Inhibition of proteolysis by the proform of eosinophil major basic protein (proMBP) requires covalent binding to its target proteinase

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Abstract **By proteolytic cleavage of insulin-like growth factor binding proteins, the metalloproteinase pregnancy-associated plasma protein-A (PAPP-A) is able to control the biological activity of insulin-like growth factors. PAPP-A circulates in pregnancy as a proteolytically inactive complex, disulfide bound to the proform of eosinophil major basic protein (proMBP). We here demonstrate that co-transfection of mammalian cells with PAPP-A and proMBP cDNA results in the formation of a covalent PAPP-A/proMBP complex in which PAPP-A is inhibited. Formation of the complex also occurs when PAPP-A and proMBP synthesized separately are incubated. Complex formation was monitored by Western blotting, and by using an immunoassay specific for the complex. Using mutagenesis, we further demonstrate that the complex forms in a specific manner and depends on the presence of two proMBP cysteine residues. Mutated proMBP, in which Cys-51 and -169 are replaced by serine, is unable to form the covalent complex with PAPP-A. Of particular interest, such mutated proMBP further lacks the ability to inhibit PAPP-A. For the first time, this conclusively demonstrates that proMBP is a proteinase inhibitor. We further conclude that proMBP inhibits PAPP-A in an unusual manner, not paralleled by other proteinase inhibitors of our knowledge, which requires proMBP to be covalently bound to PAPP-A by disulfide bonds. ProMBP binding to PAPP-A most likely either abrogates substrate access to the active site of PAPP-A or induces a conformational change in the structure of PAPP-A, as we, by further mutagenesis, were able to exclude that the inhibitory mechanism of proMBP is based on a cysteine switch-like mechanism.**

Key words: Proform of eosinophil major basic protein; Pregnancy-associated plasma protein-A; Insulin-like growth factor binding protein; Proteolysis; Proteinase inhibition

1. Introduction

Pregnancy-associated plasma protein-A (PAPP-A) is a 400 kDa dimeric protein, which belongs to the metzincin superfamily of metalloproteinases [1–3]. It regulates the availability of insulin-like growth factor (IGF)-I and -II by cleavage of IGF binding protein (IGFBP)-4 at one site, resulting in lowered affinity for IGF [4,5]. IGFBP-5 [6] and -2 [7] are also PAPP-A substrates in vitro, but their biological significance is currently less clear.

PAPP-A functions in many biological systems, including the ovary [8,9], the cardiovascular system [10,11], and the skeletal system [12,13]. During human pregnancy, PAPP-A is synthesized in the placenta [14] from which it is secreted abundantly into the maternal circulation. PAPP-A exists there primarily as a covalent, disulfide-bound 2:2 complex of 500 kDa with the proform of eosinophil major basic protein (proMBP) [15], also synthesized in the placenta, but in a different type of cell [14]. The proMBP polypeptide of 206 residues is highly glycosylated [16,17] and migrates in sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) as a smear above 50 kDa [15].

The mature, 117 residue MBP is known from the eosinophil leukocyte as a cytotoxic protein, but no evidence suggests that proMBP synthesized outside the eosinophil is proteolytically processed. In the bone marrow, MBP is generated during maturation of the eosinophils [18]. With a calculated isoelectric point of 10.8, mature MBP is highly basic, but as the propiece is highly acidic (pI = 4.0), proMBP has an isoelectric point of 6.0. The propiece is believed to mask the cytotoxicity of MBP in eosinophils prior to its deposition in cytoplasmic granules [19]. The three-dimensional structure of neither PAPP-A nor proMBP is known, but the structure of the mature MBP isolated from eosinophils was recently solved [20].

Interestingly, the PAPP-A/proMBP complex in the maternal circulation lacks proteolytic activity [21]. However, the activity of unfractionated pregnancy serum against IGFBP-4 is caused by the existence of about 1% uncomplexed PAPP-A, and possibly by a minor fraction of PAPP-A, that binds proMBP in a 2:1 stoichiometry, suggesting that proMBP inhibits the activity of PAPP-A [21]. Furthermore, the activity of endogenous PAPP-A of human fibroblasts can be inhibited by transfection with proMBP cDNA [22]. There are no re-

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Abbreviations: PAPP-A, pregnancy-associated plasma protein-A; proMBP, proform of eosinophil major basic protein; IGF, insulin-like growth factor; IGFBP, IGF binding protein

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ports of other proteins with inhibitory function towards PAPP-A.

Herein, we describe a system that we have established to generate the recombinant PAPP-A/proMBP complex in vitro. We further describe the effects of specific mutations in proMBP on complex formation and on inhibitory activity. In particular, we unequivocally demonstrate that proMBP is a proteinase inhibitor of PAPP-A, and that only covalently bound proMBP is inhibitory.

2. Materials and methods

2.1. Plasmid constructs

Human placental oligo-dT-primed cDNA [23] was used as a template to amplify cDNA encoding proMBP ([24], accession number Y00809). Specific primers containing an *Nhe*I site (5′-CGGCTAGC-TAGC**ATGAAACTCCCCCTACTTCTG**-3′) and an *Xho*I site (5′-CGCCGCTCGAG**TCAGTAGGAACAGATGAAAGG**-3′) were used, and the resulting polymerase chain reaction (PCR) product was blunt end-ligated into pCR-Blunt II-TOPO (Invitrogen). The cDNA was excised from this vector with *Nhe*I and *Xho*I and cloned into the mammalian expression vector pcDNA3.1+ (Invitrogen) to generate pcDNA3.1-proMBP. Mutagenesis of pcDNA3.1-proMBP was carried out by overlap extension PCR [25]. In brief, outer primers were 5′-CCCCATTGACGCAAATGGGCGG-3′ (5′ end) and 5′-AGGAA-AGGACAGTGGGAGTGG-3′ (3′ end) (nt 760–781 and nt 1117–1097, respectively, of pcDNA3.1+). Internal primers with an overlap of ~22 bp were used to generate mutated fragments that were digested with *Nhe*I/*Xho*I and swapped into the wild-type construct. Four mutants (proMBP-C89S, proMBP-C128S, proMBP-C147S, proMBP-C201S) with single cysteine residues substituted by serine were made. A double mutant (proMBP-C51S/C169S) was made by two consecutive rounds of PCR, in which a C51S mutant of proMBP was used as a template in the second round. All PCR reactions were carried out with *Pfu* DNA polymerase (Promega), and all constructs were verified by sequence analysis. Plasmid DNA for transfection was prepared using the QIAprep Spin Kit (Qiagen).

2.2. Tissue culture and transfection

Human embryonic kidney 293T cells (293tsA1609neo) [26] were maintained in high glucose Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 2 mM glutamine, non-essential amino acids, and gentamicin (Life Technologies). Cells were plated onto 6 cm tissue culture dishes, and were transfected 18 h later by calcium phosphate co-precipitation [27] using 10 μg of plasmid DNA. The cells were transfected with either a proMBP expression vector or the PAPP-A expression vector [21], or co-transfected with both. After a further 48 h the supernatants were harvested and cleared by centrifugation.

2.3. Visualization of PAPP-A/proMBP complex formation

Formation of the PAPP-A/proMBP complex in culture medium of co-transfected cells was visualized by Western blotting following separation by SDS–PAGE in 3–8% precast Tris-acetate gels (Invitrogen). Prior to loading of the gel, samples were incubated in loading buffer without heating. After electrophoresis, the protein was blotted onto a polyvinylidene difluoride membrane, and the blots were blocked 30 min with 2% skimmed-milk powder dissolved in 50 mM Tris, 500 mM sodium chloride, 0.1% Tween 20, pH 9.0 (TST), and then washed and equilibrated in TST. Primary antibody (mAb 234-2 for PAPP-A, and mAb 234-10 for proMBP) [28] was diluted in TST containing 2% skimmed-milk powder, and blots were incubated for 1 h at room temperature. Incubation with peroxidase-conjugated secondary antibodies (P0260, Dako) diluted in TST was done for 0.5 h at room temperature. Proteins were visualized using enhanced chemiluminescence (ECL, Amersham). Similar experiments were carried out using wild-type PAPP-A and mutated proMBP, as described in the text.

2.4. Determination of protein concentration by enzyme-linked immunosorbent assay (ELISA)

Measurements of concentrations of PAPP-A and proMBP in culture supernatants were carried out by sandwich ELISAs, in which polyclonal rabbit anti-(PAPP-A/proMBP) [16] was used for capture, and monoclonal antibodies against PAPP-A (234-2) [28] or proMBP (234-10) [29] followed by peroxidase-conjugated anti-(mouse IgG) (P0260, Dako) were used for detection. Blocking was done using phosphate-buffered saline (PBS) with 2% bovine serum albumin (BSA). For dilution of antibodies, PBS containing 0.01% Tween-20 (PBST) and 1% BSA was used. PBST was used for washing. Specific measurement of the PAPP-A/proMBP complex was carried out in a double monoclonal assay using a PAPP-A monoclonal (234-5) for capture and a biotinylated proMBP monoclonal (VRPM-2, V. Rodacker, M. Overgaaard, K. Mortensen, H. Sperling-Petersen and C. Oxvig, unpublished) and peroxidase-conjugated avidin (P0347, Dako) for detection. In the latter assay, sample dilution and washing after sample incubation were carried out using PBST to which 800 mM sodium chloride was added. All standard curves were based on the 2:2 PAPP-A/proMBP complex purified from pregnancy serum [16].

2.5. In vitro formation of the PAPP-A/proMBP complex after separate synthesis

Culture supernatants containing recombinant PAPP-A and proMBP were mixed and incubated at 37°C while shaking (800 rpm). The final concentration of PAPP-A subunit was 20 nM (4.0 μg/ml), and the final concentration of proMBP subunit (wild-type or mutant) was 200 nM (7.6 μg/ml), but lower (20–40 nM) in experiments in which the effect of proMBP concentration was analyzed. Samples were taken out and frozen at defined time points from 0 to 72 h, and PAPP-A/proMBP complex formation was visualized by Western blotting (see Section 2.3) and quantified by the complex specific ELISA (see Section 2.4). Mutants proMBP-C89S and proMBP-C128S were first purified by affinity chromatography using mAbs 234-8 and -10 [28] coupled to cyanogen bromide-activated Sepharose 4B (Amersham Pharmacia Biotech), and then mixed with medium from mock-transfected cells, as they were found to express at a lower level than wild-type proMBP.

2.6. Measurements of proteolytic activity

Proteolytic activities of PAPP-A-containing samples were assayed as described previously for IGFBP-4 and -5 [6], and IGFBP-2 [7]. In brief, PAPP-A (0.6 nM, 0.12 μg/ml) was incubated at 37°C with purified, ^{125}I-labeled IGFBP-4 or -2 (10 nM, 0.30 μg/ml) contained in 50 mM Tris, 100 mM sodium chloride, 1 mM calcium chloride, pH 7.5. Incubations were carried out in the presence of added IGF-II (50 nM, 0.35 μg/ml) (Bachem). Similarly, assays for proteolytic activity against IGFBP-5 were carried out incubating with PAPP-A (0.2 nM, 0.04 μg/ml) in the absence of added IGF-II. Samples of the reaction mixtures, taken out at time points from 0 to 30 min, were separated by non-reducing SDS–PAGE (10–20% Tris-glycine Laemmli gels). The degree of cleavage was assessed by measuring band intensities with a PhosphorImager (Molecular Dynamics), and plotted as a function of time after subtraction of background, as previously reported [6].

3. Results and discussion

3.1. In vitro formation of the PAPP-A/proMBP complex

We co-transfected 293T cells with cDNAs encoding human PAPP-A and proMBP, and analyzed the culture supernatant by Western blotting using a monoclonal antibody against PAPP-A (Fig. 1A). PAPP-A from co-transfected cells migrated with a higher molecular weight compared to the PAPP-A dimer secreted from cells transfected with PAPP-A cDNA only (Fig. 1A, lane 4 vs. lane 2), for the first time demonstrating complex formation between PAPP-A and proMBP in vitro. Compared to the native PAPP-A/proMBP complex purified from pregnancy serum (Fig. 1A, lane 3), the recombinant complex migrated slightly faster, in agreement with the known difference in glycosylation between recombinant and native PAPP-A [21], and a possible similar difference between recombinant and native proMBP. We cannot exclude that a minor fraction of PAPP-A exists in a 2:1 complex with proMBP, but no PAPP-A was detected at the position of uncomplexed PAPP-A dimer in the co-transfection experi-

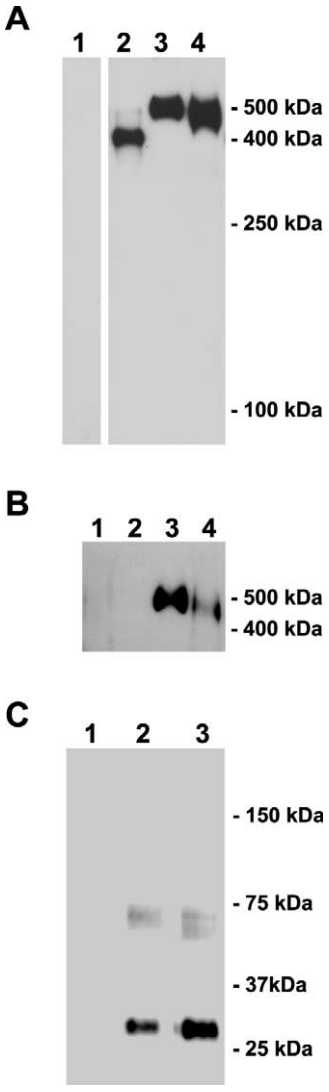


Fig. 1. In vitro formation of the PAPP-A/proMBP complex by co-expression of PAPP-A and proMBP cDNA in human embryonic kidney 293T cells. A: Culture supernatants from cells transfected with proMBP cDNA (lane 1), PAPP-A cDNA (lane 2), or PAPP-A and proMBP cDNA together (lane 4) were separated by non-reducing SDS–PAGE (3–8% gels) and analyzed by Western blotting using a monoclonal antibody against PAPP-A. Native PAPP-A/proMBP isolated from pregnancy serum is shown for comparison (lane 3). B: A similar experiment using a proMBP mAb. C: Culture supernatants from cells transfected with empty vector (lane 1), or with proMBP wild-type cDNA (lane 2) or proMBP-C51S/C169S cDNA (lane 3) were separated by non-reducing SDS–PAGE (10–20% gel) and analyzed by Western blotting using a monoclonal antibody against proMBP.

ment. The same samples were also analyzed with a monoclonal antibody against proMBP (Fig. 1B), confirming our interpretation. Analysis of proMBP expressed alone shows that the vast majority of the protein migrates as a monomer of approximately 28 kDa (Fig. 1C, lane 2). Traces of dimeric proMBP appear to be present, but no proMBP multimers can be detected.

In human pregnancy, PAPP-A and proMBP are synthesized separately in different cell types of the placenta [14]; yet the vast majority (approximately 99%) of circulating PAPP-A is complexed to proMBP [21]. In accordance with this, we find that the recombinant PAPP-A/proMBP complex is also formed specifically when PAPP-A and proMBP synthesized separately are incubated. We used 20 nM of PAPP-A and 200 nM of proMBP, reflecting the average concentrations of both components in pregnancy plasma, and also the molar ratio between the two [30]. The complex formation was monitored by SDS–PAGE (Fig. 2A) and by a complex-specific ELISA, in which a PAPP-A mAb was used for catching and a proMBP mAb for detection (Fig. 2B). Under the current conditions and concentrations used, the vast majority of PAPP-A was covalently bound to proMBP after about 48 h of incubation. The amount of complex measured at the plateau level (20 nM) is equal to the molar amount of PAPP-A added to the reaction, further demonstrating that the complex formed has a 2:2 stoichiometry. A similar experiment was carried out in which the concentration of proMBP was lowered to 40 nM (Fig. 2A). As expected, PAPP-A is converted into PAPP-A/proMBP complex at a slower rate, and the pla-

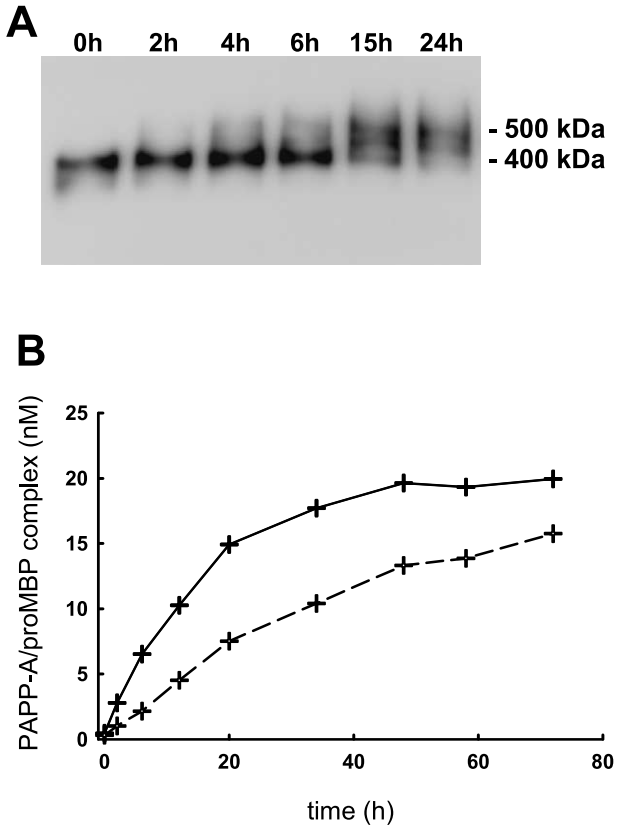


Fig. 2. In vitro formation of the PAPP-A/proMBP complex by incubation of recombinant PAPP-A and proMBP synthesized separately. A: Cells were transfected separately with PAPP-A and proMBP cDNA, and culture supernatants containing PAPP-A (20 nM) and proMBP (200 nM), respectively, were mixed and incubated at 37°C after quantification by ELISA. Samples taken out at defined time points, as indicated, were separated by SDS–PAGE and analyzed by Western blotting with a PAPP-A monoclonal antibody. After 4 h of incubation, recombinant PAPP-A/proMBP complex could be detected, and after 24 h, very little uncomplexed PAPP-A was seen. B: The amount of complex, as estimated using an ELISA specific for the PAPP-A/proMBP complex, was measured in samples from a similar experiment and plotted against time (unbroken line). The plateau level of complex, reached after about 50 h (20 nM, 4.0 μg/ml), corresponded to the total amount of PAPP-A measured at 0 h and 72 h (not shown), demonstrating that the complex formed has a 2:2 stoichiometry. A similar experiment is shown in which the concentration of proMBP was lowered to 40 nM (dashed line).

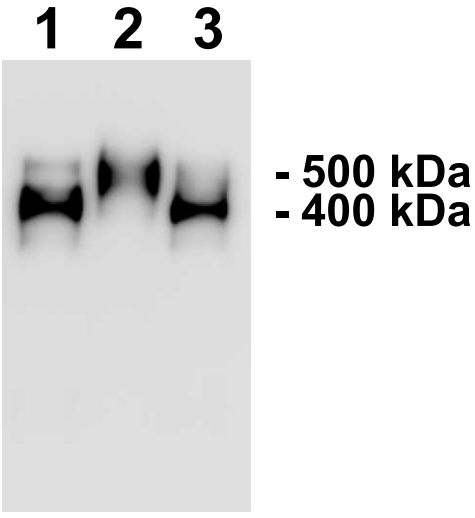


Fig. 3. Substitution of Cys-51 and Cys-169 by serine abrogates complex formation. Recombinant PAPP-A was incubated (48 h) with mock medium (lane 1), with proMBP wild-type protein (lane 2), or with a mutated variant of proMBP (proMBP-C51S/C169S) (lane 3). Protein was visualized by Western blotting using a PAPP-A monoclonal antibody.

teau level is not reached within 72 h. At equimolar concentrations (20 nM of both PAPP-A and proMBP), about 10 nM of complex had formed after 72 h (not shown).

3.2. Substitution of proMBP cysteine residues abrogates complex formation

The complete disulfide pattern of the 2:2 PAPP-A/proMBP complex isolated from pregnancy serum was recently solved [31]. Within this complex, each PAPP-A subunit is connected to a proMBP subunit by two disulfide bridges, which involve Cys-51 and Cys-169 of proMBP. Thus, to obtain a variant of proMBP unable to form those two interchain disulfides, we made an expression construct encoding mutated proMBP, in which both of these residues are substituted by serine, proMBP-C51S/C169S. Expressed alone, this mutant migrates like wild-type proMBP in SDS–PAGE (Fig. 1C, lanes 2 and 3). Importantly, when proMBP-C51S/C169S is incubated with wild-type PAPP-A, the covalent complex is not formed (Fig. 3). The same observation was made with co-expression experiments (not shown).

This result provides us with a tool to study the implications of covalent linkage of the two proteins. In addition, it also further emphasizes that the process of complex formation between PAPP-A and proMBP is highly specific, even though proMBP may contain unpaired cysteine residues with free sulfhydryl (–SH) groups [31]. In the mature MBP, such cysteine residues are known to promote (self)polymerization [32].

3.3. Inhibition of PAPP-A by proMBP requires covalent complex formation

We then compared the proteolytic activity of recombinant PAPP-A dimer with the activity of the recombinant PAPP-A/proMBP complex. In a time course experiment using IGFBP-4 as the substrate, we found that PAPP-A alone rapidly cleaved IGFBP-4, whereas the recombinant complex cleaved very little IGFBP-4 (Fig. 4A). This experiment is in agreement with our earlier finding that the specific activity of the PAPP-A/proMBP complex of pregnancy serum is negligible compared to that of the recombinant PAPP-A dimer [21]. Previously, we were unable to exclude an effect of an unidentified inhibitory component. The experiments presented here conclusively demonstrate that proMBP functions as a proteinase inhibitor of PAPP-A. The weak, residual activity of the recombinant PAPP-A/proMBP complex (see inset of Fig. 4A) is likely explained by incomplete complex formation: a small subpopulation of the PAPP-A molecules may have escaped complex formation with proMBP, although uncomplexed PAPP-A could not be detected by Western blotting. Alternatively, some PAPP-A dimers may bind only one proMBP molecule in a partially inhibited, 2:1 complex. We are currently unable to distinguish between these two possibilities. It should be stressed, as irreversibility of the process of complex formation can reasonably be assumed, that estimation of a proMBP K_i value is meaningless.

Of particular interest, when a similar experiment was carried out using the proMBP-C51S/C169S mutant, no reduction of the proteolytic activity of PAPP-A was seen (Fig. 4A). This strongly suggests that proMBP must be covalently bound to PAPP-A to function as an inhibitor. The same conclusion was drawn from similar experiments using IGFBP-5 (Fig. 4B) or IGFBP-2 (not shown) as PAPP-A substrates. Curiously, the residual proteolytic activity of the recombinant PAPP-A/proMBP complex appeared to be slightly higher towards IGFBP-5 when compared to IGFBP-4 and -2.

To eliminate the possibility that the proMBP-C51S/C169S mutant lacks inhibitory potential as a hypothetical, non-covalent inhibitor because it is altered structurally in a non-specific manner, we also carried out measurements of proteolytic activity against the IGFBPs immediately after the mixing of PAPP-A and wild-type proMBP, i.e. before a significant amount of complex had formed. As we see no difference in activity with and without wild-type proMBP (Fig. 4C), this experiment conclusively demonstrates that proMBP is only inhibitory when covalently bound to PAPP-A.

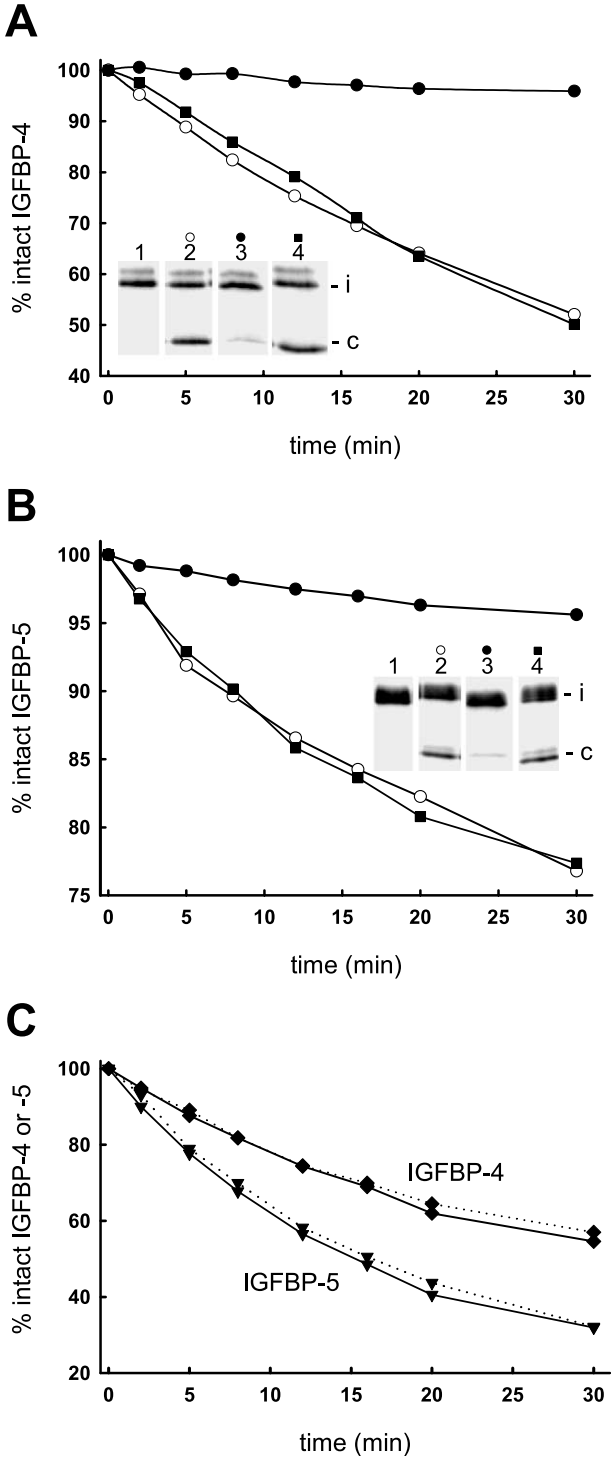
A faint band is present above the 400 kDa PAPP-A dimer in lane 1 of Fig. 3. We variably observe this in Western blots of PAPP-A (it amounts to less than 1% of the PAPP-A immunoreactivity in the similar experiment of Fig. 1A, lane 1). Based on its lack of reactivity with proMBP antibodies (Fig. 2B, lane 3), this band does not represent PAPP-A/proMBP complex, hypothetically formed from endogenous proMBP of the mammalian cell line used.

3.4. The inhibitory mechanism does not involve a cysteine switch

For protein inhibitors of proteinases, possible modes of inhibition generally include (1) binding directly to the active site in a substrate-like manner, (2) binding adjacent to the active site (or to an exosite) indirectly preventing substrate binding, or (3) binding distant from the active site thereby allosterically preventing proteolysis [33]. The inhibitory activity of the vast majority of known proteinase inhibitors is based on non-covalent interaction [33], but the serpins (serine proteinase inhibitors) [34] and α2-macroglobulin [35] are known to form covalent complexes with their target endoproteases.

What is the role of the covalent, disulfide bond connections in the inhibited PAPP-A/proMBP complex? In one simple model, the two disulfides serve to increase the binding energy between PAPP-A and proMBP. ProMBP would then function by blocking access to the active site, although it would not

bind directly to the substrate binding site of the active site. The disulfide structure of the PAPP-A/proMBP complex suggests that proMBP interacts with the proteolytic domain of PAPP-A and therefore likely is located in close proximity to the active site in the three-dimensional structure. In an alternative model, as disulfide bonds are formed and probably also broken in the process of complex formation, a conformational change of PAPP-A may allosterically cause it to become inactive. The latter model is difficult to evaluate, as the pattern of disulfide bonds is only resolved for the PAPP-A/proMBP complex, not the uncomplexed PAPP-A dimer.



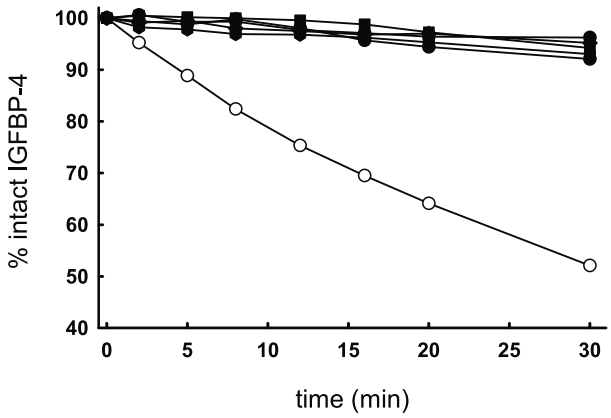


Fig. 5. The inhibitory activity of proMBP does not depend on cysteine residues with free sulfhydryl groups. PAPP-A was preincubated (48 h) with mock medium (open circles), with wild-type proMBP (filled circles), or with mutated proMBP (proMBP-C89S, filled hexagons; proMBP-C128S, filled squares; proMBP-C147S, filled triangles; proMBP-C201S, filled diamonds). The plot shows cleavage of IGFBP-4.

We speculated that a side chain sulfur atom of a proMBP cysteine residue not engaged in a disulfide bond might interact directly with the active site zinc atom of PAPP-A. Such interaction could critically contribute to positioning and/or binding of proMBP to the active site of PAPP-A, in analogy with the cysteine switch mechanism known to operate in latent collagenase [36] and other metalloproteinases [2]. As mentioned, based on the disulfide structure of PAPP-A/proMBP, specific cysteine residues of proMBP could potentially function in this manner, Cys-89, Cys-128, Cys-147, and Cys-201 [31].

We expressed proMBP mutants with those cysteines individually substituted by serine, proMBP-C89S, proMBP-C128S, proMBP-C147S, and proMBP-C201S, respectively. All mutants formed a covalent complex with PAPP-A as well as did wild-type proMBP (not shown), and we were unable to detect any difference in their inhibitory properties towards PAPP-A (Fig. 5). Based on this experiment, no cys-

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Fig. 4. Inhibition of PAPP-A by proMBP requires covalent complex formation. Measurements of proteolytic activity were carried out using ^{125}I-labeled IGFBPs, each cleaved at one site by PAPP-A into two fragments of similar size. Samples were taken out at time points from 0 to 30 min, and the percentage of intact IGFBP in each sample was determined after separation by SDS–PAGE, as illustrated in the insets. The concentrations of all reagents were kept constant in each experiment. A: The plot shows the proteolytic activity against IGFBP-4 of PAPP-A preincubated (48 h) with mock medium (open circles), with wild-type proMBP (filled circles), or with mutated proMBP (proMBP-C51S/C169S), unable to form the PAPP-A/proMBP complex (filled squares). The gel inset shows activity (at $t = 30$ min) of negative control (mock, lane 1), PAPP-A (lane 2), PAPP-A/proMBP complex (lane 3), and PAPP-A with proMBP-C51S/C169S (lane 4). Positions of intact (i) and cleaved (c) IGFBP-4 are indicated. B: A similar experiment carried out with IGFBP-5 as the PAPP-A substrate. C: A similar experiment, but without preincubation of PAPP-A and proMBP. The plot shows cleavage of IGFBP-4 (filled diamonds) in the absence (solid line) and presence (dotted line) of wild-type proMBP, and likewise cleavage of IGFBP-5 (filled triangles) in the absence (solid line) and presence (dotted line) of wild-type proMBP. Within the time frame of this experiment, no difference in proteolytic activity could be detected, in agreement with the absence of detectable covalent complex.

teine side chain of proMBP interacts directly with the zinc atom of the active site of PAPP-A.

Basic residues of both IGFBP-4 and -5 are known to be important in PAPP-A substrate recognition [37]. Even though covalent binding of proMBP to PAPP-A is required for inhibition, substrate-like interactions with basic residues of proMBP might still be important. Further studies are required to evaluate the possible influence on inhibitory activity of individual basic proMBP residues.

3.5. Concluding remarks

We have demonstrated that proMBP forms a covalent, disulfide-bound complex with PAPP-A when the two molecules are co-expressed in mammalian cells or incubated together after separate synthesis. The recombinant PAPP-A/proMBP complex forms in a specific manner, and it lacks proteolytic activity. This conclusively demonstrates that proMBP functions as a proteinase inhibitor. We further show that the ability of proMBP to function as an inhibitor depends on its covalent linkage to PAPP-A. To our knowledge, this represents a unique mode of inhibition, not paralleled by any proteinase–inhibitor pair. The system we have established will allow the process of complex formation as well as the inhibitory mechanism of proMBP to be further studied. We are currently unable to distinguish between possible inhibitory mechanisms, including steric hindrance and allosteric inhibition, but we can exclude that the inhibitory mechanism is based on an interaction between a sulfhydryl group of proMBP and the active site zinc atom of PAPP-A, in a cysteine switch-like mechanism.

Basic residues of both IGFBP-4 and -5 are known to be important in PAPP-A substrate recognition [37]. Even though covalent binding of proMBP to PAPP-A is required for inhibition, substrate-like interactions with basic residues of proMBP might still be important. Further studies are required to evaluate the possible influence on inhibitory activity of individual basic proMBP residues.

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