Pregnancy-associated Plasma Protein-A2 (PAPP-A2), a Novel Insulin-like Growth Factor-binding Protein-5 Proteinase*

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A novel metalloproteinase with similarity to pregnancy-associated plasma protein-A (PAPP-A), which we denoted PAPP-A2, has been identified. Through expression in mammalian cells we showed that recombinant PAPP-A2 polypeptide of 1558 residues resulted from processing of a 1791-residue prepro-protein. Unlike PAPP-A, PAPP-A2 migrated as a monomer (of 220 kDa) in non-reducing SDS-polyacrylamide gel electrophoresis. The prepro-parts of PAPP-A2 and PAPP-A are not homologous, but mature PAPP-A2 shares 45% of its residues with PAPP-A. Because PAPP-A specifically cleaves insulin-like growth factor-binding protein (IGFBP)-4, one of six known modulators of IGF-I and -II, we looked for a possible PAPP-A2 substrate among the members of this family. We showed that PAPP-A2 specifically cleaved IGFBP-5 at one site, between Ser-143 and Lys-144. In contrast to the cleavage of IGFBP-4 by PAPP-A that strictly requires the presence of IGF, the cleavage of IGFBP-5 by PAPP-A2 was IGF-independent. Recent data firmly establish PAPP-A and IGFBP-4 as an important functional pair in several systems. Because of its close relationship with PAPP-A, both structurally and functionally, PAPP-A2 is a likely candidate IGFBP-5 proteinase in many tissues and conditioned media where IGFBP-5 proteolysis has been reported.

Pregnancy-associated plasma protein-A (PAPP-A) has recently been shown to specifically cleave insulin-like growth factor-binding protein-4 (IGFBP-4) (1), one of six modulators of IGF-I and -II activity (2). Cleavage of IGFBP-4 causes release of IGF-I and -II activity (2). Interestingly, the cleavage of IGFBP-4 by PAPP-A strictly depends on the presence of IGF (1). It has been established that PAPP-A is the IGFBP-4 protease secreted from fibroblasts (1), osteoblasts (1, 4), marrow stromal cells (1), and vascular smooth muscle cells (5) and is present in pregnancy serum (6) and ovarian follicular fluid (7).

PreproPAPP-A is synthesized as a 1627-residue protein, which is processed into mature PAPP-A of 1547 residues (8, 9). In most systems, PAPP-A appears to exist as a homodimer of 400 kDa (1). However, in pregnancy serum, and possibly elsewhere, >99% of PAPP-A is found as a disulfide bound 2:2 complex with the highly and unusually glycosylated proform of eosinophil major basic protein (proMBP) (10–12). In this covalent complex, proMBP functions as an inhibitor of the proteolytic activity of PAPP-A (6).

No proteins with global homology to PAPP-A have been described. Here we report the identification of a novel protein, PAPP-A2, which is a homologue of PAPP-A. Expression of recombinant PAPP-A2 allowed us to establish that PAPP-A2 is an active metalloproteinase, that it specifically cleaves IGFBP-5, and that it therefore likely functions in the same growth regulatory system as PAPP-A.

EXPERIMENTAL PROCEDURES

Identification and Cloning of a Nucleotide Sequence Encoding PAPP-A2

Searching (13) public databases for DNA sequences that when translated showed homology to the preproPAPP-A protein sequence revealed two genomic clones, AL031734 and AL031290, containing coding sequence stretches corresponding to ~25% of the N-terminal amino acid sequence of PAPP-A (residues 96–493), and 15% of the C-terminal (residues 1393–1593). Based on their co-localization on chromosome 1 (1q24), we hypothesized the existence of a novel protein similar to PAPP-A, which we named PAPP-A2. We first established a coding cDNA sequence aligning with the ~60% remaining, central region of PAPP-A; cDNA was synthesized using human placental mRNA as a template and a primer derived from AL031290 (5′-GCTCACACACCA-CAGGAAATG-3′). With primers derived from AL031734 (5′-GGGTGAT- GTGCACAGCAAGTGCTC-3′) and AL031290 (5′-GCAACCCGTACTTAA-CAGG-3′), a PCR product was generated that corresponded to the central region, a total of 908 amino acids (PAPP-A2 residues 665–1572). Manual inspection of the genomic sequence of AL031734 revealed that the open reading frame of the sequence stretch aligning with PAPP-A continued further in the 5′ direction; nt 102846–103566 encode a polypeptide sequence of 307 residues that starts with a methionine residue. Thus, the same cDNA preparation was used to obtain a contiguous cDNA sequence encoding the N-terminal portion of PAPP-A2 using primers from AL031734 (5′-GAAATGTGACTCTGCTGTCTGAT-3′) and from the central region (5′-CCCTGGGAAAGGCGAATCGTGCGC-3′). A stop codon was present in the sequence of AL031290 corresponding to PAPP-A residue 1618. Therefore, cDNA was synthesized using plasmid mRNA and a primer originating from AL031290 further 3′ to this stop codon (5′-GACATCTTAAAGATCCGTCATGC-3′). A contiguous...
cDNA corresponding to the C-terminal of PAPP-A2 was obtained in a PCR with primers from the central region (5′-GAGACGTCTGGGACTTTATGCTGC-3′) and from AL031290 (5′-CTTAAACTGCTTGAAGGACTGG-3′). All PCRs were carried out with Pfu polymerase (Stratagene). The three overlapping PAPP-A2 cDNA fragments obtained were cloned into the vector pBluntII-TOPO (Invitrogen), and referred to as p2N, p2Mid, and p2C, respectively. The entire sequence of 5376 nucleotides encodes preproPAPP-A2 of 1791 residues.

Identification of EST Sequences

A cluster of EST sequences matching the genomic sequence of AL031290 was identified around nt 64000–66000 of AL031290 starting ~1.2 kb from the 3′ end of the PAPP-A2 encoding sequence. The existence of mRNA connecting the coding region of PAPP-A2 and this cluster was verified by PCR using primers from AL031290 (5′-GGAGAAAGCCAGATGTCCACCATCCATC-3′) and (nt 64900–64879 of AL031290), the PAPP-A2 encoding sequence (5′-CGCTGTTGCTACAGCTCCATC-3′, nt 20499–20519 of AL031290 and nt 5171–5191 of AF311940), and oligo(dT)-primed placental cDNA as a template (12). As expected, the size of the resulting product was 2.2 kb, further demonstrating the existence of a PAPP-A2 mRNA with a 3′ untranslated region of ~3 kb.

Plasmid Construction

pPA2, Encoding Wild-type PreproPAPP-A2—The NotI-BamHI fragment from p2C was cloned into pBlueScriptIIKS+ (Stratagene) to obtain p2CBlue. The NotI-SpeI fragment from p2N and the SpeI-BclI fragment were ligated into the NotI/BclI sites of p2CBlue to obtain p2MidBlue containing the entire PAPP-A2 cDNA. The NotI-Apal fragment of pBlueScriptIIKS+ was ligated into the NotI/Apal sites of the mammalian expression vector pcDNA3.1+ (Invitrogen) to obtain a modified version of this vector, pcDNA-NA. The full-length cDNA was then excised from p2MidBlue with XhoI and XhoI and cloned into pcDNA-NA to obtain pPA2. pPA2—By overlap extension PCR (14), a construct encoding an inactive variant of pPA2, pPA2-KO, was made in which Gln-734 is replaced with a Gln (E734Q). Outer primers were 5′-GCCATCTGGGAGGCGAATCAACCACTGCACATCC-3′; primer content of the central region (5′-GACAGCTGTCCGATCAGG-3′), ends of the mammalian expression vector pcDNA3.1+ (Invitrogen) to obtain pPA2. pPA2-mH—Two primers (5′-GGAGGCCCTGGGACACGGAG-3′, nt 4906–4926 of AF311940, and 5′-GACCTAAAGCCTTGTATGTTCTCTTCTGCTGTTG-3′, nt 5373–5354 of AF311940, preceded by a HindIII site) were used in a PCR with p2C as the template to generate a nucleotide fragment encoding the C-terminal 156 residues of PAPP-A2 with the stop codon replaced by a HindIII site for in-frame ligation into the vector pcDNA3.1+Myr-His(B). The PCR product was digested with EcoRI and HindIII and cloned into the EcoRI/HindIII sites of the vector to generate pPA2-mH. The NotI-XhoI fragment and the XhoI-EcoRI fragment were excised from pPA2 and cloned into the NotI/EcoRI sites of pPA2-mH. The resulting construct, pPA2-mH, encoded PAPP-A2 followed by residues KLGP, the c-myc epitope (EQKLISEEDL), residues NSAVD, and six histidine residues.

Measurement of PAPP-A2 Activity

A proteinase assay based on ligand blotting (15) with radiolabeled IGF-II (Bachem) was used initially (for Fig. 3) to test for activity against IGFBP-1 (from HepG2–conditioned medium), rIGFBP-2 (GroPep), rIGFBP-3 (gift of D. Powell), rIGFBP-4 (Austral), rIGFBP-5 (gift of D. Andress), and rIGFBP-6 (Austral). For further analysis, recombinant, c-myc- and His-tagged IGFBP-5 (rIGFBP-5) was produced in mammalian cells. In brief, human placental oligo(dT)-primed cDNA (12) was used as a template to amplify cDNA encoding human IGFBP-5 (M65062). Primers containing an XhoI site (5′-TCCGGCTGGATGTTGTTGCTACACCCGCGGTT-3′) and a HindIII site (5′-GCAATACGCTTCTCGGATCTGCGTTC-3′) were used, and the resulting PCR product was cloned into the XhoI/HindIII sites of pcDNA3.1+Myr-His(B) (Invitrogen). Expression was performed as described above. Cleavage analysis was carried out by Western blotting. Briefly, rIGFBP-5 as contained in cell culture medium (2 μl, ~10 ng of rIGFBP-5) was incubated with culture supernatants from cells transfected with pPA2 or pPA2-KO (10 μl, ~2 ng of rPA2 and PAPP-A2) or with the same amount of culture supernatant from cells transfected with empty vector. Phosphate-buffered solution and inhibitors as specified in the main text were added to a final volume of 25 μl. After incubation at 37 °C for 12 h, 10 μl of the reaction mixture was separated by reducing SDS-PAGE and blotted onto a PVDF membrane, and intact rIGFBP-5 and the C-terminal cleavage product of rIGFBP-5 were detected with monoclonal anti-c-myc (clone 9E10, ATCC) using peroxidase-conjugated secondary antibodies (P260, DAKO), and ECL (Amersham Pharmacia Biotech).

Miscellaneous Procedures

SDS-PAGE was performed in Tris/glycine gels (10–20% or 16%). A metal chelate affinity column (1 ml, Amersham Pharmacia Biotech) was charged with nickel ions and used for affinity purification of His-tagged recombinant bovine PAPP-A2 and recombinant bovine PAPP-A2 as eluted with 10 mM EDTA in phosphate-buffered solution containing 500 mM NaCl and further dialyzed against 20 mM HEPES, 100 mM NaCl, and 1 mM CaCl2, pH 7.4. For cleavage site determination, affinity-purified rIGFBP-5 (20 μg) was digested (37 °C for 12 h) with purified rPA2 (1 μg) immobilized via anti-c-myc to recombinant protein G-agarose (Life Technologies, Inc.). Edman degradation was performed as described earlier (16). The amount of purified protein was determined by amino acid analysis (17). Estimation of the amount of rPA2 and rIGFBP-5 when non-purified was done by comparing the responses in Western blotting (using anti-c-myc) with the responses of known molar amounts of purified rIGFBP-5.

To ensure the complete absence of IGF-I or -II in the preparation of rIGFBP-5, affinity-purified material (100 μg) was further loaded onto a Superdex 75 (Amerham Pharmacia Biotech), equilibrated, and eluted with 50 mM formic acid at 0.5 ml/min. At the acidity of the solvent (pH < 1), any possible bound IGF would dissociate from rIGFBP-5 and elute separately, 18 (18). Next, IGF-BP-5-containing fractions were loaded directly onto a reversed-phase high pressure liquid chromatography (RP-HPLC) column (4 × 250 mm Nucleosil C4 500-7, Macherey-Nagel). A linear gradient was formed from 0.1% (v/v) trifluoroacetic acid (solvent A) and 0.075% (v/v) trifluoroacetic acid in 90% (v/v) acetonitrile (solvent B), increasing the amount of solvent B to 2.3%/min. The column was equilibrated with 5% solvent B, operated at 50 °C and with a flow rate of 0.5 ml/min, and the separation was monitored at 226 nm. Bound IGF-BP-5 eluted at ~42% solvent B. The HPLC step alone was not sufficient as IGF-II (Bachem) eluted close to IGF-BP-5 on this column as seen in separate runs. The pure protein was lyophilized, resuspended in solvent A, and further diluted three times in 50 mM Tris, pH 7.5. This material was prepared for experiments to demonstrate that cleavage of IGF-BP-5 by PAPP-A2 (using ~0.1 μg purified PAPP-A2 0.22 μg IGF-BP-5) occurs in the absence of IGF and is not promoted by IGF.

RESULTS AND DISCUSSION

We have identified and cloned a novel cDNA sequence encoding a protein of 1791 residues with homology to PAPP-A as detailed under “Experimental Procedures”. We name this protein preproPAPP-A2. Alignment of preproPAPP-A2 with preproPAPP-A (Fig. 1) demonstrates its similarity to mature PAPP-A; there is no homology between the prepro-peptides. In this alignment, 46% of the residues of mature PAPP-A are also found in PAPP-A2. Like PAPP-A, the amino acid sequence of PAPP-A2 contains three -20 nt motif specific five short consensus repeats (SCR1–5) (Fig. 1). All 82 cysteine residues of mature PAPP-A are also found in PAPP-A2, but PAPP-A2 has four additional cysteines not present in PAPP-A. In addition, the zinc binding site and a putative Met-turn are conserved between the two proteins (Fig. 1), classifying PAPP-A and PAPP-A2 as metzincins (20). Interestingly, the residue immediately following the third histidine of the zinc binding consensus (Val-744 in PAPP-A2) and one residue close

2The entire sequence of 5376 nucleotides encoding preproPAPP-A2 (1791 residues) is deposited in the GenBank™ data base under accession number AF311940.
to the methionine (Asn-805 in PAPP-A) are conserved within each of the four recognized metzincin superfamily members: the astacins, the reprolysins, the serralysins, and the matrix metalloproteinases (20). However, PAPP-A and PAPP-A2 do not fit in this pattern, they do not show homology to any members of the four families, and further, the linear distance between the third histidine and the methionine is much longer in PAPP-A and PAPP-A2. PAPP-A and PAPP-A2 are therefore reasonably classified as members of a new, fifth metzincin family, which we tentatively designate the pappalysins.

Edman degradation, performed on purified rPAPP-A2 (Fig. 2, lane 5) blotted onto a PVDF membrane, revealed the N-terminal residue of mature PAPP-A2 (Ser-234).

FIG. 1. Amino acid sequence of preproPAPP-A2 aligned with preproPAPP-A. The amino acid sequence of preproPAPP-A2 (PA2) was aligned with the sequence of preproPAPP-A (PA) using CLUSTALW (29). Because the prepro-part of PAPP-A did not show significant identity with the corresponding region of PAPP-A2, the alignment was manually adjusted to emphasize difference in length of pro-peptides. Arrows indicate the N-terminal residues of the mature proteins as found earlier for PAPP-A (8) (Glu-81), and here for PAPP-A2 (Ser-234). Putative signal peptides, strongly predicted using SignalP V2.0 (30), are shown with lowercase letters. The pro-part of PAPP-A2 contains one other candidate initiation codon corresponding to Met-168, but no signal peptide was predicted following this residue using SignalP. The sequence motifs of PAPP-A (8) are also found in PAPP-A2; the catalytic zinc binding motif (residues 733–743) and residues of the putative Met-turn (around Met-807) are underlined and bolded in both sequences. Lin-notch motifs (LNR1–3) and short consensus repeats (SCR-1–5) are boxed. All cysteines (shaded) of mature PAPP-A are also found in PAPP-A2. In addition, the secreted form of PAPP-A2 has four cysteine residues (Cys-343, Cys-533, Cys-618, and Cys-1268) with no counterpart in PAPP-A.

To obtain recombinant PAPP-A2 (rPAPP-A2), 293T cells were transiently transfected with an expression vector encoding c-myc tagged PAPP-A2 (pPA2-mH). Western blotting of the culture medium showed a single band of ~220 kDa (Fig. 2, lane 2), absent in medium from mock transfected cells (Fig. 2, lane 1). Reduction of disulfide bonds did not cause a visible change in band migration (Fig. 2, lane 4). Thus, in contrast to PAPP-A, PAPP-A2 is not a disulfide bound dimer.
The activity of PAPP-A2 against IGFBP-5. Medium from 293T cells transfected with empty vector (lane 1), cDNA encoding wild-type PAPP-A2 C-terminally tagged with the c-myc peptide (pPA2-mH), (lane 2, non-reduced), or cDNA encoding PAPP-A2 with an inactivating E734Q mutation (pPA2-KO-mH), (lane 3, non-reduced). Lane 4, as lane 2 but reduced. Recombinant PAPP-A2 was purified by nickel affinity chromatography from serum-free medium of cells transfected with pPA2-KO-mH (lane 5, reduced, Coomassie-stained).

To further analyze the cleavage of IGFBP-5 by PAPP-A2, IGFBP-5 was expressed in mammalian cells. Wild-type PAPP-A2 was purified by nickel affinity chromatography from serum-free medium of cells transfected with pPA2-KO-mH (lane 3). The activity of PAPP-A2 was abolished by cleavage product. In the absence of inhibitors, wild-type rPAPP-A2 c-myc, intact rIGFBP-5; sheared rIGFBP-5 was expressed in mammalian cells. Wild-type PAPP-A2 was purified by nickel affinity chromatography from serum-free medium of cells transfected with pPA2-KO-mH (lane 5, reduced, Coomassie-stained).

Because the two motifs responsible for the metalloproteolytic activity of PAPP-A are conserved in PAPP-A2, we hypothesized that PAPP-A2 is a proteolytic enzyme. Because IGFBP-4 is the only known PAPP-A substrate, we looked for a PAPP-A2 activity of PAPP-A are conserved in PAPP-A2, we hypothesized that cleavage was not induced by traces of IGF bound to PAPP-A2 (1 µg/20 µg of IGFBP-5). A Western blot of this digest using anti-c-myc recognizing the C-terminal cleavage product, is also shown (lane 3). Sequence analysis of protein blotted onto a PVDF membrane revealed that PAPP-A2 cleaves IGFBP-5 at one site between Ser-143 and Lys-144.

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4 IGFBP-5 is numbered with the N-terminal Leu of the mature protein as residue 1.
can on the N-terminal cleavage fragment is likely to cause it to smear around the two distinct, C-terminal fragments. Sequence analysis directly on the reaction mixture (>100 pmol) without SDS-PAGE separation showed only the same two IGFBP-5 sequences in equimolar amounts. Thus, PAPP-A2 cleaves IGFBP-5 at one site between Ser-143 and Lys-144.

Probing a blot with mRNA from several human tissues, previously used for PAPP-A (12), resulted in a signal from the human placenta only (not shown). However, using the blast algorithm (13), a total of 98 human EST sequences were identified that matched the 3′ untranslated region of the PAPP-A2 mRNA sequence. Of these, 39% originated from placenta, 21% from pregnant uterus, 11% from fetal liver/spleen, and 5% from kidney. Several other tissues were represented but with fewer EST sequences. Hence, like PAPP-A (12), PAPP-A2 expression is neither limited to the placenta nor to pregnancy.

Proteolytic activity against IGFBP-5 has been widely reported from several sources, e.g., pregnancy serum (22), seminal plasma (23), culture media from smooth muscle cells (24), granulosa cells (25), osteosarcoma cells (26), and also from osteoblasts (27) and fibroblasts (28). Matrix metalloproteinase-2 and the serine protease complement C1s have been reported to cleave IGFBP-4 physiologically. It is therefore very tempting to attribute in several systems. No other substrate has been found for PAPP-A, and no other protease has been shown to cleave IGFBP-4 physiologically. It is therefore very tempting to speculate that the pair of PAPP-A2 and IGFBP-5 plays an analogous role in a number of the tissues mentioned above. Interestingly, incubating IGFBP-5 with smooth muscle cells-conditioned medium resulted in cleavage between Ser-143 and Lys-144 (24), the same cleavage site as found here with purified PAPP-A2. This immediately identifies PAPP-A2 as an obvious candidate IGFBP-5 protease for this tissue.

After completion of the experimental work presented here, data base searching revealed that additional genomic sequences (AC027620 and AL139282) and a partial cDNA sequence (AJ279348) had appeared. From none of these, however, can the complete cDNA sequence of PAPP-A2 be deduced.

In conclusion, we have identified, cloned, and expressed a novel protein with homology to PAPP-A, and we have demonstrated the putative proteolytic activity of this protein. We named the protein PAPP-A2 to signify its close relationship with PAPP-A both structurally and functionally. With PAPP-A, PAPP-A2 defines a new, fifth family of the metzincin superfamily of metalloproteinases, the pappalysins. Further, we have identified a natural substrate for PAPP-A2, IGFBP-5, analyzed its cleavage, and indicated several tissues where PAPP-A2 may be of physiological relevance.

Note Added in Proof—Following the acceptance of this manuscript we have become aware that the partial sequence of database entry AJ279348 has been presented in a publication (Farr et al., 2000) Biochim. Biophys. Acta 1493, 356–362. In this paper, residues 147–1791 of PAPP-A2 is named PAPP-E.

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