The metzincin metalloproteinase pregnancy-associated plasma protein-A (PAPP-A, pappalysin-1) promotes cell growth by proteolytic cleavage of insulin-like growth factor-binding proteins 4 and 5, causing the release of bound insulin-like growth factors. PAPP-A binds an unknown cell-surface heparan sulfate proteoglycan, suggesting that it controls insulin-like growth factor signaling spatially. In human pregnancy, the majority of PAPP-A circulates as a disulfide-bonded complex with its inhibitor, the proform of eosinophil major basic protein (proMBP). Interestingly, Ser-62 of proMBP is substituted with a glycosaminoglycan (GAG) chain, possibly a heparan sulfate type, and the PAPP-A-proMBP complex is unable to bind to the cell surface. We show here that proMBP detaches surface-bound PAPP-A in a process that depends on the proMBP GAG and also on the formation of intermolecular disulfide bonds between PAPP-A and proMBP. Unlike what was expected, we demonstrate that the GAG of proMBP is not required for PAPP-A-proMBP complex formation and that proMBP residues His-137, Ser-178, Arg-179, and Asn-181 are important for the recognition of PAPP-A. Using a mouse model, we find that the half-life of circulating PAPP-A and proMBP in complex is severalfold higher than both of the uncomplexed proteins, further suggesting that the PAPP-A-proMBP complex is formed at the cell surface in vivo rather than in the circulation. Further supporting this, we show that formation of the PAPP-A-proMBP complex at the cell surface proceeds rapidly compared with the slow rate of complex formation in solution. Because both PAPP-A and proMBP are expressed ubiquitously, this model may be applicable to many tissues in which insulin-like growth factor bioavailability is locally regulated.

The metzincin metalloproteinase pregnancy-associated plasma protein-A (PAPP-A<sup>2</sup>, pappalysin-1) cleaves insulin-like growth factor-binding proteins 4 and 5 (1, 2), which generally function to antagonize the activity of insulin-like growth factors (IGF)-I and -II. The cleavage of insulin-like growth factor-binding protein in complex with IGF results in the release of bioactive IGF, and the responsible proteinase is consequently an important modulator of IGF activity (3). Accordingly, the proteolytic activity of PAPP-A is believed to be involved in a number of physiological and pathological processes known to involve IGF signaling, e.g. ovarian follicular development (4), human implantation (5), fetal development (6–8), wound healing (9), and atherosclerosis (10).

The binding of PAPP-A to an unknown heparan sulfate proteoglycan directs its proteolytic activity to the surface of cells (11), suggesting that surface binding controls PAPP-A-mediated IGF release and consequently IGF signaling spatially. Mapping experiments localized the site responsible for specific surface binding to complement control protein (CCP) modules 3 and 4 in the C-terminal region of PAPP-A, which contains five consecutive CCP modules (11, 12). These modules, originally known from proteins associated with the complement system, are responsible for interactions with glycosaminoglycans (GAGs) in several other proteins (13, 14).

The proteolytic activity of PAPP-A is inhibited physiologically by the proform of eosinophil major basic protein (proMBP) (15) that also binds to a cell surface heparan sulfate proteoglycan (16). The inhibitory activity of proMBP requires the formation of a disulfide-mediated covalent proteinase-inhibitor complex, denoted PAPP-A-proMBP, a 2:2 heterotetramer of 500 kDa held together by 7 intermolecular disulfide bonds (15, 17, 18). Complex formation between PAPP-A and proMBP is a relatively slow process in vitro but is accelerated severalfold by micromolar concentrations of reductant (17). The PAPP-A-proMBP complex was first discovered in the cir...
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culation of pregnant women (19) where its origin is the placenta (20), but both PAPP-A and proMBP are ubiquitously expressed at lower levels (21). Other known covalent proteinase-inhibitor complexes, e.g. complexes of serpins or α2-macroglobulin with their target proteinases, are rapidly cleared from circulation (22, 23). However, it is unknown whether complex formation with proMBP mediates the clearance of PAPP-A.

ProMBP is substituted with a single GAG chain, possibly of the heparan sulfate type, covalently bound to Ser-623 (24). Such covalent modification of soluble proteins is unusual and restricted to a distinct set of proteoglycans, e.g. bikunin, a component of inter-α-trypsin inhibitor, which is substituted with a chondroitin sulfate GAG that functions to bind the additional subunits (25). The function of the proMBP GAG is unknown, but the PAPP-A-proMBP complex does not exhibit cell surface binding, and surface binding can be regained after the treatment of the PAPP-A-proMBP complex with heparinase (11).

In this study we demonstrate that proMBP detaches PAPP-A from the cell surface in a process that depends on GAG covalently bound to proMBP and requires the formation of intermolecular PAPP-A-proMBP disulfide bonds. Unlike what was expected, the recognition between PAPP-A and proMBP is not mediated by protein-GAG interactions. Rather, we identify proMBP residues His-137, Ser-178, Arg-179, and Asn-181 as part of a site responsible for the recognition of PAPP-A. The PAPP-A-proMBP complex is formed rapidly at the cell surface compared with the slow rate of complex formation in solution, indicating that the redox potential of the tissue microenvironment controls the process. Using a mouse model, we find that the half-life of the PAPP-A-proMBP complex is severalfold higher than the uncomplexed components. This further supports that the circulating PAPP-A-proMBP complex in human pregnancy is formed at the surface of cells within the placenta rather than in the circulation.

EXPERIMENTAL PROCEDURES

Mutagenesis—Site-directed mutagenesis of proMBP cDNA was carried out by overlap extension PCR (26) using proMBP cDNA contained in pcDNA3.1+ (pcDNA3.1-proMBP (15)) as the template. Outer primers derived from pcDNA3.1+ were 5′-CCCCA-TTGACGCAAATGGGCGG-3′ (5′ end) and 5′-AGGAAAGGACGATGGGAGTGG-3′ (3′ end). Internal primers with an overlap of ~22 bp were used to generate mutated fragments that were digested with Nhel/Xhol and swapped into the wild-type construct. PCRs 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). At least two independent clones of each construct were used for further analysis.

Cell Culture and Transfection—Human embryonic kidney 293T cells (293tsA1609neo) (27) were maintained in high glucose Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 2 mM glutamine, nonessential amino acids, and gentamicin (Invitrogen). Cells (4.5 × 10⁶) were plated onto 6-cm culture dishes and transfected 18 h later by calcium phosphate co-precipitation (28) using 10 μg of plasmid DNA. The cells were transfected with either a proMBP or a PAPP-A expression vector, and the medium was harvested 48 h post-transfection. In different experiments cells transfected with PAPP-A cDNA or co-transfected with both PAPP-A and proMBP cDNAs were harvested and analyzed by flow cytometry. All proMBP mutants expressed at a level of 10–20 μg/ml, similar to the level of wild-type proMBP, as determined by enzyme-linked immunosorbent assay (ELISA) (see below).

In Vitro Formation of the PAPP-A-ProMBP Complex—PAPP-A-proMBP complex formation experiments were performed as described (15). In brief, culture medium from 293T cells transfected separately with PAPP-A or proMBP cDNAs were mixed to final concentrations of 20 nM PAPP-A and 200 nM proMBP. The proteins were incubated at 37 °C, and samples were taken out and frozen at defined time points between 0 and 48 h. Complex formation was quantitatively monitored by an ELISA specific for the complex (see below). The effect of GAGs on PAPP-A-proMBP complex formation was studied by the addition of 0 or 100 μg/ml heparan sulfate (16,500 g/mol), chondroitin sulfate (14,900 g/mol), or dermatan sulfate (17,800 g/mol) (gifts of Kristian Johansen, LEO Pharma) to the reaction mixtures.

Increasing concentrations of NaCl were also added to the mixture to study the effect of increasing ionic strength on PAPP-A-proMBP complex formation. PAPP-A-proMBP complex formation was also studied using purified PAPP-A (variant E483A (29)) and proMBP diluted to final concentrations of 20 and 200 nM, respectively, in 50 mM Tris, 100 mM NaCl, 100 μM CaCl₂, pH 7.4. PAPP-A and proMBP were purified by immunoaffinity chromatography using a PAPP-A specific (mAb 234-5 (30)) or a proMBP specific (mAb 234-10 (30)) monoclonal antibody immobilized to CNBr-activated Sepharose 4B (GE Healthcare). Complex formation at the surface of cells was analyzed in 96-well culture plates (Nunc) with ~60,000 cells/well. After 24 h, the culture medium was replaced with conditioned medium from cells expressing PAPP-A and incubated for 1 h on ice. The cells were washed 3 times with culture medium and subsequently incubated at 37 °C with conditioned medium from cells expressing proMBP or proMBP variants. Samples of culture medium were frozen at defined time points (0–16 h) and subjected to the complex-specific ELISA. The cells of each well were detached with 50 μl of PBS supplemented with 5 mM EDTA and 1 M NaCl to measure surface-bound PAPP-A by ELISA. In other experiments complex formation was monitored using 20 nM PAPP-A and 100 nM proMBP without preincubation on ice and without washing the cells.

ELISA—The concentrations of PAPP-A and proMBP were measured by sandwich ELISAs in 96-well microtiter plates (Nunc) using polyclonal rabbit anti-(PAPP-A-proMBP) (31) for capture. Monoclonal antibodies against PAPP-A (PA-1A) or proMBP (PM-5A) followed by peroxidase-conjugated anti-

[^3]: The numbering of the 222-residue proproMBP polypeptide (Swiss-Prot accession number P13727) is used. The numbering of the 1547-residue mature PAPP-A polypeptide is used in this report. Glu-1 of mature PAPP-A corresponds to position 81 of proPAPP-A (Swiss-Prot accession number Q13219).

PBS containing 0.01% Tween 20 supplemented with 800 mM and washing after sample incubation were carried out using an ELISA specific for the complex that was based on PAPP-A and proMBP monoclonal antibodies (15). Complex formation is expressed relative to the amount of complex formed after 48 h (100%). The gel inset shows a Western blot of PAPP-A incubated with wild-type proMBP for 0 h (lane 1) or 48 h (lane 2) using a PAPP-A specific monoclonal antibody. B, complex formation by the incubation of PAPP-A with either wild-type proMBP (circles) or proMBP(S62A) (squares) and monitored over time by ELISA. The gel inset shows wild-type proMBP (lane 1) and proMBP(S62A) (lane 2) analyzed by reducing SDS-PAGE followed by Western blotting using polyclonal antibodies. GAG-substituted proMBP migrates as a high molecular weight species, and it is seen that wild-type proMBP is only partially GAG-substituted. In contrast, proMBP(S62A) migrates exclusively as the low molecular weight species not substituted with GAG (16). Complex formation is expressed relative to the amount of complex formed after 48 h (100%). C, PAPP-A:proMBP complex formation using purified PAPP-A and purified wild-type proMBP (filled circles) or purified proMBP(S62A) (filled squares) contained in 50 mM Tris, 100 mM NaCl, 100 μM CaCl2, pH 7.4. Complex formation is expressed relative to the amount of complex formed after 48 h (100%). D, PAPP-A:proMBP complex formation after 48 h in the presence of increasing concentrations of NaCl. Results are the averages of at least three independent experiments. S.D. are indicated.

Fig. 1. PAPP-A:proMBP recognition is not mediated by protein-GAG interactions. A, PAPP-A:proMBP complex formation in the absence (filled circles) or presence (empty circles) of heparan sulfate (0 or 100 μg/ml). NaCl to avoid non-covalent association. A dilution series of the PAPP-A:proMBP complex purified from pregnancy serum (31) was used to establish standard curves.

Western Blotting—Western blotting of wild-type proMBP and proMBP(S62A) was performed after separation by SDS-PAGE (10–20% Tris-glycine gels). After electrophoresis, proteins were blotted onto a polyvinylidene difluoride membrane and blocked in 2% skimmed-milk powder diluted in 50 mM Tris, 500 mM sodium chloride, 0.1% Tween 20, pH 9.0 (TST). After washing and equilibration in TST, the membrane was incubated with a PAPP-A specific monoclonal (mAb 234-5) or polyclonal rabbit anti-PAPP-A:proMBP (diluted to 1 or 2.5 μg/ml, respectively, in TST containing 2% skimmed-milk powder, and incubated for 1 h at room temperature. Incubation with peroxidase-conjugated secondary antibodies (P0217, DAKO) diluted in TST containing 2% skimmed-milk powder was done for 0.5 h at room temperature. The blots were developed using enhanced chemiluminescence (ECL, Amersham Biosciences), and images were captured with an Eastman Kodak Co. Image Station 1000.

Circular Dichroism (CD) Spectroscopy—Wild-type proMBP and selected mutants were purified by immunoaffinity chromatography (see above). The purity of the proteins was assessed by SDS-PAGE, and the purified proteins were dialyzed against 20 mM NaH2PO4, 20 mM NaF, pH 7.4. Protein concentrations were determined by amino acid analysis (32). Three CD spectra were recorded at 25 °C for each protein on a Jasco J-810 spectropolarimeter. (Jasco Spectroscopic, Japan) using a polypeptide concentration of ~0.1 mg/ml and a cuvette of 2-mm path length. CD data were obtained in the range from 260 to 190 nm at a resolution of 1 nm using a bandwidth of 1.0 nm. The scan speed was 100 nm/min, and the response time was 1 s. To assess the stability of the wild-type and mutant proteins, temperature scans from 30 to 90 °C at intervals of 10 °C in the range from 240 to 200 nm were also carried out. Δε (expressed in millidegrees × cm⁻¹ × m⁻¹) were calculated on the basis of a mean molar mass of 110 g/mol/residue.

Flow Cytometry—Transfected cells detached with PBS containing 5 mM EDTA were washed with cold Dulbecco’s modified Eagle’s medium (Invitrogen) containing 2% fetal bovine serum (Dulbecco’s modified Eagle’s medium/fetal bovine serum) and incubated on ice for 1 h with a PAPP-A-specific (mouse IgG) (P0260, DAKO) were used for detection. The wells were blocked by incubation with PBS containing 2% bovine serum albumin. Antibodies were diluted in PBS containing 0.01% Tween 20 (PBST) and 1% bovine serum albumin. PBST was used for washing. To assure that the binding of monoclonal antibody PM-5A to the proMBP mutants was not altered as a result of the amino acid substitutions, the expression levels of proMBP were also verified by ELISA using rabbit anti-(PAPP-A:proMBP) polyclonal antibody for capture and biotinylated rabbit anti-(PAPP-A:proMBP) followed by incubation with peroxidase-conjugated avidin (P0347, DAKO) for detection. The amount of PAPP-A:proMBP complex formed under different conditions was specifically measured as described (15). In brief, a PAPP-A specific monoclonal antibody (PA-1A) was used as the catching antibody, and the PAPP-A:proMBP complex was detected with a biotinylated, proMBP specific monoclonal antibody (PM-5A) followed by incubation with peroxidase-conjugated avidin. In this assay, sample dilution and washing after sample incubation were carried out using PBS containing 0.01% Tween 20 supplemented with 800 mM NaCl to avoid non-covalent association. A dilution series of the PAPP-A:proMBP complex purified from pregnancy serum (31) was used to establish standard curves.
Detachment of Cell Surface-bound PAPP-A by Its Inhibitor

A

TCRYLLVRSLQTPFQAMTWCRCTGKNLVIWNVWNYQCCVSLNHDNYFGRT

110 120 130 140 150 160

170 180 190 200 210 220

GSQRFFQVVDGSNWFATWAAHPWSRGCGTCGLCTAGSYMRCACRLLRPPP

B

Cell surface binding

C

R171 S166 R168

Cell surface binding site

mAb (234-5) (10 μg/ml). After three washes in Dulbecco’s modified Eagle’s medium/fetal bovine serum, the cells were incubated for 30 min on ice with fluorescein isothiocyanate goat anti-mouse IgG (1:50) (Zymed Laboratories Inc.). After 3 washes, the cells were suspended in PBS and analyzed on a Beckman Coulter CytoFacs FC 500 MPL flow cytometer. In other experiments non-transfected cells were detached and incubated (1 h on ice) with culture medium containing PAPP-A (10 μg/ml) in the absence or presence of purified proMBP (100 μg/ml) before staining.

C

PAPP-A recognition site

RESULTS

The ProMBP GAG Does Not Mediate Recognition between PAPP-A and ProMBP—In one model formation of the covalent proteinase-inhibitor complex between PAPP-A and proMBP (PAPP-A-proMBP) requires an initial interaction between the proMBP GAG chain and CCP-3 and -4 of PAPP-A (11). However, the presence of heparan sulfate GAG (100 μg/ml) did not affect the formation of the covalent PAPP-A-proMBP complex (Fig. 1A). Similarly, we found that chondroitin or dermatan sulfate did not have any effect on complex formation (data not shown). In a different experiment the amount of PAPP-A-proMBP complex formed over time was similar for wild-type proMBP and proMBP(S62A), in which the single GAG-substituted residue of proMBP, Ser-62, is replaced with alanine (16) (Fig. 1B). This experiment was also carried out with purified proteins with similar results (Fig. 1C). Therefore, the recognition between PAPP-A and proMBP in the process of complex formation...
most likely does not depend on protein-GAG interactions. However, because increasing concentrations of salt inhibited PAPP-A-proMBP complex formation (Fig. 1D), other types of ionic interactions are likely to be important in the process.

**Mapping of ProMBP Residues Involved in the Recognition of PAPP-A**—Several basic residues of insulin-like growth factor-binding proteins 4 and 5 are known to be important for PAPP-A substrate recognition (33). We, therefore, speculated that basic residues of the highly basic MBP domain of proMBP might be involved in the proteinase-inhibitor interaction between PAPP-A and proMBP. Thus, the rate of PAPP-A-proMBP complex formation for a set of proMBP mutants with all surface-exposed basic residues substituted individually or in pairs of two for alanine was compared with wild-type proMBP (Fig. 2A and B). All mutants expressed at levels similar to wild-type proMBP, as measured by ELISA using polyclonal antibodies for capture and a proMBP-specific monoclonal antibody (PM-5A) for detection. The expression levels were verified using biotinylated polyclonal antibodies for detection, suggesting that the epitope of mAb PM-5A was not altered as a result of amino acid substitutions. Of the mutants, only proMBP(R179A) showed reduced complex formation (60%) compared with wild-type proMBP (Fig. 2B). Arg-179 is in proximity with His-137, Ser-178, and Asn-181 in the crystal structure of the MBP domain (34) (Fig. 2A and C). Accordingly, when these three residues were substituted individually by alanine, the ability to form the PAPP-A-proMBP complex was reduced by 50–60% (Fig. 2B). These mutants and wild-type proMBP migrated similarly in non-reducing SDS-PAGE (Fig. 3A), and all showed similar CD spectra (Fig. 3B). In addition, temperature scans were performed from 30 to 90 °C in the range from 240 to 200 nm for both wild-type proMBP (Fig. 3C) and the proMBP mutants (data not shown). The wild-type and mutant proteins all showed similar gradual loss of secondary structure with increasing temperature, measured at 208 nm (Fig. 3D), further suggesting that the substitutions did not affect the stability of proMBP. In addition, all
proteins were refolded after cooling from 90 °C, as the temperature scanning experiments could be largely reproduced for all proteins (data not shown). Therefore, the recognition of PAPP-A by proMBP is based on protein-protein interactions, and residues His-137, Ser-178, Arg-179, and Asn-181 are part of a PAPP-A recognition site of proMBP.

wild-type proMBP, the amount of remaining surface-bound PAPP-A was detected over time by ELISA (Fig. 6A). In contrast, no decrease in surface-bound PAPP-A was detected for cells incubated with proMBP(S62A) (Fig. 6C). This suggests that the proMBP GAG is required for proMBP to prevent PAPP-A surface binding.

Covalent PAPP-A-ProMBP Complex Formation Is Required for Surface Detachment of PAPP-A—We speculated that the proMBP GAG might compete with the PAPP-A receptor for binding to CCP-3 and -4. However, under conditions (1 h on ice) where no covalent PAPP-A-proMBP complex was formed, PAPP-A surface binding was not reduced by the presence of proMBP, as demonstrated using cells incubated with PAPP-A in the absence (Fig. 5A) or presence of excess wild-type proMBP (Fig. 5B) or proMBP(S62A) (Fig. 5C). We consequently tested if surface-bound PAPP-A can be detached by proMBP under conditions (0–16 h at 37 °C) that allow covalent complex formation using cells incubated first with PAPP-A and subsequently with wild-type proMBP, proMBP(S62A), or a proMBP mutant, proMBP(C51S/C169S) (15), unable to bind covalently to PAPP-A. For cells incubated with
incubated with wild-type proMBP, whereas only a small increase in the amount of complex was detected for cells incubated with either proMBP(S62A) or proMBP(C51S/C169S) (Fig. 6B). This conclusively demonstrates that 1) surface-bound PAPP-A is targeted and detached by proMBP, 2) the cell surface detachment of PAPP-A is mediated by the proMBP GAG, and 3) detachment requires the formation of intermolecular PAPP-A-proMBP disulfide bonds. In addition, the amount of PAPP-A-proMBP complex detected at the cell surface after 1 h of incubation was significantly higher for cells incubated with proMBP(S62A) than for cells incubated with wild-type proMBP (Fig. 6C), further suggesting that the covalent PAPP-A-proMBP complex is formed while the components are bound to the surface. Accordingly, the rate of PAPP-A-proMBP complex formation in the presence of cells was accelerated several-fold compared with formation of the complex under cell-free conditions (Fig. 6D). Thus, surface binding may serve to increase the effective concentrations of PAPP-A and proMBP and, hence, the rate of complex formation.

**Rapid Clearance of Uncomplexed PAPP-A and ProMBP in Mice**—Other known covalent proteinase-inhibitor complexes are rapidly removed from the circulation (22, 23). Using female C57BL/6jbm mice as a model, we found that uncomplexed PAPP-A and proMBP disappeared from the circulation with half-lives in the fast phase (α-phase \( t_{1/2} \)) of 0.670 and 2.49 min, respectively (Fig. 7A and Table 1). The vast majority of both PAPP-A (97.2%) and proMBP (88.1%) was cleared in the fast phase, and the half-lives of PAPP-A and proMBP in the slow phase (β-phase \( t_{1/2} \)) were 27.9 and 98.5 min, respectively. In striking contrast, the PAPP-A-proMBP complex disappeared only slowly from the circulation with an α-phase \( t_{1/2} \) of 34.8 min and a β-phase \( t_{1/2} \) of 1.48 × 10^3 min (Fig. 7B and Table 1). Furthermore, the amount of PAPP-A-proMBP cleared in the two phases was more equally distributed, with only 62.0% clearing in the α-phase. Similar results were obtained in eight female BALB/c mice. In these mice PAPP-A and proMBP cleared with half-lives of ~1 and 2–3 min, respectively, whereas the majority of the PAPP-A-proMBP complex was still found in the circulation after 30 min when the experiment was terminated.

These results demonstrate that the PAPP-A-proMBP complex formation does not promote clearance of PAPP-A. The rapid clearance of the uncomplexed components further suggests that the circulating PAPP-A-proMBP complex is formed at the surface of cells, not after secretion into the circulation of uncomplexed PAPP-A and proMBP.

**DISCUSSION**

In the present study we demonstrate that proMBP abrogates PAPP-A surface binding and that this requires proMBP to be substituted with GAG at Ser-62 (Fig. 4). Unlike expected, we find that the proMBP GAG does not mediate the recognition of PAPP-A (Fig. 1). This is in contrast to the formation of another covalent proteinase-inhibitor pair, thrombin-antithrombin-III, which critically depends on the binding of heparin-like glycosaminoglycans, serving as a template on which proteinase and inhibitor can interact (35, 36). Using site-directed mutagenesis, we further show that the recognition of PAPP-A by proMBP is based on protein-protein interactions rather than protein-

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**TABLE 1**

**Kinetic parameters characterizing the clearance of the PAPP-A-proMBP complex and its uncomplexed components in mice (C57BL/6jbm)**

The percentages of the administered sample cleared in the fast (\( C_1 \)) and the slow (\( C_2 \)) phase, and the values of the kinetic rate constants (\( k_1 \) and \( k_2 \)) of each phase were determined by fitting the clearance data to Equation 1 (see “Experimental Procedures”). The half-lives of the fast phase (α-phase \( t_{1/2} \)) and the slow phase (β-phase \( t_{1/2} \)) were defined as α-phase \( t_{1/2} = 0.693/k_1 \) and β-phase \( t_{1/2} = 0.693/k_2 \), respectively. S.E. are shown in parentheses.

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<th>( k_1 ) (min (^{-1}))</th>
<th>( t_{1/2} ) (min)</th>
<th>( C_2 ) (%)</th>
<th>( k_2 ) (min (^{-1}))</th>
<th>( t_{1/2} ) (min)</th>
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<td>38.0 (3.91)</td>
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<td>PAPP-A</td>
<td>97.2 (2.97)</td>
<td>1.03 (0.0339)</td>
<td>0.670</td>
<td>2.80 (0.260)</td>
<td>0.0248 (0.00577)</td>
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<td>ProMBP</td>
<td>88.1 (2.72)</td>
<td>0.278 (0.0202)</td>
<td>2.49</td>
<td>11.9 (1.73)</td>
<td>0.00704 (0.00204)</td>
<td>98.4</td>
</tr>
</tbody>
</table>
**Detachment of Cell Surface-bound PAPP-A by Its Inhibitor**

GAG interactions and identify proMBP residues His-137, Ser-178, Arg-179, and Asn-181 as part of a site responsible for the recognition of PAPP-A. These residues are located in the extended L2 loop and in the loop between S2 and H2 close to one another in the crystal structure of the MBP domain (Fig. 2, A and C) (34). The MBP domain of proMBP belongs to the C-type lectin super family (34). Known ligands of this family include both carbohydrates and proteins, and the ligand binding region in C-type lectins is usually formed by three extended loops and a β-strand, corresponding to L1, L3, L4, and S7 (34) (Fig. 2A). However, both the PAPP-A recognition site and the recently identified cell surface binding site (16) are located on the opposite side of the MBP domain compared with the C-type lectin ligand binding region (Fig. 2C), suggesting that proMBP is not a typical C-type lectin.

Heparan sulfate GAGs compete with the PAPP-A receptor for PAPP-A surface binding (11). However, we find that under experimental conditions where no covalent complex is formed, excess proMBP did not affect PAPP-A surface binding (Fig. 5). Conversely, under conditions that allowed covalent complex formation, surface-bound PAPP-A was rapidly targeted and detached by wild-type proMBP. In contrast, proMBP mutants proMBP(S62A) and proMBP(C51S/C169S) were unable to detach PAPP-A from the surface (Fig. 6, A and B), demonstrating that both GAG substitution at Ser-62 of proMBP and the formation of intermolecular PAPP-A/proMBP disulfide bonds are required for detachment of surface-bound PAPP-A.

The covalent PAPP-A-proMBP complex is formed at the cell surface (Fig. 6C). As a result, the proMBP GAG may be localized in close proximity to CCP-3 and -4, allowing it to effectively compete with the PAPP-A receptor, which in turn results in surface detachment of PAPP-A. Interestingly, the rate of PAPP-A-proMBP complex formation at the cell surface was accelerated severalfold compared with formation of the complex in the absence of cells (Fig. 6), suggesting that surface binding may serve to increase the effective concentrations of PAPP-A and proMBP. The complex formation is a multistep process of disulfide rearrangements that is influenced by the redox potential (17), and cell surface-associated factors, such as disulfide isomerases (37), potentially participate in the process. Consequently, pathological conditions of altered redox potential, e.g. placental hypoxia in preeclampsia, may change the balance between complexed and uncomplexed PAPP-A and, hence, affect IGF signaling.

How does proMBP lose affinity for its surface receptor before release of the PAPP-A-proMBP complex from the cell surface? Cys-169 is located in the loop recently identified as part of the proMBP surface binding site (16) (Fig. 2C). However, upon complex formation Cys-169 switches from participating in an intramolecular proMBP disulfide bond to the formation of the first intermolecular PAPP-A-proMBP disulfide bond with Cys-652 in PAPP-A (17). We speculate that this causes a conformational change of the loop resulting in the abrogation of proMBP surface binding.

The disulfide rearrangements upon formation of the PAPP-A-proMBP complex were recently delineated (17). Based on these and our present findings, we depict a model of how surface-bound PAPP-A is targeted and detached by proMBP (Fig. 8). In this model, proMBP binds a cell surface heparan sulfate proteoglycan and recognizes the surface-bound PAPP-A dimer by non-covalent protein–protein interactions. The initial recognition is followed by the formation of intermolecular PAPP-A/proMBP disulfide bonds accompanied by the reduction of an intramolecular proMBP disulfide bond, resulting in the inhibition of PAPP-A proteolytic activity (15), and therefore, the release of bound IGF cannot occur. Only one of the two subunits of the dimeric PAPP-A is shown. S—S and SH symbolize disulfide bonds and free sulfhydryls, respectively. Further details are given under “Discussion.”
A-proMBP disulfide bonds and the abrogation of proMBP surface binding. The redox balance upon complex formation is maintained, as the formation of the two intermolecular disulfides is accompanied by the reduction of another intramolecular proMBP disulfide bond (17). Covalent complex formation localizes the proMBP GAG in close proximity to CCP-3 and -4 of PAPP-A and allows it to effectively compete with the PAPP-A receptor, leading to the detachment of one PAPP-A subunit. A second proMBP then reacts with the uncomplexed PAPP-A subunit and dimerizes with the complexed proMBP subunit by the isomerization of two intramolecular disulfide bonds (17), causing detachment of the heterotetramer.

Covalent complexes of the serpins or α2-macroglobulin with their target proteinases are rapidly cleared from the circulation compared with their uncomplexed components (22, 23). In striking contrast, using a mouse model we find that the half-life of PAPP-A and proMBP in complex is increased severalfold compared with the rapid clearance of the uncomplexed components (Fig. 7). The half-life in the fast phase (∝ t½) of the PAPP-A-proMBP complex was 34.8 min, whereas uncomplexed PAPP-A and proMBP cleared with half-lives of 0.670 and 2.49 min, respectively (Table 1). For comparison, the half-lives in mice of the human thrombin-antithrombin-III complex and α2-macroglobulin-proteinase complexes are ∼3 and 2–5 min, respectively (22, 23), whereas uncomplexed α2-macroglobulin is removed slowly from the circulation with a half-life of several hours (38). Thus, the slow clearance rate of PAPP-A-proMBP may reflect an unknown function for the circulating complex. Interestingly, depressed levels of total PAPP-A in the circulation of pregnant women are associated with Down syndrome (8) and low birth weight (7), although it is unclear how this relates to known PAPP-A function. In this view, it will be interesting to assess the diagnostic value for pregnancy outcome of distinguishing between both complexed and uncomplexed circulating PAPP-A.

The rapid clearance of uncomplexed PAPP-A and proMBP further supports that the circulating PAPP-A-proMBP complex in human pregnancy is formed at the surface of placental cells rather than in the circulation. Of interest, a minor fraction (∼1%) of PAPP-A from human pregnancy serum is uncomplexed and proteolytically active (39). Our clearance data indicate that the turnover of uncomplexed PAPP-A is higher than would be expected from this fraction. During pregnancy, the main site of PAPP-A synthesis is the syncytiotrophoblasts and the extravillous trophoblasts of the placenta, whereas only extravillous trophoblasts synthesize proMBP (20). Thus, it is possible that circulating PAPP-A-proMBP complex originates primarily from the extravillous trophoblasts.

In conclusion, we have shown that proMBP recognizes and detaches cell surface-bound PAPP-A, and we have mapped the PAPP-A recognition site of proMBP. Based on our results, we depicted a hypothetical model of the process of PAPP-A cell surface detachment and inhibition (Fig. 8). First, proMBP recognizes PAPP-A by protein-protein interactions. Second, the intermolecular disulfide bonds between PAPP-A and proMBP are formed at the cell surface. Third, covalent complex formation allows the GAG of proMBP to effectively compete with the PAPP-A receptor for binding to CCP-3 and -4 of PAPP-A, leading to the detachment of the proteolytically inhibited PAPP-A-proMBP complex. Because both PAPP-A and proMBP are expressed ubiquitously, this model may be applicable to many tissues in which IGF bioavailability is locally regulated.

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Detachment of Cell Surface-bound PAPP-A by Its Inhibitor
Cell Surface Detachment of Pregnancy-associated Plasma Protein-A Requires the Formation of Intermolecular Proteinase-Inhibitor Disulfide Bonds and Glycosaminoglycan Covalently Bound to the Inhibitor

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