Cell surface targeting of pregnancy-associated plasma protein-A proteolytic activity.

Reversible adhesion is mediated by two neighboring short consensus repeats*

Lisbeth S. Laursen‡, Michael T. Overgaard‡, Kathrin Weyer‡, Henning B. Boldt‡, Peter Ebbesen§, Michael Christiansen¶, Lars Sottrup-Jensen‡, Linda C. Giudice#, and Claus Oxvig‡**

From the ‡Department of Molecular Biology, Science Park, University of Aarhus, Gustav Wieds Vej 10C, DK-8000 Aarhus C, Denmark, the §Department of Virus and Cancer, The Danish Cancer Society, DK-8000 Aarhus C, Denmark, the ¶Department of Clinical Biochemistry, Statens Serum Institut, DK-2300 Copenhagen S, Denmark, and the #Department of Gynecology and Obstetrics, Stanford University, Stanford, CA 94305.

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**To whom correspondence should be addressed (e-mail co@mb.au.dk).
SUMMARY

The activities of insulin-like growth factor (IGF)-I and -II are regulated by IGF binding proteins (IGFBPs). Cleavage of IGFBP-4 by the metalloproteinase pregnancy-associated plasma protein-A (PAPP-A) causes release of bound IGF, and has been established in several biological systems, including the human reproductive system. Using flow cytometry, we first demonstrate that PAPP-A reversibly binds to the cell surface of several cell types analyzed. Heparin and heparan sulfate, but not dermatan or chondroitin sulfate, effectively compete for PAPP-A surface binding, and, as incubation of cells with heparinase abrogated PAPP-A adhesion, it is probably mediated by a cell surface heparan sulfate proteoglycan. Furthermore, the proteolytic activity of PAPP-A is preserved while bound to cells, suggesting that adhesion functions to target its activity to the vicinity of the IGF receptor, decreasing the probability that released IGF is captured by another IGFBP molecule before receptor binding. This mechanism potentially functions in both autocrine and paracrine regulation, as PAPP-A need not be synthesized in a cell to which it adheres. A truncated PAPP-A variant, without the five short consensus repeats (SCRs) in the C-terminal third of the 1547-residue PAPP-A subunit, lacked surface binding. We also show that PAPP-A2, a recently discovered IGFBP-5 proteinase with homology to PAPP-A, does not bind cells. This finding allowed further mapping of the PAPP-A adhesion site to SCR modules 3 and 4 by the expression and analysis of nine PAPP-A/PAPP-A2 chimeras. Interestingly, the proteolytically inactive, disulfide bound complex of PAPP-A and the proform of eosinophil major basic protein (proMBP), PAPP-A/proMBP, shows only weak surface binding, probably because the adhesion site of PAPP-A is occupied by heparan sulfate, known to be covalently bound to proMBP. This hypothesis was further substantiated by demonstrating that heparinase treatment of PAPP-A/proMBP restores surface binding. We finally propose a model, in which IGF bioactivity is regulated by reversible cell surface binding of PAPP-A, which in turn is regulated by proMBP.
INTRODUCTION

Insulin-like growth factors (IGF)-I and -II have potent anabolic and mitogenic actions in vivo and in vitro, mediated primarily through the type 1 IGF receptor (1). Their biological activities are regulated by six different, homologous IGF-binding proteins (IGFBP-1 to -6), which bind the IGFs with high affinities and thus, in some cases, prevent them from binding to the receptor (2). As a regulatory mechanism, proteolytic cleavage in the central region of the IGFBPs causes dissociation of bound IGF (3).

The identities of the proteinases responsible for cleavage in vitro of individual IGFBPs are often unknown. However, it has recently been established that the metalloproteinase pregnancy-associated plasma protein-A (PAPP-A) is responsible for cleavage of IGFBP-4 (4,5). This was first found using conditioned medium from human fibroblasts and osteoblasts (4), but it has also been demonstrated that PAPP-A is the IGFBP-4 proteinase secreted from vascular smooth muscle cells (6,7) and granulosa cells (8). In addition, recent data suggests the involvement of PAPP-A and IGFBP-4 in human implantation (9), and in ovarian follicular development (10-12). Furthermore, it has been found that IGFBP-5 is also a PAPP-A substrate, but unlike the cleavage of IGFBP-4 by PAPP-A, which is dramatically increased by IGF, the cleavage of IGFBP-5 by PAPP-A is slightly inhibited by IGF (13). Where, and to what extent, PAPP-A functions as an IGFBP-5 proteinase in vivo is currently not known. Interestingly, IGFBP-5, but not IGFBP-4, is known to interact with the cell surface (14).

The metzincin superfamily of metalloproteinases is a diverse group of zinc endopeptidases, which includes the astacins, the reprolysins, the serralysins, and the matrix metalloproteinases (MMPs) (15). The proteolytic domains of metzincins have common sequence elements, including an elongated zinc-binding motif (HEXXHXXGXXH) (16), and they share a common fold, but specific structural features distinguish families from one another (15). The proteolytic domain of PAPP-A fulfills the criteria of a metzincin, but cannot be grouped into one of the mentioned four families. It rather belongs, as a founder member, to a fifth metzincin family, the pappalysins (17).
In addition to the proteolytic domain of about 300 residues (17), the PAPP-A subunit contains three lin-notch modules (LNR-1-3, each of 26-27 residues), two of which are inserted into the proteolytic domain, and five short consensus repeats (SCR-1-5, each of 57-77 residues) located in the C-terminus of the protein (18). The SCR module is also known as the complement control protein (CCP) module. It occurs frequently in complement proteins, but within the metzincins it is unique to the pappalysins. PAPP-A shares its domain organization only with PAPP-A2, a recently discovered second member of the pappalysin family (19,20). PAPP-A and PAPP-A2 are both synthesized as prepro-proteins of 1627 (21) and 1791 (19) residues, respectively. There is no homology between their prepro-peptides, but 46% of the residues of mature PAPP-A are also present in mature PAPP-A2. PAPP-A2 cleaves IGFBP-5 (and -3), not IGFBP-4, as determined by analysis of recombinant protein (19).

During human pregnancy, the 1547-residue PAPP-A circulates as a disulfide bound 2:2 complex with the proform of eosinophil major basic protein (proMBP) (22). In the PAPP-A/proMBP complex, proMBP functions as an inhibitor of PAPP-A enzymatic activity (5). Measurable PAPP-A activity of pregnancy serum results from a minor fraction (< 1%) of PAPP-A which exists as a homodimer, not complexed to proMBP (5), and possibly also from partially inhibited 2:1 complexes. In the human placenta, PAPP-A and proMBP mRNAs are primarily synthesized in different cell types, and therefore the covalent PAPP-A/proMBP complex is formed in the extracellular compartment (23). Compared to other tissues, the placental levels of PAPP-A and proMBP mRNAs are relatively high (24), in accordance with the circulating levels in pregnancy.

Uncomplexed, native proMBP has never been isolated, but the proMBP subunit has been prepared from reduced and carboxymethylated PAPP-A/proMBP complex by denaturing gel filtration, and found to be extensively substituted (39% (w/w)) with N- and O-linked carbohydrates (25). Of particular interest, Ser-62 of the 222-residue preproMBP is substituted with a heparan sulfate glycosaminoglycan (GAG) (26).

We here demonstrate and analyze cell surface interactions of PAPP-A. We find that PAPP-A, but not PAPP-A2, binds glycosaminoglycan on the surface of cells, and by using PAPP-A/PAPP-A2 chimeras, the binding site is located to SCR-3 and -4. A functional role of PAPP-A proteolytic activity in proximity to the
cell surface is suggested. Further, we propose a model in which the adhesion and the activity of PAPP-A are both regulated by proMBP.
Plasmid Construction - An expression plasmid encoding the proform of human PAPP-A, proPAPP-A, was constructed by a procedure similar to the construction of an expression plasmid for the mature form of human PAPP-A (pcDNA3.1-PAPP-A), previously described in detail (5). A series of oligonucleotides encoding the 2 C-terminal residues of the signal peptide (5), the propeptide of preproPAPP-A (residues 23-80), and the first five residues of the mature PAPP-A were used to generate template DNA (5’- CaaGacCGGCaCGtACGAGAACCtGaGCtG GaCGAC-3’, 5’- CtcCtaGcctGCtGCtGaCCtGCACCTGctGCtAC-3’, 5’- CaGacG GCCCtaGcCcTaGcGtTaGCaGcGCgGC-3’, 5’- GcGacGCaGCaGcGtcGtACGTGcGaGTaCc-3’, 5’- CaCGtCgtCGGCAGCAGaGAaGCTAGGGGC GCCCAT-3’, and 5’- tCTGCTGCGCaCGaCGtACtACtCACaCCTCCCAt-3’, 5’- GTcTgtCCtGCtGCtGCcGCtGCtGCtGCtACaCttG-3’, 5’- GaGGtCtaGGCCTCGGTaGCACAGTGcGtCCaGtCC-3’, 5’- aGCaGcGtcGtCtaGGGTCgCtcGaCtGdraGgtCT-3’, 5’- CTaCGrGCcGCtCGrTGCTCCCGCGGTACTACGT-3’). Nucleotides corresponding to residues 23-80 of preproPAPP-A are underlined. To facilitate sequence analysis and amplification by the polymerase chain reaction (PCR), the content of G/C nucleotides were decreased without changing the encoded amino acid sequence. Changes made, compared to the proPAPP-A cDNA sequence, are indicated by lower cases letters. Primers for the PCR were 5’-AGTACGTagCCCGcGGAAcGaC-3’ (nucleotides of preproPAPP-A are underlined, SacII site is in bold) and 5’-ATGGCGCCCCCtGCTCC-3’ (nucleotides of preproPAPP-A are underlined, Bbel site is in bold). The resulting PCR product was cloned into the SacII/Bbel sites of pcDNA3.1-PAPP-A, to generate the proPAPP-A expression plasmid (pcDNA3.1-proPAPP-A).
A plasmid construct (pA-C1210STOP) encoding a PAPP-A variant with a stop codon introduced just N-terminal to the five SCR modules was made with QuickChange (Stratagene) using the PAPP-A plasmid pB989-1547 as a template, as previously described (17). The primers used were 5'-
CAGTTTTTCTCATGCGAAGTGACGCAGC-3' and 5'-
CACTTCGATGAGAAAACCTGACTGTCAGAGC-3' (mutated nucleotides are shown in lowercase). The mutated PAPP-A cDNA fragment was then swapped into a variant of pcDNA3.1-PAPP-A, encoding a proteolytically inactive protein, in which Glu-563 is substituted with Gln (17).

Constructs encoding chimeric PAPP-A/PAPP-A2 subunits with N-terminal PAPP-A sequence and C-terminal PAPP-A2 sequence were prepared by overlap extension PCR (27) using the PAPP-A expression plasmid pPA-BspEI (17) and the PAPP-A2 expression plasmid pPA2-mH (19) as templates. In brief, outer primers were (5'-CATCATCGGACAGCCACGACATC-3') (corresponding to PAPP-A residues 1041-1047) and (5'-TTGCCTCTAGAGcaactaagggcagctg-3') (lowercase sequence correspond to nt 1114-1135 of pcDNA3.1/Myc-His(-)A (Invitrogen), XbaI site is in bold). The resulting PCR products were swapped into the KpnI/XbaI sites of pcDNA3.1-PAPP-A. Internal primers were (nucleotides derived from PAPP-A2 are lowercase): 5’-GGAGATGCAAaggaatgccacccaccccc-3’ and 5’-
agcattctcTTGCATCTCCTGGCGACATG-3’ for chim1 (PAPP-A residues 1-1475), 5’-
TTGTGTTCTGtgggtgtgagccacc-3’ and 5’-ggcattctcAGGAACACAAGCTCCCTCTTG-3’ for chim2 (PAPP-A residues 1-1412), 5’-GTGTGAGCTGAgtgtgtgtcagctcccc-3’ and 5’-
catcacacteGAGCTCACACAGGGCCTCTG-3’ for chim3 (PAPP-A residues 1-1344), 5’-
CTTGAGCCAggggattggtttcgcrtg-3’ and 5’-ccagtcacTGGGCTCACTGCACACCTGC-3’ for chim4 (PAPP-A residues 1-1282), and 5’-GAAAACCTGACagctgtcctcctgcctgac-3’ and 5’-
aagcaagctGTCAGTTTTCTCACATGCGA-3’ for chim5 (PAPP-A residues 1-1214).

Using these constructs (chim5, chim3, chim2, and chim1) as templates, a second series of PAPP-A/PAPP-A2 chimeras with internal PAPP-A2 sequence were prepared using the same 5' outer primer and a 3’ outer primer derived from the vector sequence of pcDNA3.1-PAPP-A (5’-
GCAAAACAGATGGGTGCAACTAG-3’) (nt 1037-1062 of pcDNA3.1+, Invitrogen). Internal primers were (nucleotides derived from PAPP-A2 are lowercase): 5’-CACAGGTCAAggaatgcagctg-3’ and
5’-ctgcattcctGTGACCTGTGACCCACCTC-3’ for chim6 (PAPP-A residues 1-1344 + 1413-1627), 5’-AGCACTGGCCttgagattctcacacaact-3’ and 5’-gaatctgcaaGGCCAGTGCTCGGTTCCAAAC-3’ for chim7 (PAPP-A residues 1-1412 + 1476-1627), 5’-AGCACTGGCCttgagattctcacacaact-3’ and 5’-gaatctgcaaGGCCAGTGCTCGGTTCCAAAC-3’ for chim8 (PAPP-A residues 1-1344 + 1476-1627), and 5’-CGAGGCACATcaacttgcagtagacttcag-3’ and 5’-ctgcaagttgATGTGCCTCGCTCCAC-3’ for chim9 (PAPP-A residues 1-1214 + 1345-1475). All plasmid constructs were verified by sequence analysis.

_Tissue Culture and Transfection_ - Human embryonic kidney 293T cells (293tsA1609neo) were maintained in high glucose DMEM medium supplemented with 10% fetal bovine serum, 2 mM glutamine, nonessential amino acids, and gentamicin (Invitrogen) (5). Cells were plated onto 6 cm tissue culture dishes, and were transfected 18 h later by calcium phosphate co-precipitation using 10 µg of plasmid DNA (5). Plasmid constructs used for transfection were: pcDNA3.1-proPAPP-A, pcDNA3.1-PAPP-A, pPA2 (encoding preproPAPP-A2), pA-C1210STOP, and chimeric PAPP-A/PAPP-A2 constructs as detailed above. In other transfection experiments, cells were co-transfected with plasmid DNA encoding the αM (CD11b) and the β2 (CD18) integrin subunits (28), which associate as the leukocyte integrin Mac-1 on the cell surface. Other cell lines (JAR, BHK-21, JEG-3) were obtained from American Type Culture Collection (ATCC) and cultured in the same medium. A primary culture of normal human skin fibroblasts was obtained and cultured as described (29).

_ELISA_ - The levels of recombinant PAPP-A in the supernatants were measured by a standard sandwich ELISA. PAPP-A polyclonal antibodies, anti(PAPP-A/proMBP) (25), were used for capture, and a PAPP-A monoclonal antibody (mAb 234-2) (30) followed by peroxidase conjugated anti(mouse IgG) (P260, DAKO) for detection. PAPP-A/proMBP purified from pregnancy serum (25) was used to establish standard curves.

_Flow Cytometry_ - Transfected 293T cells were detached with phosphate-buffered saline (PBS) containing 5 mM EDTA 48 h post transfection, and washed with cold L15 medium (Sigma) containing 2% FBS (L15/FBS), and incubated on ice with primary antibody against PAPP-A (234-2 or 234-5 (30), in all cases with similar results) or integrin Mac-1 (2LPM19c, DAKO) at 10 µg/ml for 1 h. After washing three
times in L15/FBS, the cells were incubated with fluorescein isothiocyanate goat antimouse IgG (Zymed) diluted 1 to 50 for 30 min. After three washes, the cells were suspended in PBS with 2% paraformaldehyde and analyzed on a Becton-Dickinson flow cytometer. At least 5.000 cells were analyzed. In other experiments, non-transfected cells (293T, JAR, BHK-21, JEG-3, human fibroblasts) were detached with PBS/5 mM EDTA 24 h after splitting, incubated on ice for 1 h with culture supernatant (100 µl) containing 0-5 µg/ml PAPP-A or 0-200 µg/ml PAPP-A/proMBP complex purified from pregnancy serum (25), and then washed prior to incubation with primary antibody. The number of cells were kept constant in all series of experiments. The effect of glycosaminoglycans on PAPP-A surface binding was studied by preincubation of the PAPP-A containing culture supernatant for 30 min on ice with 0-100 µg/ml low molecular weight (approximately 6000 g/mol) heparin (Sigma), heparan sulfate (16500 g/mol), chondroitin sulfate (14900 g/mol), or dermatan sulfate (17800 g/mol) (gifts of Kristian Johansen, LEO Pharma). The effect of heparinase (heparinase III (EC number 4.2.2.8) purchased from Sigma, or heparinase I (EC number 4.2.2.7) purified from a culture of Flavobacterium heparinum, a gift of Kristian Johansen) was studied by preincubation of cells for 1 h with 0.3 mIU/ml of enzyme at 37 °C followed by washing of the cells. The effect of preincubation of PAPP-A or PAPP-A/proMBP complex with heparinase was also studied: Recombinant PAPP-A containing supernatant or purified PAPP-A/proMBP complex (0.25 mg) was incubated for 1 h with 1 mIU/ml of heparinase I at 37 °C. To separate PAPP-A or PAPP-A/proMBP from the heparinase, PAPP-A mAb 234-2 immobilized on protein G agarose beads (Life Technologies) was added to the digest, followed by washing in 50 mM NaCl, 50 mM Tris, pH 7.5. PAPP-A antigen was then eluted with 0.1M glycine, pH 3.0, and the pH was adjusted to 7.5 by the addition of 0.5 M Tris. The concentrations of purified protein were measured by amino acid analysis. The absolute fluorescence varied for similar samples with the batch of secondary antibodies used, and with instrument settings, but all comparisons were made with these parameters kept constant.

Western Blotting - For immunovisualization of PAPP-A, protein separated by nonreducing SDS-PAGE (5-15% Tris-glycine gels), were blotted onto a polyvinylidene difluoride membrane (Millipore). Blots were allowed to dry, and then incubated with primary antibody (polyclonal anti(PAPP-A/proMBP) (25))
diluted in 10 mM sodium phosphate, 150 mM sodium chloride, 0.01% Tween 20, pH 7.2 (PBST) containing 2% (w/v) skimmed milk powder for 1 h at room temperature, according to the protocol of the manufacturer (Millipore). Blots were washed in PBST, and further incubated with secondary antibody (P217, DAKO) diluted in PBST with 2% (w/v) skimmed milk powder, and then washed again with PBST. The blots were developed using enhanced chemiluminescence (ECL, Amersham). Immunovisualization of proMBP following reducing SDS-PAGE was carried by the same procedure.

Measurement of Proteolytic Activity - Purified recombinant IGFBP-4 (3 µg) (17) was 125I-labeled, and cleavage analysis was performed as previously described (13). In brief, approximately 5 ng labeled (50,000 cpm) and 0.5 µg unlabeled IGFBP-4, with or without added molar excess of IGF-II (Bachem), was used for each reaction. PAPP-A bound to the surface of cells was prepared by incubating 293T cells (detached with PBS/5 mM EDTA, and preincubated with 1% paraformaldehyde for 12 h at 4 °C to minimize possible shedding of receptors) with PAPP-A (5 µg/ml) for 1 h on ice following thorough washing in 100 mM NaCl, 1 mM CaCl2, 50 mM Tris, pH 7.5 to remove unbound enzyme. The PAPP-A-binding cells were incubated with labeled IGFBP-4 and IGF-II in 100 mM NaCl, 1 mM CaCl2, 50 mM Tris, pH 7.5, for 1 h at 37 °C, and cleavage was visualized by reducing SDS-PAGE followed by autoradiography using a PhosphorImager (Molecular Dynamics). As a control, the PAPP-A activity contained in the supernatant following a 1 h incubation at 37 °C (without IGFBP-4 and IGF-II) was also measured. The experiment was also carried out with IGFBP-5 as the substrate (13), and a similar experiment was performed using PAPP-A2 and IGFBP-5 (19).
RESULTS

Surface binding of proPAPP-A and PAPP-A - A proPAPP-A expression construct was made from a previously constructed plasmid encoding the 1547-residue mature form of PAPP-A (5). The construct was prepared by using oligonucleotides derived from the propeptide cDNA sequence (21), because we were unable to amplify the extremely GC-rich cDNA encoding the 58-residue propeptide by PCR. The GC-content of the oligonucleotides used was decreased without making changes in the encoded amino acid sequence.

When the proPAPP-A plasmid was expressed transiently in 293T cells, the level of PAPP-A antigen in the culture medium was found to be several fold lower than from cells transfected with cDNA encoding mature PAPP-A (data not shown). Further, since PAPP-A antigen has previously been detected in solubilized membranes of stimulated JAR cells (21), it had been speculated that the propeptide might be involved in binding to the cell surface, and that soluble, mature PAPP-A results from proteolytic shedding. To test this hypothesis, 293T cells transiently transfected with proPAPP-A cDNA were detached and analyzed for the presence of surface bound PAPP-A antigen by flow cytometry, using a PAPP-A specific monoclonal antibody (mAb) (Fig. 1A). This experiment clearly demonstrated that PAPP-A antigen is present on the surface of the transfected 293T cells. The majority (> 90%) of cells appeared to bind PAPP-A, as only a minor fraction showed fluorescence in the range of mock-transfected cells.

In a parallel experiment, we analyzed the transient surface expression of integrin Mac-1, a transmembrane receptor, and found Mac-1 to be present only on about 50% of the cells (Fig. 1B), which accounts for the fraction of cells successfully transfected. It thus appeared that cells in which proPAPP-A is not synthesized do also bind PAPP-A antigen, a finding supported by the marked difference in the distribution of cells in the two histograms (Fig. 1A vs. 1B). We therefore analyzed cells transfected with cDNA encoding the mature PAPP-A polypeptide, known to be secreted into the medium. Because this experiment also demonstrated PAPP-A binding to the vast majority of the cells analyzed (Fig. 1C), we conclude that the mature PAPP-A polypeptide is sufficient for cell surface binding.
When non-transfected 293T cells were incubated with culture supernatant containing recombinant PAPP-A, surface binding to all cells was evident, as determined by flow cytometry (Fig. 2A). Surface binding of PAPP-A was also observed when this experiment was carried out with JAR cells (Fig. 2B), with BHK-21 cells (Fig. 2C), or with JEG-3 cells (data not shown). In addition to these cell lines, we probed a primary culture of normal human skin fibroblasts (known to express very low levels of PAPP-A) (4). With these cells, PAPP-A surface adhesion was also demonstrated (Fig. 2D).

In summary, we have shown that PAPP-A is capable of adherence to the cell surface, and that PAPP-A needs not be synthesized in a cell to which it binds. All cell types analyzed appeared to have the ability to bind PAPP-A.

Adhesion is mediated by the C-terminal third of PAPP-A - The amino acid sequence of PAPP-A can roughly be divided into 1) an N-terminal third, including the proteolytic domain (17), 2) a central third without similarity to other sequences (except PAPP-A2), and 3) the C-terminal third, including the five SCR modules (18). Introducing a stop codon before the first SCR module potentially would allow a preliminary localization of the region in the PAPP-A amino acid sequence responsible for surface binding. By flow cytometry of transfected 293T cells, we found that a truncated PAPP-A variant, C1210STOP², lacking the C-terminal third of the PAPP-A sequence, was unable to bind to the cell surface (Fig. 3). Importantly, the mAb used for flow cytometry did recognize the C1210STOP mutant in ELISA, verifying that PAPP-A surface binding, not the antibody epitope, had been lost. Expression of the C1210STOP mutant was also verified by Western blotting (not shown).

Heparin and heparan sulfate inhibits surface binding of PAPP-A - As some SCR domains of other proteins are known to bind glycosaminoglycan (GAG) (31), we tested the ability of heparin to inhibit surface binding of PAPP-A. Analysis by flow cytometry were carried out as previously, using non-transfected 293T cells, but prior to incubation with the cells, PAPP-A was preincubated with heparin. As we found that heparin has a dramatic effect on PAPP-A adhesion (Fig. 4A), the effect of other GAGs was also analyzed: The effect of heparan sulfate was very similar to that of heparin. In both cases, PAPP-A binding was reduced > 95% at a GAG concentration of 25µg/ml (Fig. 4A and 4B). In contrast, the effect of both dermatan sulfate
and chondroitin sulfate were relatively weak (Fig. 4B). Thus, PAPP-A adhesion can be effectively inhibited by competing for binding to a GAG binding site, which appears to reside in the SCR region of PAPP-A. Accordingly, we also found that removal of GAG from the cell surface, by pretreatment of the cells with heparinase, completely abrogated PAPP-A surface binding (Fig. 4C). Furthermore, surface binding was also abrogated when cells were incubated first with PAPP-A, and then with heparin (not shown), demonstrating reversibility of PAPP-A surface binding.

**PAPP-A attached to the surface of cells is proteolytically active** - Next, we asked whether PAPP-A retained its proteolytic activity while bound to the cell surface. Detached 293T cells were preincubated with PAPP-A, and washed to remove unbound protein. Cells in suspension were then incubated with radiolabeled IGFBP-4 and -5 substrates, with or without added IGF, and proteolytic cleavage was assessed (Fig 5A, lanes 1-3, and 5B, lanes 1-2). Cleavage of IGFBP-4 and -5 was also carried out using soluble PAPP-A that might have been released from the cells while incubating, possibly by receptor shedding (32) (Fig. 5A, lanes 4-6, and 5B, lanes 3-4). However, the activities in the supernatants were much lower than the activities on the cell surface (Fig. 5A, lanes 3 vs. 6, and 5B, lanes 2 vs. 4). We concluded that surface bound PAPP-A is active, and that it generates the same IGFBP-4 fragments whether immobilized to the cell surface or in solution.

A similar experiment was carried out using PAPP-A2 and IGFBP-5 as the substrate. A suspension of cells preincubated with PAPP-A2 was proteolytically inactive against IGFBP-5 (Fig. 5B, lanes 5-6), demonstrating that, unlike PAPP-A, PAPP-A2 does not bind to the cell surface. This experiment is of particular importance, as we were unable to investigate the possible surface binding of PAPP-A2 by flow cytometry, because PAPP-A2 antibodies are not yet available. When added to the reaction mixture, PAPP-A2 cleaved IGFBP-5 as shown previously (19).

**Localization of the GAG binding site to SCR-3 and -4 using chimeras of PAPP-A and PAPP-A2** - The strategy used above for roughly delineating the GAG binding site of PAPP-A has possible limitations, in particular in the case of the PAPP-A SCR region: From the studies of other proteins, it is known that the structure and stability of SCR modules are not entirely independent of context (33), and therefore the introduction of stop codons after individual domains might result in incorrectly folded protein. As an alternative strategy to map the glycosaminoglycan binding site within the SCR region of PAPP-A, we took
advantage of the fact that PAPP-A2 is homologous (46% identical) to PAPP-A, but does not exhibit surface binding. We constructed two series PAPP-A/PAPP-A2 chimeras, in which variable sequence stretches of PAPP-A were replaced with the corresponding PAPP-A2 sequence.

First, five chimeras (chim1-5) were made, in which a progressively longer portion of the C-terminal PAPP-A sequence was replaced with PAPP-A2 sequence (Fig. 6A). Points of transition between PAPP-A and PAPP-A2 sequence were the N-terminal end of each of the SCR modules (Fig. 6A). As judged by Western blotting, all chimeras expressed as dimeric proteins, like wild-type PAPP-A (Fig. 7). Chim1, containing PAPP-A2 SCR-5, bound 293T cells as well as wild-type PAPP-A. Chim2, in which SCR-4 is also substituted, showed only weak surface binding, and chim3, chim4, and chim5 did not show any surface binding (Fig. 6A). Therefore, this experiment points at the involvement of SCR-3 and -4 in adhesion of PAPP-A.

Second, to further substantiate this interpretation, we replaced SCR-3 and -4 alone or together (chim6-8) (Fig. 6B). Substituting PAPP-A SCR-3 domain alone with PAPP-A2 sequence completely abrogated surface binding (chim6), whereas replacement of SCR-4 alone resulted in a chimera with relatively weak binding (chim7), compared to wild-type PAPP-A. As expected, when SCR-3 and -4 were replaced at the same time (chim8), surface binding was lost. Finally, in chim9, sequence stretches on both sides of SCR-3 and -4 were replaced with PAPP-A2 sequence. This PAPP-A/PAPP-A2 chimera bound to the cell surface as well as did wild-type PAPP-A (Fig. 6B). We conclude that the domains SCR-3 and -4 are not only required, but also sufficient, for efficient surface binding of PAPP-A.

PAPP-A complexed to proMBP does not adhere to cells - In pregnant women, PAPP-A circulates as a covalent (disulfide bound) 2:2 complex with the proform of eosinophil major basic protein (proMBP), denoted PAPP-A/proMBP (22), in which proMBP functions as an inhibitor of PAPP-A activity (5). Of particular interest, residual PAPP-A activity in pregnancy serum stems from about 1% of noncomplexed, dimeric PAPP-A, and possibly from a small fraction of partially inhibited 2:1 complexes (5). Previous biochemical characterization of proMBP, isolated from reduced and denatured PAPP-A/proMBP complex, has revealed that it is substituted with one heparan sulfate GAG chain (25,26). In one possible scenario, based on the above results, the GAG of proMBP competes for binding to the GAG binding site of PAPP-A,
and thereby reduces or abrogates binding of PAPP-A to the cell surface. We therefore compared the surface binding of PAPP-A and PAPP-A/proMBP complex, isolated from pregnancy serum, by incubating cells with increasing amounts of protein. The fluorescence intensity observed with 200 µg/ml complex was less than half of the fluorescence obtained with 2 µg/ml of dimeric PAPP-A, clearly demonstrating compromised binding of PAPP-A when complexed to proMBP (Fig. 8).

**Surface binding of heparinase treated PAPP-A/proMBP complex** - To further substantiate a hypothesis that the proMBP GAG prevents surface binding of the PAPP-A/proMBP complex, we sought to excise the proMBP GAG by digesting purified PAPP-A/proMBP with heparinase. Prior to incubation with cells, the heparinase was removed by affinity purification of the PAPP-A/proMBP complex to avoid damage to cell surface proteoglycans. We found a dramatic increase in surface binding of heparinase treated PAPP-A/proMBP complex (Fig. 9A). A control experiment showed that heparinase treatment did not have any effect on the binding of uncomplexed PAPP-A (Fig. 9B).

Heparinase digested PAPP-A/proMBP complex was also analyzed by Western blotting after reducing SDS-PAGE. ProMBP released from untreated complex migrates as a smear of 50-90 kDa, which is not visible in Coomassie-stained gels (22). However, although the protein still migrated as a smear, heparinase treatment caused a slight shift in its position (not shown), in agreement with the loss of the proMBP GAG with a calculated (average, without sulfate) molecular weight of 3 kDa (26).
DISCUSSION

We have demonstrated and analyzed the interaction between PAPP-A and the cellular surface. We have shown that 1) mature PAPP-A is capable of binding reversibly to the surface of all cell types analyzed, and that it can adhere to cells in which it is not synthesized, 2) heparin and heparan sulfate effectively compete for the binding site on PAPP-A, and 3) heparinase treatment of cells abrogates their capacity to bind PAPP-A. We further 4) locate the binding site of PAPP-A to its C-terminal third by analysis of a truncated PAPP-A mutant, and 5) narrow down the binding site to SCR modules 3 and 4 using chimeras of PAPP-A and PAPP-A2. The use of PAPP-A2 was made possible by our finding that PAPP-A2, unlike PAPP-A, is unable to adhere to cells. Finally, 6) we demonstrate that the PAPP-A/proMBP complex shows only weak surface binding, which can be restored by the treatment of the complex with heparinase.

Proteolysis of IGFBP-4/IGF complexes in the interstitial fluid, at a distance from the IGF receptor, is likely ineffective in terms of promoting IGF actions: Once released, IGF may be captured by an intact IGFBP molecule, effectively antagonizing the effect of proteolysis. In contrast, proteolysis of IGFBP-4/IGF complexes at the cell surface immediately brings released IGF in close proximity to the IGF receptor, decreasing the probability that IGF is captured and inactivated before it binds to the receptor.

Therefore, our finding that PAPP-A binds to the surface of cells (Fig. 1) suggests that this plays a part in targeting of its proteolytic activity. We demonstrated that PAPP-A is enzymatically active while bound to cells (Fig. 5), and that PAPP-A synthesized in one cell can adhere to another (Fig. 2). Importantly, this allows possible functions of PAPP-A in both autocrine and paracrine mechanisms.

Based on preliminary localization of the PAPP-A binding site to the C-terminal third of PAPP-A (Fig. 3), we speculated that glycosaminoglycan (GAG) is implicated in the surface binding of PAPP-A. Indeed, we found that heparin effectively competes for the binding site on PAPP-A. The effect of heparan sulfate was very similar to that of heparin, but dermatan sulfate and chondroitin sulfate both competed inefficiently (Fig. 4). These results suggest that a cell surface heparan sulfate proteoglycan (HSPG), possibly a syndecan or glypican family member, is involved in binding of PAPP-A. HSPGs are particularly
abundant on the surface of cells, and are known to participate in binding of many different proteins (34,35). In some cases, cell surface HSPGs function as co-receptors, efficiently increasing the interaction between a ligand, for example a growth factor, and its signaling receptor (36). Although HSPG do not appear to function as a co-receptor for IGF, adhesion of PAPP-A indirectly increases the effective concentration of IGF in the vicinity of its receptor. However, the existence of a PAPP-A signaling receptor cannot be ruled out. Further, an internalization receptor, or a non-signaling co-receptor for PAPP-A, providing binding energy and/or specificity, may also exist.

We previously observed PAPP-A antigen in solubilized membranes of stimulated JAR cells (21), and other data have indicated that PAPP-A associates with preparations of membrane made from human placenta (37). Although these data were carried out with polyspecific PAPP-A antibodies, known to recognize different antigens (22,38) they are in agreement with our data. Furthermore, we have recently demonstrated that PAPP-A antigen present in a preparation of solubilized membranes from human trophoblasts is proteolytically active.

A functional role in vivo of surface adhesion of a soluble proteinase has been demonstrated for matrilysin (MMP-7). MMP-7 binding of heparan sulfate was first established (39), and it was then shown that CD44, a facultative cell surface proteoglycan, binds both MMP-7 and its substrate, the precursor of heparin-binding epidermal growth factor (pro-HB-EGF). Within this assemblage, mature HB-EGF is generated in close proximity to its receptor, ErbB4 (40). Another example, also with similarity to the system of IGF, IGFBP-4, and PAPP-A, is the activation of transforming growth factor-β (TGF-β) by surface bound MMP-9 (41). Other nontransmembrane metzincins have been found to associate with the cell surface, although they do not appear to function in growth factor activation: MMP-2 binds integrin αvβ3 promoting cellular invasion (42), and MMP-2 binds a clearance receptor, proposed to regulate extracellular activity of this enzyme (43). Adhesion of MMP-19 has also been shown, although its function is not known (44). Further, the serine proteinase plasmin, potentially activated at the cell surface, has been shown to be capable of degrading IGFBPs, independent of the presence of IGF (45,46), but the physiological significance of plasmin in the IGF system is not known.
To further define the GAG binding site of PAPP-A, nine chimeras of PAPP-A and PAPP-A2 were constructed and analyzed (Fig. 6). We found that both SCR-3 and -4 are involved in PAPP-A surface binding. There may be a separate GAG binding sites on each of these domains, or there may be single site, to which both SCR-3 and -4 make contributions. In either case, the residues of SCR-3 appear to be more important, as chim6, in which SCR-3 alone is substituted with PAPP-A2 sequence, did not bind, and as chim7, in which SCR-4 only is replaced, showed weak binding (Fig. 6). The true modularity of the SCR domain is debated (33), and we cannot exclude that inter-domain interactions are disturbed in some of the chimeras. It is therefore important that no result contradicts the above interpretation, which is based on both knock-out and knock-in experiments.

SCR domains (also called CCP modules) are best known from their presence in complement control proteins such as factor H (fH), which is composed of 20 SCR modules alone (33); SCR domain 16 of fH was the first to have its three-dimensional structure solved (47). The SCR domain is a five-stranded β-sandwich structure, and individual domains are linked in a head-to-tail manner with varying angels between the axes of each domain (33).

Factor H is known to bind heparin, and the heparin binding has been mapped to individual SCR domains (7, 13, and 20) (48). Complexes between SCR domain(s) and GAG have not yet been studied at atomic resolution, but the binding of heparin to other proteins, such as antithrombin and fibroblast growth factors, has been studied in detail by X-ray crystallography (49). It is therefore known that residues with positively charged side chains interact with GAG sulfate groups. Vaccinia virus complement control protein (VCP) contains four SCR domains, and is also known to bind heparin. Its recently reported crystal structure (31) reveals two patches of positive residues. Interestingly, the one is at the end of domain 4, the other straddles domains 1 and 2.

SCR-3 of PAPP-A contains 13 residues with basic side chains (18), whereas SCR-3 of PAPP-A2 contains only six (19). The average number of basic residues in the 10 SCR modules of PAPP-A and PAPP-A2 is five; PAPP-A SCR-4 has five basic residues. These figures clearly point at direct interactions between...
GAG and residues of PAPP-A SCR-3, but further mutagenesis, focusing at single amino acids, will be required to define the PAPP-A-GAG interactions in greater detail.

As proMBP is known to be substituted with a heparan sulfate GAG (26), we hypothesized that, in the disulfide bound PAPP-A/proMBP complex, this occupies the GAG binding site of PAPP-A, and therefore antagonizes PAPP-A surface binding. Comparison of dimeric PAPP-A with heterotetrameric PAPP-A/proMBP complex indeed revealed compromised surface binding of the complex (Fig. 8). The weak binding observed may be caused by the presence of a small fraction of 2:1 complexes in the PAPP-A/proMBP preparation, capable of surface binding because it has two GAG binding sites, but only one proMBP GAG. A 2:1 complex, but not dimeric PAPP-A, would co-purify with the predominant 2:2 complex in chromatography (5). Alternatively, the limited PAPP-A/proMBP binding can be explained by competition between cell surface GAG and proMBP GAG about binding to the binding site of the PAPP-A subunit. Of particular importance, demonstrating that the GAG of proMBP inhibits adhesion, treatment of the PAPP-A/proMBP complex with heparinase restored cell surface binding (Fig. 9A), whereas binding of uncomplexed PAPP-A was not affected (Fig. 9B). Thus, PAPP-A, still part of the disulfide bound PAPP-A/proMBP complex, was able to bind to the cell surface after removal of the proMBP GAG.

The activities of IGF are antagonized by IGFBP-4, whose effect is abrogated when cleaved by PAPP-A. This regulatory system appears to function in several tissues, including the ovary and the human placenta; recent data show that first trimester serum levels of PAPP-A correlate positively with birth weight (50). We have previously demonstrated that proMBP, in turn, controls the enzymatic activity of PAPP-A, as it functions as a proteinase inhibitor (5). The levels of both PAPP-A and proMBP are relatively high in the human placenta, but all tissues analyzed synthesize mRNAs encoding both molecules (24), suggesting that both the proteinase (PAPP-A) and its inhibitor (proMBP) function together.

We have depicted the possible role of surface bound PAPP-A in promoting IGF activity by cleaving IGFBP-4 in proximity to the IGF receptor (Fig. 10A), and further, the possible role of proMBP in effectively antagonizing this effect by both detachment and inhibition of PAPP-A (Fig. 10B). This model comprises the components known to function with PAPP-A, directly or indirectly. The elements of their dynamics, such as
attachment, proteolytic activity, detachment, complex formation, and inhibition, are all supported by experimental data.

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FOOTNOTES

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**To whom correspondence should be addressed (e-mail co@mb.au.dk).

1The abbreviations used are: PAPP-A, pregnancy-associated plasma protein-A; proPAPP-A, proform of pregnancy-associated plasma protein-A; IGF, insulin-like growth factor; IGFBP, insulin-like growth factor binding protein; proMBP, the proform of eosinophil major basic protein; PAPP-A2, pregnancy-associated plasma protein-A2; SCR, short consensus repeat; GAG, glycosaminoglycan; PCR, polymerase chain reaction; ELISA, enzyme-linked immunosorbent assay; mAb, monoclonal antibody; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis.

2The numbering of preproPAPP-A (AAC50543) (21) and preproPAPP-A2 (AF311940) (19) is used in this paper. (Glu-1 of the mature PAPP-A polypeptide is at position 81 of preproPAPP-A).

FIGURE LEGENDS

FIG. 1. Flow cytometry of transfected cells. A, human embryonic kidney 293T cells were mock-transfected with empty vector or with vector containing proPAPP-A cDNA. The cells were detached 48 h post transfection and incubated with a PAPP-A specific monoclonal antibody (mAb 234-2). After washing, the cells were incubated with secondary fluorescein isothiocyanate labelled antibodies, and then analyzed by flow cytometry to visualize surface bound antigen. The immunofluorescence of the two population of cells is displayed in histograms (the number of cells (Events) at a given Fluorescence Intensity). B, in a similar experiment, cells were co-transfected with vectors containing CD11b cDNA (encoding the α-subunit of integrin Mac-1) and CD18 cDNA (encoding the β-subunit of Mac-1) (28). A Mac-1 specific monoclonal antibody (mAb 2LPM19c) was used for detection of surface bound antigen. C, the same experiment as in A, but using a vector with cDNA encoding the mature PAPP-A subunit.

FIG. 2. Flow cytometry of non-transfected cells incubated with PAPP-A. A, detached, non-transfected human embryonic kidney 293T cells were incubated for 1 h with culture supernatant harvested from 293T cells transfected with empty vector or with vector containing PAPP-A cDNA. The supernatants contained 0 (-PAPP-A) and 5 (+PAPP-A) µg/ml of PAPP-A, respectively. The cells were processed for flow cytometry using a monoclonal antibody (mAb 234-2) specific for PAPP-A followed by secondary fluorescein isothiocyanate labelled antibodies. Binding of PAPP-A to the cell surface is displayed in histograms. B, a similar experiment performed with JAR cells. C, a similar experiment performed with BHK-21 cells. D, a similar experiment performed with a primary culture of human skin fibroblasts. The fluorescence intensities cannot be quantitatively compared between experiments, primarily because different batches of secondary antibodies were used.
FIG. 3. C-terminally truncated PAPP-A does not bind the cell surface. Cells (293T) were transfected with empty vector (left histogram), vector containing PAPP-A wild-type cDNA (center histogram), or vector containing PAPP-A cDNA truncated at Cys-1210 (right histogram). The mAb (234-2) used for flow cytometry recognized the C1210STOP mutant in ELISA (not shown), verifying that PAPP-A surface binding, not the antibody epitope, had been lost. Schematic diagrams comparing the PAPP-A wild-type subunit with the truncated C1210STOP mutant, in which the codon encoding Cys-1210 is replaced by a stop codon, are shown above the histograms.

FIG. 4. The effect of glycosaminoglycans on PAPP-A surface binding. A, detached, non-transfected human embryonic kidney 293T cells were incubated (1 h) with culture supernatant containing PAPP-A (5 µg/ml), which had been preincubated for 30 min with heparin (0-100 µg/ml). The cells were further incubated with a PAPP-A monoclonal antibody (mAb 234-2) followed by secondary fluorescein isothiocyanate labelled antibodies, and then analyzed by flow cytometry. Mean specific fluorescence intensity relative to the value at 0 µg heparin per ml is plotted. Values are averages of three experiments. Standard deviations are shown by vertical bars. Before calculations, values were subtracted background (defined as the fluorescence observed without PAPP-A incubation). The inserted histograms shows fluorescence without PAPP-A incubation (upper), with PAPP-A but without heparin incubation (middle), and with both PAPP-A and heparin (100 µg/ml) incubation (lower). B, similar experiments were carried out using heparan sulfate (HS, filled circles), chondroitin sulfate (CS, filled squares), and dermatan sulfate (DS, filled triangles). C, the effect of heparinase treatment of cells. The left histogram shows untreated 293T cells not incubated with PAPP-A; the center histogram shows untreated cells incubated with PAPP-A; the right histogram shows cells treated (1 h) with heparinase prior to incubation with PAPP-A.
**FIG. 5. Activity of surface bound PAPP-A.** A, radiolabeled IGFBP-4 was incubated (1 h at 37 °C) with suspended 293T cells, first incubated with mock medium (lane 1), or with PAPP-A followed by washing (lanes 2-3). Experiments were performed in the absence or presence of added IGF-II, as indicated under each lane (- or +). The proteolytic activity released from the cells while digesting was measured by analyzing supernatant following 1 h incubation in a parallel experiment without IGFBP-4 and IGF-II (lanes 4-6, marked 'sup'). B, a similar experiment was performed with radiolabeled IGFBP-5 as the substrate, using PAPP-A (lanes 1-4) or PAPP-A2 (lanes 5-6). All reaction mixtures were analyzed by SDS-PAGE followed by autoradiography. C-terminal tags on both protein substrates causes their cleavage products to co-migrate, and thus appear as one band (13).

**FIG. 6. Localization of surface binding site to PAPP-A domains SCR-3 and -4.** Based on the result of Fig. 3, expression plasmids encoding chimeric PAPP-A/PAPP-A2 molecules were constructed. PAPP-A sequence stretches C-terminal to the beginning of SCR-1 were exchanged, and transfected 293T cells were prepared for flow cytometry using PAPP-A mAb 234-2. A, histograms of mock-transfected cells, cells transfected with PAPP-A wild-type cDNA, and cells transfected with cDNA encoding chim1-5. The schematic diagrams show the PAPP-A sequence stretch replaced with PAPP-A2 sequence (shaded regions) in chim1-5. B, histograms of mock-transfected cells, cells transfected with PAPP-A wild-type cDNA, and cells transfected with cDNA encoding chim6-9. The schematic diagrams show the PAPP-A sequence replaced with sequence of PAPP-A2 (shaded regions) in chim6-9.
FIG. 7. Western blotting of PAPP-A/PAPP-A2 chimeras. Nonreducing Western blot of culture supernatants from transfected 293T cells using polyclonal PAPP-A antibodies. Like wild-type PAPP-A, all PAPP-A/PAPP-A2 chimeras constructed expressed as dimeric proteins. A variable trace of monomer at 200 kDa was observed with both the wild-type and the chimeric proteins. Lane 1, mock transfected cells; lane 2, PAPP-A wild-type; lane 3, chim1; lane 4, chim2; lane 5, chim3; lane 6, chim4; lane 7, chim5; lane 8, chim9. Similar results were obtained with chim6, chim7, and chim8 (not shown). The identities of the chimeric proteins are depicted in Fig. 6.

FIG. 8. The PAPP-A/proMBP complex does not bind the cell surface. Detached, non-transfected human embryonic kidney 293T cells were incubated (1 h) with culture supernatant containing PAPP-A (0, 1, 2.5, or 5 µg/ml, open squares) or PAPP-A/proMBP complex purified from pregnancy serum (0, 5, 10, 25, 50, 100, 150, 200 µg/ml, filled squares) contained in culture medium. Analysis of PAPP-A and PAPP-A/proMBP surface binding was carried out as detailed in the legend of Fig. 4. Specific fluorescence intensity, relative to the value obtained with 5 µg/ml of PAPP-A, is plotted. Values are averages of three experiments, and were subtracted background, defined as the fluorescence observed without PAPP-A incubation. Standard deviations are shown by vertical bars.
FIG. 9. Surface binding of heparinase treated PAPP-A/proMBP complex. A, non-transfected 293T cells were incubated with culture medium (mock), culture medium containing untreated PAPP-A/proMBP complex (10 µg/ml) or heparinase treated PAPP-A/proMBP complex (10 µg/ml). Surface binding was analyzed by flow cytometry using PAPP-A mAb 234-2. B, a similar control experiment carried out using recombinant PAPP-A (3 µg/ml). All samples were affinity purified prior to incubation with cells to remove the heparinase.

FIG. 10. Hypothetical model of the function of PAPP-A and proMBP at the cell surface. A target cell with IGF receptors and a GAG-substituted receptor (unidentified) is outlined. A, interaction between surface bound GAG and SCR domains 3 and 4 immobilizes PAPP-A to the cellular surface (top of figure). Here, proteolysis of IGFBP-4 causes release of IGF in close proximity to the IGF receptor. Unbound PAPP-A (bottom of figure) proteolyses IGFBP-4 at a distance from the cell surface, and released IGF is bound by another molecule of IGFBP-4 rather than by its receptor. B, the scenario in A is altered by proMBP, which forms a covalent (disulfide bound) complex with attached PAPP-A. The GAG chain of proMBP now binds to SCR domains 3 and 4, thereby releasing inhibited PAPP-A from the cell surface. Soluble components of the figures are IGF, IGFBP-4, the IGF/IGFBP-4 complex, PAPP-A, and proMBP. PAPP-A exists as a disulfide bound dimer, but depicted here as a monomer containing only the proteolytic domain and the five SCR domains. Likewise, the 2:2 PAPP-A/proMBP complex is shown as a 1:1 complex. All symbols used are named on the figure.
Figure 1

A

Fluorescence Intensity

mock (empty vector)

proPAPP-A cDNA

B

Fluorescence Intensity

mock (empty vector)

Mac-1 cDNA

C

Fluorescence Intensity

mock (empty vector)

PAPP-A cDNA
Laursen et al.
Figure 2

A

- PAPP-A

+ PAPP-A

B

- PAPP-A

+ PAPP-A

C

- PAPP-A

+ PAPP-A

D

- PAPP-A

+ PAPP-A
Figure 3
Laursen et al.
Figure 5

A

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IGF-II: - - + - - +

29 kDa - 20 kDa - 14 kDa -

B

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IGF-II: - - - - -

43 kDa - 29 kDa - 20 kDa -
Laursen et al.
Figure 6

A

PAPP-A residues in construct

1-1627
1-1475
1-1412
1-1344
1-1282
1-1214

SCR1-5

wt

chim1

chim2

chim3

chim4

chim5

LNR3

B

PAPP-A residues in construct

1-1627
1-1344 + 1413-1627
1-1412 + 1476-1627
1-1344 + 1476-1627
1-1214 + 1345-1475

SCR1-5

wt

chim6

chim7

chim8

chim9

LNR3

mock

PAPP-A wt

Fluorescence Intensity

Fluorescence Intensity

Fluorescence Intensity

Fluorescence Intensity

Fluorescence Intensity

Fluorescence Intensity

Events

128

128

256

128

256

128

Events

128

256

128

256

128

Events

128

256

128

256

128
Laursen et al.
Figure 7
Laursen et al.
Figure 9
Laursen et al.
Figure 10

A

TARGET CELL

- Signaling
- IGF receptor
- IGFBP-4/IGF complex (inactive)
- Proteolytic fragment of IGFBP-4
- ProMBP
- GAG

- SCR1-5
- PAPP-A proteolytic domain
- Active & soluble PAPP-A
- Cleavage of IGFBP-4 distant from the cell surface

B

- Inactive & detached PAPP-A/proMBP complex
- ProMBP
- ProMBP protein
- IGF
- IGFBP-4 binds and inactivates released IGF
Cell surface targeting of pregnancy-associated plasma protein-A proteolytic activity. Reversible adhesion is mediated by two neighboring short consensus repeats

Lisbeth S. Laursen, Michael T. Overgaard, Kathrin Weyer, Henning B. Boldt, Peter Ebbesen, Michael Christiansen, Lars Søtrup-Jensen, Linda C. Giudice and Claus Oxvig

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