants were prepared using a recently established system for mammalian expression of the recombinant protein [15]. The expression and functionality of all mutants were evaluated by SDS/PAGE and by ligand blotting using radiolabelled IGF-I. All mutants were expressed at levels similar to that of the wild-type protein, and they all bound IGF-I efficiently (Figures 2A and 2B). Visualization of intact and cleaved IGFBP-4 after incubation with PAPP-A was performed by Western blotting with a monoclonal antibody recognizing the C-terminal c-myc tag of the recombinant IGFBP-4 (Figure 2C). Based on the indistinguishable sizes of the two generated fragments, all cleavable mutants of IGFBP-4 appeared to be cleaved at the same peptide bond as wild-type IGFBP-4.

Cleavage analysis of IGFBP-4 mutants

In the first series of mutants (B4-1 to B4-13; Figure 3A), single residues of IGFBP-4 were replaced by alanine. Except for two glycine residues (at positions 132 and 133), all amino acids in a 14-residue stretch (residues 126–139) spanning the PAPP-A cleavage site were substituted. The cleavage of mutants was semi-quantitatively compared with the cleavage of wild-type IGFBP-4 under conditions that resulted in approx. 50 % degradation of the latter (Figure 2C), as detailed above. Surprisingly, no single residue had an appreciable effect on the cleavage reaction, but individual substitution of four residues located next to one another, with two residues on each side of the cleavage site (Lys-134, Met-135, Lys-136 and Val-137), resulted in slightly decreased proteolysis. Compared with wild-type IGFBP-4, the amount of cleavage of each of the mutants was decreased by less than 20 % (B4-7 to B4-10 in Figure 3A; results for mutants B4-8 and B4-9 are also shown in Figure 2C).

To analyse possible synergistic effects of amino acid side chains within the selected 14-residue stretch, we expressed a series of double and triple mutants, and one mutant with four residues substituted (B4-14 to B4-28; Figure 3A). Most of the mutants involved substitution of at least one of the four residues identified above, but we also included Arg-126 and Arg-128, since they are conserved in IGFBP-5 (as discussed below), and because basic peptides are known to inhibit the proteolysis of IGFBP-4 [9,25–27].

Pairwise combination of the substitutions of B4-7 to B4-9 further decreased the degree of proteolysis (mutants B4-19 to B4-21). Interestingly, with mutant B4-16, in which Arg-126 is substituted together with Lys-136, a substantial effect of substitution of a side chain distant from the cleavage site is evident. None of the triple mutants analysed showed complete resistance to cleavage, but clearly demonstrated that the observed effects on proteolysis are not additive: mutant B4-16 (in which Arg-126 and Lys-136 are substituted) is a poor substrate, but mutant B4-

Table 1 Cleavage of synthetic peptides derived from IGFBP-4

The positions of each peptide in the sequence of IGFBP-4 are given in parentheses. The individual peptides (5 μM) were incubated with PAPP-A (20 nM) for 24 h at 37 °C. Cleavage is expressed in %, as determined by measuring peak heights (see Figure 4). The arrow indicates the cleavage site as found in intact IGFBP-4 and in peptides P9–13.

Peptide no.	Peptide sequence	Cleavage (%)
P1 (129–140)	STSGGKM↓KVNGA	0
P2 (129–143)	STSGGKM↓KVNGAPRE	0
P3 (128–143)	RSTSGGKM↓KVNGAPRE	0
P4 (127–143)	DRSTSGGKM↓KVNGAPRE	0
P5 (126–143)	RDRSTSGGKM↓KVNGAPRE	0
P6 (125–143)	IRDRSTSGGKM↓KVNGAPRE	0
P7 (124–143)	KIRDRSTSGGKM↓KVNGAPRE	0
P8 (123–143)	AKIRDRSTSGGKM↓KVNGAPRE	0
P9 (122–143)	FAKIRDRSTSGGKM↓KVNGAPRE	4
P10 (121–143)	HFAKIRDRSTSGGKM↓KVNGAPRE	10
P11 (120–143)	KHFAKIRDRSTSGGKM↓KVNGAPRE	21
P12 (119–143)	QKHFAKIRDRSTSGGKM↓KVNGAPRE	51
P13 (118–143)	LQKHFAKIRDRSTSGGKM↓KVNGAPRE	51
IGF-II added (5 μM)		
P11 (120–143)	KHFAKIRDRSTSGGKM↓KVNGAPRE	21

23, in which Arg-128 is also substituted, is a better substrate. Similarly, the combined substitution of Lys-134 and Met-135 (B4-19) results in substantial resistance to cleavage, but the triple mutant B4-26, also including substitution of Arg-128, is cleaved as efficiently as wild-type IGFBP-4. However, the combined substitution of the four residues Arg-126, Arg-128, Lys-134 and Lys-136 with alanine resulted in a mutant (B4-24) that was not cleaved by PAPP-A (Figure 2C, lane 7).

Cleavage of synthetic peptides derived from IGFBP-4

Based on the above data, we first synthesized a 12-residue peptide derived from the sequence of IGFBP-4, and spanning the PAPP-A cleavage site (peptide P1; Table 1). Incubation of 5 μM peptide with 20 nM PAPP-A for 20 h at 37 °C did not result in the occurrence of detectable cleavage fragments, as analysed by RP-HPLC of the reaction mixture (results not shown). Likewise, a second peptide (P2), further extended by three C-terminal residues, was not cleaved. We thus synthesized and analysed a series of peptides, derived from peptide P2, that were progressively extended at the N-terminal end (Table 1). None of peptides P3–P8 showed any sign of degradation, but peptide P9, comprising 22 residues, was cleaved at Met-135/Lys-136 (Figure 4). With P10, and further with P11 and P12, the rate of cleavage increased, but cleavage of peptide P13 did not occur faster than that of P12 (Table 1). Thus efficient cleavage of a peptide substrate derived from IGFBP-4 requires at least the presence of 16–17 residues on the N-terminal side of the cleavage site, including Lys-120.

We also tested whether the addition of IGF-II to PAPP-A and peptide P11 increased the rate of cleavage, but found no effect of IGF (Table 1). Importantly, this finding strongly supports recent data demonstrating that IGF does not interact directly as a cofactor with PAPP-A, and that IGF promotes proteolysis by binding to IGFBP-4, thereby making it a better PAPP-A substrate [4].

Further mutagenesis of IGFBP-4

Because the above analysis of synthetic peptides suggested that Lys-120 and/or residues in its vicinity are critical for binding to
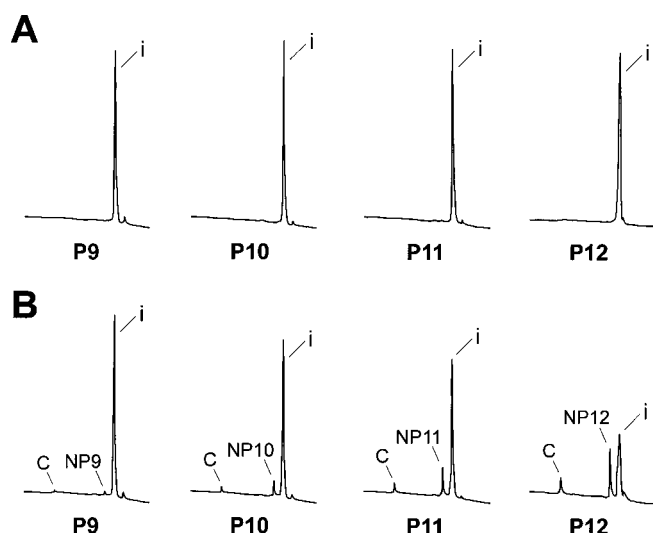
A

P9   P10   P11   P12

B

C NP9   C NP10   C NP11   NP12 C

P9   P10   P11   P12

Figure 4   Cleavage of synthetic peptides derived from IGFBP-4

Synthetic peptides (P9–P12; 5 μM) derived from IGFBP-4 were incubated (37 °C, 20 h) without (**A**) or with (**B**) PAPP-A (20 nM), and the cleavage reactions were analysed by RP-HPLC on a Nucleosil C18 column. An 11 min section of each chromatogram is shown, corresponding to the time interval from 12.5 min to 23.5 min, and a change in actonitrile concentration at the column inlet from 11.3 % to 29.5 %. The maximum peak height shown corresponds to an absorbance of 0.08 at 215 nm. The disappearance of the added peptide, along with the appearance of shorter fragments thereof, demonstrates proteolytic cleavage by PAPP-A. Eluted peptides were identified by MS. Intact, undigested peptides (P9–P12) are labelled 'i'. The common C-terminal fragment of eight residues (KVNGAPRE) is labelled 'C', and the different N-terminal fragments resulting from cleavage are labelled 'NP9–N12'. Cleavage analysis and sequences of all peptides tested are summarized in Table 1.

PAPP-A, we mutated this residue, along with His-121, to evaluate the effects of substitution in the full-length substrate. Although important for cleavage of the isolated peptides, and although the amount of cleavage was decreased for the mutant compared with the wild-type, the side chains of these residues in the intact protein were not indispensable (B4-29; Figure 3A). We also mutated the two basic residues on the C-terminal side of the scissile bond, i.e. Arg-142 and Arg-146, which had not been analysed previously, but did not observe any effect (B4-30; Figure 3A).

Cleavage analysis of IGFBP-5 mutants

Recent data demonstrated that, in addition to IGFBP-4, IGFBP-5 is also a PAPP-A substrate, and that cleavage of this binding protein occurs at Ser-143/Lys-144 [4]. Compared with the N- and C-terminal regions, the mid-regions of IGFBP-4 and -5 show little identity, but when aligned according to their cleavage sites,
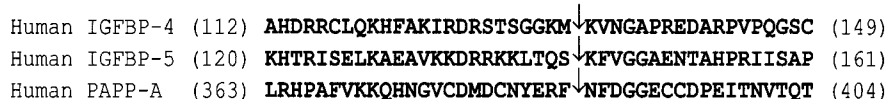

Figure 6   Cleavage of mutated IGFBP-5 by PAPP-A

Wild-type and mutated IGFBP-5 were expressed in mammalian cells and analysed for their ability to be cleaved by PAPP-A. Reactions loaded in lanes 1–2 contained wild-type (wt) IGFBP-5, and reactions loaded in lanes 3–10 contained mutated IGFBP-5 (B5-1 to B5-8 respectively; see Figure 3B), as specified below individual lanes. Reactions loaded in lanes 2–10 contained PAPP-A, as indicated. Intact (i) and cleaved (c) IGFBP-5 were visualized by immunoblotting using anti-c-myc antibody, which recognizes the C-terminal tag.

certain similarities are apparent (Figure 5). Basic residues of IGFBP-4 at positions -16, -10, -8, -2 and $+1$ (relative to the scissile bond) appear to be important for cleavage of IGFBP-4 by PAPP-A, and, interestingly, except for the residue at position -2, all corresponding positions are also occupied by a basic residue in IGFBP-5 (Figure 5). We therefore asked whether these residues of IGFBP-5 are important for specific proteolysis of this substrate.

A series of eight IGFBP-5 mutants in which selected residues were substituted by alanine was prepared (B5-1 to B5-8; Figure 3B). All mutants expressed and bound radiolabelled IGF as effectively as wild-type protein (results not shown), verifying correct protein folding. Cleavage analysis of these mutants is shown in Figure 6, and is also summarized in Figure 3(B). First, two mutants with substitutions of residues close to the cleavage site (B5-7 and B5-8) showed a limited effect on the cleavage reaction (Figure 6, lanes 9 and 10 compared with lane 2). This is in fair agreement with the results with the IGFBP-4 mutants B4-20 and B4-9 respectively, although the effect of double substitution was greater for IGFBP-4. Secondly, substitution of both Lys-134 and Arg-136 (mutant B5-4; corresponding to B4-14) did not result in a reduced cleavage rate. When this double substitution was combined with the substitutions of mutants B5-7 and B5-8 (in mutants B5-6 and B5-5 respectively), still no decrease in cleavage was observed. This result was highly surprising, because mutant B5-6 exactly matches the IGFBP-4 mutant B4-24, which is resistant to cleavage by PAPP-A. Curiously, cleavage of mutant B5-4 occurred more rapidly than that of wild-type IGFBP-5

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Human IGFBP-4 (112) AHDRRCLQKHFAKIRDRSTSGGKM↓KVNGAPREDARPVPQGSC (149)
Human IGFBP-5 (120) KHTRISELKAEAVKKDRRKKLTQS↓KFVGGAENTAHPRIISAP (161)
Human PAPP-A  (363) LRHPAFVKKQHNGVCDMDCNYERF↓NFDGGECCDPEITNVTQT (404)
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Figure 5   Alignment of sequence stretches spanning sites of cleavage by PAPP-A

Sequence stretches derived from human IGFBP-4, IGFBP-5 and PAPP-A are aligned according to the positions of the peptide bonds cleaved by PAPP-A. The cleavage site (vertical arrow) of IGFBP-4 is identified in the present paper. The cleavage site of IGFBP-5 [4] and the autocleavage site of PAPP-A [15] were identified recently.
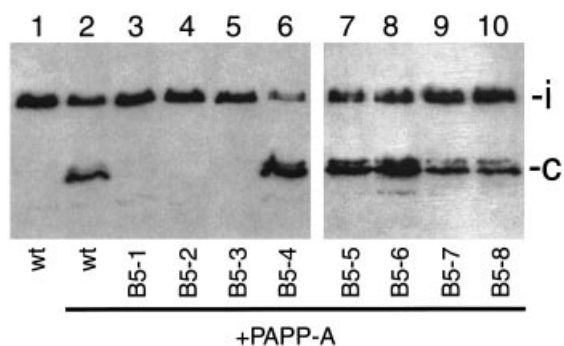
(Figure 6, lane 6 compared with lane 2). Thirdly, all three mutants that included substitution of Lys-128 (B5-1, -2 and -3) were not cleaved by PAPP-A. Even mutant B5-1, in which only Lys-128 is substituted into alanine, showed complete resistance towards proteolysis (Figure 6). This is also surprising, because substitution of the corresponding residue in IGFBP-4 (Lys-120; substituted in B4-29) caused only a limited decrease in cleavage.

DISCUSSION

We first determined that PAPP-A cleaves IGFBP-4 at a single site, between Met-135 and Lys-136. Secondly, we analysed the influence of ionic strength, pH and zinc ion concentration on the cleavage reaction. Thirdly, we sought to delineate the roles of individual residues of IGFBP-4 in PAPP-A cleavage, by the construction and analysis of 30 IGFBP-4 mutants. Fourthly, cleavage analysis of synthetic peptides derived from IGFBP-4 allowed us to conclude that PAPP-A substrate recognition is complex and involves residues distant from the scissile bond. Finally, we extended our analysis of PAPP-A substrate specificity by analysing eight mutants of IGFBP-5, the second PAPP-A substrate.

Before the identification of PAPP-A as an IGFBP-4 proteinase, different cleavage sites in IGFBP-4 had been reported using non-purified protein, mainly conditioned medium, as the sources of the proteinase. The peptide bond between Met-135 and Lys-136 had most often been identified as the scissile bond, either alone [5,28–30] or in combination with Lys-120/His-121 [31] or Phe-122/Ala-123 [32]. In one report, however, Lys-120/His-121 was pointed out as the only cleavage site [33]. Therefore, as a prerequisite for the further study of specificity, we analysed the cleavage products resulting from incubation of IGFBP-4 with highly purified PAPP-A. Only one cleavage site, Met-135/Lys-136, was found.

Our finding that PAPP-A is most active slightly above physiological pH, and that it loses activity at around pH 6, most probably because it loses its active-site metal due to protonation of histidine residues, is compatible with PAPP-A being a zinc peptidase (Figure 1B). It is surprising, however, that PAPP-A shows pronounced sensitivity to ionic strength, and that its optimum does not appear to be close to physiological conditions, but rather much lower (Figure 1A). This will be discussed further below. Interestingly, although a zinc ion is required for proteolysis as an essential part of the active site, less than 10% activity remained at 10 μM Zn^{2+}, and at 50 μM Zn^{2+} PAPP-A almost ceased to be active. Very similar behaviour was first observed with thermolysin [34], and explained by the binding of a second zinc ion close to the active site, preventing the substrate from reaching the latter [35], but whether this explanation is also valid for PAPP-A is unknown. These results dictate that comparisons of PAPP-A activity are carried out under carefully controlled conditions of pH and ionic strength, and that zinc is not added to the reaction.

For efficient interaction between an endopeptidase and its substrate, side chains of residues in the vicinity of the cleavage site typically play critical roles. We therefore initially substituted two residues on each side of the Met-135/Lys-136 peptide bond in a set of four mutants (B4-7 to B4-10). However, compared with the cleavage of wild-type IGFBP-4, substitution of these residues by alanine had a very limited effect on the ability of PAPP-A to cleave the protein (Figures 3A and 2C). Further, none of the other mutants in the entire set of mutants with single amino acid substitutions (B4-1 to B4-13) showed reduced cleavage. Proceeding as detailed above, we analysed IGFBP-4 mutants with two, three or four residues substituted by alanine (B4-14 to

B4-28). Interestingly, the effects of mutations were not always additive. For example, mutant B4-18 (K134A, M135A) clearly showed decreased cleavage, whereas B4-26 (R128A, K134A, M135A) was cleaved as efficiently as wild-type IGFBP-4. Although substantial decreases in the ability to be cleaved by PAPP-A were seen with several mutants, only one (B4-24) showed complete resistance to cleavage by PAPP-A (Figures 3A and 2C). In this mutant, arginine and lysine residues at positions −10, −8, −2 and +1 (relative to the scissile bond) are substituted by alanine.

Mutants with other combinations of the substituted residues, or mutants with alanine-substituted residues at other positions, may show resistance to cleavage by PAPP-A. Also, residues with side chains larger than that of alanine may interfere with or abrogate substrate binding if included at certain positions. However, mutants in which the residues at positions −2, −1 and +1 were individually substituted by residues with large, but chemically different, side chains did not result in a pronounced decrease in proteolysis [28]. A variant of IGFBP-4 with a large deletion (His-121–Pro-141) showed resistance to cleavage [5], but a proteinase-resistant mutant with fewer residues substituted, such as B4-24, has been lacking.

The importance of several basic residues (Arg-126, Arg-128, Lys-134 and Lys-136; all substituted in mutant B4-24) for efficient interaction between PAPP-A and IGFBP-4 was supported by cleavage analysis of synthetic peptides derived from IGFBP-4. However, for cleavage to occur, the peptide must extend to at least position −14, as in peptide P9, the shortest peptide with which proteolysis was observed (Table 1). This result supports an important role in enzyme–substrate interaction for residues N-terminal to those substituted in mutant B4-24, in agreement with the data obtained with mutant B4-29 (Figure 3A). Cleavage of synthetic peptides has not been reported previously for PAPP-A. Importantly, in addition to information about enzyme–substrate interactions, our finding that PAPP-A is capable of cleaving a peptide without any influence of IGF (Table 1) clearly demonstrates that IGF is not an activator of PAPP-A. Rather, enhanced proteolysis of IGFBP-4 by PAPP-A occurs because IGFBP-4 becomes a better substrate when it binds IGF, as found recently with intact IGFBP-4 [4].

Because IGFBP-5 is also a PAPP-A substrate [4], and because most of the residues found to be important for proteolysis of IGFBP-4 are also present in IGFBP-5 (Figure 5), we further asked whether our results are relevant to the proteolysis of this substrate. Eight IGFBP-5 mutants with residues substituted by alanine were analysed (Figures 3B and 6). Of interest, mutant B5-6 showed cleavage that could not be distinguished from cleavage of wild-type IGFBP-5. This is very surprising, since the four residues substituted in this mutant correspond precisely to the residues substituted in IGFBP-4 mutant B4-24, which could not be cleaved. However, in IGFBP-5 it appears that Lys-128 (at position −16) plays a more important role than the corresponding residue of IGFBP-4, Lys-120. All three mutants in which Lys-128 was replaced (B5-1, -2 and -3) completely resisted proteolysis by PAPP-A (Figures 3B and 6).

Previously, a proteinase-resistant IGFBP-5 mutant was reported in which Lys-138 and Lys-139 were both replaced by asparagine [36]. The inability of this mutant to be cleaved (by an unknown proteinase) may be the result of a direct effect of the substitutions, as discussed above. However, it may also be caused by steric hindrance of carbohydrate groups, as a potential glycosylation site is introduced with this mutation. In the mutated protein, Thr-141 and the two preceding residues conform to the standard signature for N-linked carbohydrate attachment, i.e. Asn-Xaa-Ser/Thr. The latter explanation is supported by the

observed slower migration of this mutant on SDS/PAGE compared with wild-type IGFBP-5 [36]. However, although the cause of the resistance may be uncertain, this mutant still has proven very useful in the study of cellular effects of the IGFBP-5 molecule [36]. Recent data demonstrate that mutated IGFBP-5 with both Lys-138 and Lys-139 substituted by alanine is cleaved as efficiently as wild-type IGFBP-5 (V. Rodacker, L. Laursen, M. Overgaard and C. Oxvig, unpublished work), pointing to steric hindrance as the explanation for the resistance of the K138N/K139N mutant.

Can a motif be defined that is recognized by PAPP-A? As discussed above, certain similarities exist between IGFBP-4 and -5 around their cleavage sites (Figure 5). In the stretch from position −16 to the scissile bond, both substrates have several basic residues in the same positions, and it is tempting to speculate that these residues play analogous roles, although their relative importance may vary. In both IGFBP-4 and -5, the lysine residue at position −16 is possibly the most critical residue in substrate recognition and/or cleavage. It has recently been shown that wild-type PAPP-A is capable of cleaving a specific peptide bond of its own by autoproteolysis (Figure 5) [15]. Interestingly, alignment of the relevant sequence stretches of PAPP-A, IGFBP-4 and -5, according to the cleavage sites (Figure 5), shows that only one position, −16, is occupied by a basic residue in all three sequences.

No general picture exists of the substrate specificity of the other metzincins, except that it appears that an extended conformation of a substrate is required, and that residues in positions from −3 to +3 interact with residues of the active-site cleft. Different metzincin families do show more or less well defined preferences at individual positions, none of which matches the sequence in the vicinity of Met-135/Lys-136 of IGFBP-4 [16]. Of particular interest, metzincins in general can cleave relatively short synthetic peptide substrates [19]. It has been found that a peptide substrate for astacin requires five residues as a minimum, and that an optimal astacin substrate has seven or more residues, which is considered unusual [37].

Thus, to our knowledge, the finding that peptide residues distant from the scissile bond are critical in substrate binding is unusual. However, PAPP-A shows very interesting similarities with a proteinase of the mitochondrial matrix, MPP [38]. MPP recognizes and cleaves the N-terminal presequence of nuclear-encoded proteins targeted for the mitochondria. With other members of the so-called inverzincin group of zinc metalloproteinases [39], which include pitrilysin and insulin-degrading enzyme, MPP shares the so-called inverted zinc-binding motif (HXXEH rather than HEXXH) [40]. The natural substrates of MPP are a wide variety of presequence-containing proteins, in which no simple motif exists. However, studies with synthetic peptide substrates and mutated protein substrates have revealed that several residues of a presequence are critical for efficient cleavage [38,41–43]. Based on such studies, binding of the substrate in a flexible conformation [44] has been proposed. This model is supported by recent crystallographic data showing binding of a presequence peptide substrate in an extended conformation [45].

Of specific interest, positively charged residues are abundant in presequences, and they appear to be critical for presequence cleavage. In particular, positively charged residues at position −2 or −3, and at about position −10, are often present and important for efficient cleavage, as is the length of the peptide substrate, but no stringent rules have been defined [38,42]. In one study, the minimal length of a peptide required for its effective cleavage was 16 residues (extending from positions −11 to +5) [42]. Interestingly, conserved acidic residues of MPP are known

to be involved in substrate recognition [45,46], and proteolysis is sensitive to ionic strength, indicating the importance of electrostatic interactions [47].

We thus believe that PAPP-A and MPP may share an unusual mode of substrate interaction, based primarily on the presence of several basic residues on the N-terminal side of the scissile bond, some of which are located distant from the cleavage site. In both systems, it appears to be difficult to estimate the importance of individual residues at specific positions. Further, equivalent enzyme–substrate interactions may be possible for particular basic residues of the substrate, as long as they are positioned within a given distance from the bond that is cleaved. Interestingly, PAPP-A and MPP are both very sensitive to ionic strength, pointing at interactions between the basic substrate residues and acidic residues of PAPP-A. More work is required to delineate details of the interaction of individual residues in the different PAPP-A substrates. This may lead to the formulation of a set of specific requirements to be fulfilled for a PAPP-A substrate that currently remain undefined.

While our data strongly support critical roles for distant residues, we cannot distinguish between roles in *bona fide* substrate binding and roles in the process of substrate recognition. It is possible that certain substrate residues contribute to a required presentation of the substrate to the proteolytic domain. It is also possible that substrate residues interact with a hypothetical substrate-binding exosite of PAPP-A. This would be by analogy with the proteolysis of collagen by collagenase [48]. In this system, substrate recognition is mediated through the non-proteolytic hemopexin-like domain of the enzyme. Further, it has recently been discovered that the hemopexin domain of gelatinase A plays an important role in the cleavage of chemokines by direct interaction with substrate [49,50].

We recently determined that proMBP functions as a physiological inhibitor of PAPP-A [14]. The C-terminal half of proMBP, corresponding to the mature 117-residue MBP as found in eosinophil leucocytes, is extremely basic (pI ≈ 11) [51], and, in the covalent complex between proMBP and PAPP-A, proMBP is disulphide-bound to a cysteine residue present in the proteolytic domain of PAPP-A [13,15]. No mechanism has been established that explains the inhibitory properties of proMBP, but, based on the findings presented here, a model in which the basic portion of proMBP interferes with the binding of substrate to PAPP-A should be considered. Further, inhibition of proteolysis of IGFBP-4 has also been shown in the presence of basic, possibly heparin-binding, peptides [9,25–27], which may also function competitively to prevent substrate binding.

The data presented herein represent our initial analysis of enzyme–substrate interactions of a new metzincin family of metalloproteinases, the pappalysins, of which PAPP-A is a founder member [15]. Our conclusions are based on analyses of two different mutated substrates and of synthetic peptides. All data concurrently suggest a complex mode of substrate recognition and/or binding, pointing at important roles for basic residues at certain positions. Although PAPP-A and MPP do not share the exact same determinants of specificity, the parallels between these two enzymes are intriguing, and may be useful in future experimental designs aimed at understanding the mechanisms of PAPP-A proteolysis. Analogies with other proteinase systems in which the substrate interacts with an exosite of the enzyme should also be considered. Furthermore, the proteinase-resistant variants of IGFBP-4 and -5 (mutants B4-24 and B5-1) will be useful for the study of proteolysis in cell-based systems. Our finding that a synthetic peptide can be cleaved by PAPP-A provides the basis for the development of quantitative assays for the investigation of the kinetic parameters of PAPP-A. In

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