Members of the pappalysin family of metzincin metalloproteinasases, pregnancy-associated plasma protein-A (PAPP-A, pappalysin-1) and PAPP-A2 (pappalysin-2), regulate the bioavailability of insulin-like growth factors (IGFs) by specific proteolytic inactivation of IGF-binding proteins (IGFBPs). PAPP-A cleaves IGFBP-4 and IGFBP-5, whereas PAPP-A2 cleaves only IGFBP-5. The pappalysins contain three Lin12-Notch repeat (LNR1–3) modules, previously considered unique to the Notch receptor family in which they function to regulate receptor cleavage. In contrast to the Notch receptor where three LNR modules are tandemly arranged, LNR3 is separated by more than 1000 residues from LNR1–2 in the pappalysin sequence. Each of the three LNR modules of PAPP-A is required for proteolysis of IGFBP-4, but not IGFBP-5. However, we here find that a C-terminal truncated variant of PAPP-A, which lacks LNR3 and therefore activity against IGFBP-4, cleaves IGFBP-4 when co-expressed with a PAPP-A variant, which is mutated in the active site. This suggests that LNR3 from the inactive subunit interacts in the active site. This work was supported by grants from the Lundbeck Foundation and the Danish Medical Research Council. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The metalloproteinase pregnancy-associated plasma protein-A (PAPP-A, pappalysin-1, EC 3.4.24.79) is secreted as a disulfide-linked 400-kDa homodimer (1) that cleaves insulin-like growth factor binding proteins (IGFBPs) (2) and IGFBP-5 (3). Through this proteolytic activity, PAPP-A causes the release of active IGF and thereby regulates its bioavailability in several biological systems. In particular, the role of PAPP-A has been studied in ovarian follicular development (4), implantation (5), fetal development (6–8), wound healing (9), and atherosclerosis (10, 11).

PAPP-A is the founding member of the pappalysin family (12, 13) within the metzincin superfamily of metalloproteinasases (14, 15), also including its only known homologue, PAPP-A2, which cleaves IGFBP-5, but not IGFBP-4 (16). The 1558-residue PAPP-A2 shares 46% of its residues with the 1547-residue PAPP-A (17), and they display the same domain organization with a laminin G-like domain and a proteolytic domain in the N-terminal part (18), a central region of ~500 residues with unknown domain composition, and a C-terminal part with five complement control protein (CCP) modules that mediate cell surface adhesion of PAPP-A (19, 20). The pappalysins also contain three Lin12-Notch repeat (LNR) modules, previously considered unique to the Notch receptor family. But in contrast to the Notch receptors, which invariably contain three tandemly arranged LNR modules, two of the LNR modules of the pappalysins (LNR1–2) are located together within the sequence of the proteolytic domain, and the third (LNR3) is located C-terminal to the CCP modules, separated by more than 1000 residues from LNR1–2.

In the Notch receptor, several studies indicate that the LNR modules are involved in the regulation of ligand-induced receptor cleavage by metzincin metalloproteinasases of the ADAM family (21, 22). This cleavage is critical for the subsequent intramembrane processing of the receptor by γ-secretase and hence Notch signaling (23, 24). Although the underlying mechanism is unknown, mutation or deletion of the LNR modules has been found to cause ligand-independent cleavage of the Notch receptor (25, 26), whereas removal of the ligand-binding sites results in receptors that are resistant to proteolysis (27, 28). It is therefore believed that the LNR modules function to...
restrain access to the cleavage site when ligand is not bound and that receptor ligand binding renews this effect.

Recent data demonstrated that each of the three LNR modules is required for PAPP-A proteolysis of IGBP-4, but not IGBP-5 (29). It was therefore speculated that the three LNR modules of PAPP-A, although separated in the primary structure, interact with each other to determine PAPP-A substrate specificity. We here report data suggesting that within the disulfide-linked PAPP-A homodimer, such a functional LNR unit is formed in trans by the interaction between LNR1–2 of one subunit and LNR3 of the other subunit. By means of PAPP-A/PAPP-A2 chimeras, we show that the LNR1–2 modules, but not LNR3, are functionally conserved. We also identify a region C-terminal to LNR3, and several charged residues within this region, as critical for PAPP-A LNR functionality.

EXPERIMENTAL PROCEDURES

Plasmid Construction and Mutagenesis—Single (PA(K1509A), PA(K1511A), PA(K1512A), PA(D1521A), PA(D1525A), PA(R1529A), PA(D1530A), PA(E1535A), PA(R1538A), PA(K1539A), PA(D1540A), PA(R1542A)) and triple (PA(C381A/C652A/C1130A)) point mutants of the 1547-residue human PAPP-A2 followed by the c-Myc epitope and a His tag (16), BspEI and the PAPP-A2 expression plasmid pPA2-mH, encoded at residue 407. Outer primers, derived from pBlueScript II SK(+), were 5′-GAACGGTTTC-3′ (corresponding to nucleotides 1114–1135 of pcDNA3.1/H11001/H11032-TCTAGA-3′ (lowercase sequence corresponds to nucleotides 1036–1061 of pcDNA3.1+ (Invitrogen)).

PA-P2(LNR1–2), where PAPP-A residues 334–393 were exchanged by the corresponding residues of PAPP-A2 (16), was prepared using pPA-BspEI and pPA2-mH as templates, and vector-derived outer primers (5′-TAATACGACTCACTATA-GGG-3′ (T7) and 5′-TAAGAGGCCACAGTCGAGG-3′ (BGH REV)). PA-P2(LNR3), where PAPP-A residues 1476–1504 were exchanged by the corresponding residues of PAPP-A2, was prepared using pPA-P2(1476–1547) as template, and 5′-CATCATCGGACAGGCCACAGCATC-3′ (corresponding to nucleotides 1036–1061 of pcDNA3.1+ (Invitrogen)) as outer primers. Using construct PA-P2(LNR1–2) as template, PA-P2(LNR1–2,3) (with residue 1–1477), PA(E483A/D1521A), PA(E483A/R1529A), and PA(E483A/D1530A).

The internal primers used to generate mutated or chimeric fragments all had an overlap of 15–20 bp. The resulting PCR products for chimeric constructs were swapped into the BspEI/XbaI or KpnI/XbaI sites of pPA-BspEI.

Expression constructs encoding variants of PAPP-A mutants PA(1–1477) (29), PA(D1521A), PA(R1529A), and PA(D1530A) further active site-inactivated by substituting Glu with Ala, were constructed by swapping the BspEI/KpnI fragment of PA(E483A) (12) into the BspEI/KpnI site of the mutants. Thus, the resulting expression constructs encoded PA(E483A/1–1477), PA(E483A/D1521A), PA(E483A/R1529A), and PA(E483A/D1530A).

Constructs encoding C-terminal PAPP-A fragments PA(1476–1547), PA(1393–1547), PA(1330–1547), PA(1263–1547), PA(1201–1547), PA(1133–1547), PA(1108–1547), PA(1077–1547), and PA(1061–1547) were prepared by PCR using pPA-BspEI as a template. 5′-outer primers contained a signal peptide cleavage site optimized using SignalP 3.0 (31) and included an Xhol site (located in the signal peptide of pPA-BspEI and shown here in bold). The 5′-outer primer was 5′-TCTGCACTCATCACTATAGGG-3′ (corresponding to PAPP-A residues 380–387) and 5′-TTGCTTCAAGcactagaaagagcatcgagc-3′ (lowercase sequence corresponds to nucleotides 1114–1135 of pcDNA3.1/Myc-His(−)A (Invitrogen) and an XbaI site is shown in bold). For chimeras PA-P2(106–1477), PA-P2(1099–1547), PA-P2(1135–1547), PA-P2(1333–1547), PA-P2(1476–1547), PA-P2(1504–1547), and PA-P2(1529–1547), outer primers were 5′-CATCATCGGACAGGCCACAGCATC-3′ (corresponding to PAPP-A residues 961–968) and 5′-TTGCTTCAAGcactagaaagagcatcgagc-3′ (lowercase sequence corresponds to nucleotides 1114–1135 of pcDNA3.1/Myc-His(−)A (Invitrogen); an XbaI site is shown in bold). Using constructs PA-P2(1064–1477), PA-P2(1088–1477), PA-P2(1476–1547), or PA-P2(1504–1547) as templates, a second and 5′-outer primer was 5′-GCAAACAACAGATGG-
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CTGCAACTAG-3’ (corresponding to nucleotides 1036–1061 of pCDNA3.1+ (Invitrogen)). The resulting PCR products were swapped into the XhoI/XbaI site of pPA-BspEI. An expression construct encoding mutant PA(937–1547) was prepared by overlap extension PCR using pPA-BspEI as a template. Outer primers were 5’-GACGCTAGCTTTAGAGATTCCCCTG-3’ (corresponding to the signal peptide of pPA-BspEI; a HindIII site located within the signal peptide of pPA-BspEI is shown in bold), and 5’-TCAACGCTAGCTAGCCATGGCTGTATCCGCGAG-3’ (corresponding to residues 1541–1547 of PAPP-A; an XbaI site is shown in bold). The resulting PCR product was swapped into the HindIII/XbaI site of pPA-BspEI. All plasmid constructs were verified by sequence analysis. At least two clones of each construct were used for further analysis.

Cell Culture and Expression of Recombinant Proteins—Human embryonic kidney 293T cells (293tsA1609neo) (32) 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 were plated onto 6-cm tissue culture dishes and transfected 18 h later by calcium phosphate co-precipitation using 10 μg of plasmid DNA (33), or in the case of co-transfections 10 μg of plasmid DNA of each PAPP-A variant, prepared by QIAprep Spin Kit (Qiagen). After 48 h, the culture media were harvested, cleared by centrifugation, and replaced with QIAprep Spin Kit (Qiagen). After 48 h, the culture media were harvested, cleared by centrifugation, and replaced by serum-free medium to be harvested after another 48 h.

Western Blotting—For immunovisualization, culture media containing recombinant wild-type or mutant protein were separated by non-reducing SDS-PAGE (5–15% Tris glycine gels) and blotted onto a polyvinylidene difluoride membrane (Millipore). The blots were dried and blocked in 5% bovine serum albumin (BSA). After sample incubation, a PAPP-A-specific monoclonal antibody (mAb 234-5 or 234-2 (35), which recognizes the N-terminal portion and the C-terminal portion of PAPP-A, respectively,4 followed by peroxidase-conjugated anti-mouse IgG (P260, DAKO), was used for detection. Antibodies were diluted in PBS containing 0.01% Tween 20 (PBST) and 1% bovine serum albumin. PBST was used for washing. A double monoclonal ELISA was used for detection of the PAPP-A dimer. In this assay, plates were coated with mAb 234-5 and sample incubation (100 μl containing 25 nm of PAPP-A or PAPP-A mutant) was followed by incubation (1 h at 37 °C) with PBS to which 0–1 mM NaCl was added. Biotinylated mAb 234-5, followed by incubation with peroxidase-conjugated avidin (P0347, DAKO), was used for detection. Washing after incubation with peroxidase-conjugated avidin was carried out using PBST supplemented with 200 mM NaCl to avoid nonspecific binding. Otherwise, washing and blocking was as described above. Dilution series of the PAPP-A/pro-MBP complex purified from pregnancy serum (34) were used in both ELISAs to establish standard curves.

Measurement of Proteolytic Activity—The proteolytic activity of wild-type PAPP-A or PAPP-A mutants was compared by incubating with purified 125I-labeled substrates, as described for IGFBP-4 (3, 12) and IGFBP-5 (3). The expression levels of wild-type PAPP-A and PAPP-A mutants were determined by ELISA (as described above) and the amount of proteinase was adjusted to equimolar levels by dilution of the culture medium. Briefly, reactions (total sample volume of 20 μl) were carried out in 50 mM Tris-HCl, 100 mM NaCl, 1 mM CaCl2, pH 7.5, at an enzyme:substrate ratio of 1:160 (0.1 nm proteinase and 16 nm substrate), in the presence (IGFBP-4) or absence (IGFBP-5) of 50 nm IGF-II (Diagnostic Systems Laboratories). Following incubation for 2 h at 37 °C, reactions were stopped by addition of 10 mM EDTA and stored at −20 °C. Cleavage products were separated by 10–20% SDS-PAGE and visualized by autoradiography. The degree of cleavage was determined by quantification of band intensities using a Typhoon imaging system (Amersham Biosciences), and background levels (mock signal) were subtracted. The degree of cleavage by wild-type PAPP-A under these conditions (2 h incubation) was ~60%. The affect on proteolysis at PA(1133–1547) was analyzed by the addition of ~100 nm to the reaction. To assay the proteolytic activity of dissociated wild-type PAPP-A or the PA(C381A/C652A/C1130A) mutant dimer, a 96-well plate coated with mAb 234-5 was incubated (1 h at 37 °C) with 100 μl of culture medium containing 25 nm proteinase, washed in PBST, and incubated (1 h at 37 °C) with PBS with or without an additional 0.5 mM NaCl. Following washing and equilibration in 50 mM Tris-HCl, 100 mM NaCl, 1 mM CaCl2, pH 7.5, reaction mixtures containing radiolabeled substrate (as described above) were added directly to the wells (total sample volume of 50 μl). After incubation for 2 h at 37 °C, 10 μl of reaction sample was stopped by the addition of 10 mM EDTA and stored at −20 °C. When assaying the IGFBP-4 proteolytic activity of PAPP-A/PAPP-A2 chimeric proteins, proteinase levels were adjusted to have the same activity against IGFBP-5 (40% cleavage after 2 h incubation), and were tested for IGFBP-4 proteolytic activity by time course experiments (reaction mixtures as described above using a total sample volume of 70 μl). Samples of 10 μl were stopped (as described above) at each time point (from 0 to 24 h). The initial cleavage rate of the chimeras was determined as the inclination of the linear phase of curves from the IGFBP-4 time course experiments (% cleavage of IGFBP-4 plotted as a function of 4 R. Soe and C. Oxvig, unpublished result.
time), and plotted relative to the wild-type PAPP-A IGFBP-4 cleavage rate.

**Chemical Cross-linking**—Proteins contained in serum-free culture medium (~20 μg/ml) and dialyzed against 50 mM Hepes, 100 mM NaCl, pH 7.5, were incubated with 0.05 or 0.5 mM EGS (ethylene glycolbis(succinimidyl succinate), Pierce) dissolved in dimethyl sulfoxide (Me2SO) for 30 min at room temperature. Samples (30 μl) of the reaction mixtures were quenched with 5 μl of 1 M Tris-HCl, 1 M glycine, pH 7.5, and mixed with SDS-PAGE loading buffer with reductant (20 mM dithiothreitol). Cross-linked adducts were separated by 5–15% SDS-PAGE and visualized by Western blotting as described above. The positions of intact and cleaved IGFBP-4 are indicated. The faint upper band represents a glycosylated variant of IGFBP-4 (3). The positions of intact IGFBP-5 and the cleavage products, which migrate close to each other, are indicated. Because of heterogeneous glycosylation, the intact IGFBP-5 appears as a broad band (3). The results shown are representative of four independent experiments. C, Illustration of the three types of dimers formed by co-expression of PA(E483A) and PA(1–1477), and their respective proteolytic activities against IGFBP-4 and -5 as determined in A and B. Only the proteolytic domain (rectangle) and the LNR modules (circles) are indicated. Inactivation of the proteolytic domain by the E483A mutation is indicated by an asterisk (*). See main text for further details.

**Native PAGE**—Proteins were loaded on a native 5–10% Tris-glycine gels (36) prepared without SDS, electrophoresed for 3 h at 10 mA, and visualized by Western blotting as described above using an anti-c-Myc antibody (mAb 9E10) as primary antibody and secondary peroxidase-conjugated antiserum IgG (P260, DAKO). To allow detection of PAPP-A by anti-c-Myc mAb, PA-P2(1529–1547), containing PAPP-A2 residues 1550–1558 followed by the c-Myc epitope in the C terminus was used.

**Surface Plasmon Resonance Analysis**—Surface plasmon resonance experiments were carried out on a BIAcore T100 instrument (BIAcore AB, Uppsala, Sweden) using series S CM5 sensor chips and coupling reagents supplied by the manufacturer. Immobilization of anti-c-Myc (9E10) was performed by first activating the surface of the sensor chip with a mixture of coupling reagents EDC/NHS followed by injection of purified mAb 9E10 (30 μg/ml) in 10 mM sodium acetate, pH 5.0) at 25 °C. Remaining activated groups were blocked by 1 M ethanolamine. The level of mAb 9E10 immobilization corresponded to 10,000 resonance units. Purified c-Myc-tagged PAPP-A LNR Modules Interact in Trans

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**Size Exclusion Chromatography**—Samples (600 μl) of PAPP-A wild-type and the PAPP-A C381A/C652A/C1130 mutant contained in diluted (1:2) culture medium were loaded onto a Superose 6 10/300 GL column (Amersham Biosciences) equilibrated with 50 mM Tris-HCl, 50 mM NaCl, 2 mM CaCl2, pH 7.5. The flow rate was 0.3 ml/min and 0.3-ml samples were collected. The amount of protein contained in each fraction was measured by ELISA (as described above).

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background signal, determined by the injection of PAPP-A variants over a surface containing mAb 9E10 only.

RESULTS

LNR Modules Interact in Trans within the PAPP-A Dimer—Based on analogy to the tandemly arranged Notch receptor LNRs, we hypothesize two scenarios of PAPP-A LNR interactions. In one model, PAPP-A is folded so that LNR3 interacts with LNR1–2 of the same polypeptide. Alternatively, as PAPP-A is a dimeric protein, LNR1–2 from one subunit interacts with LNR3 of the other subunit within the PAPP-A dimer. To distinguish between these two possibilities, cells were co-transfected to express a C-terminal truncated variant of PAPP-A lacking LNR3, PA(1–1477) (29), and a proteolytically inactive PAPP-A variant with the active-site glutamic acid substituted with alanine, PA(E483A) (12). As expected, when either PAPP-A variant was expressed alone, no activity against IGFBP-4 could be detected in the culture medium (Fig. 1A, lanes 3 and 4), and activity against IGFBP-5 was detected only with culture medium containing the truncated PAPP-A variant, PA(1–1477) (Fig. 1B, lane 3). However, upon co-expression, proteolytic activity against IGFBP-4 was rescued, corresponding to the formation within the dimer of one proteolytic domain with activity toward IGFBP-4 (Fig. 1A, lane 5). This is most likely explained by an interaction of LNR3 from the inactive subunit (E483A) with LNR1–2 from the truncated PAPP-A subunit, PA(1–1477), suggesting that the three LNR modules interact in trans within the PAPP-A dimer (Fig. 1C).

Dimerization, but Not Intersubunit Disulfide Bond Formation, Is Required for PAPP-A LNR Functionality—The above findings lead us to analyze the functional implications of disrupting the covalently associated PAPP-A dimer. An expression construct encoding a PAPP-A mutant, PA(C381A/C652A/C1130A), which is unable to form the Cys1130–Cys1130 disulfide bond responsible for covalent dimerization (37), was constructed. To avoid possible unspecific polymerization through Cys381 or Cys652, known to be unpaired (38), alanine substitutions of these residues were included in the mutant. This PAPP-A mutant displayed wild-type level activity against IGFBP-4 (Fig. 2A, lane 3), suggesting that covalent dimerization is not required for LNR functionality. To test if the PAPP-A mutant forms a non-covalent dimer, we subjected it to chemical cross-linking. Upon reducing SDS-PAGE, the cross-linked mutant was found to co-migrate with cross-linked wild-type PAPP-A (Fig. 2B), demonstrating that the mutant is able to dimerize non-covalently. This finding was confirmed by size exclusion chromatography, in which PA(C381A/C652A/C1130A) and PAPP-A wild-type co-eluted (data not shown).

When the PA(C381A/C652A/C1130A) mutant was immobilized on plastic by means of a monoclonal antibody, increasing ionic strength dissociated the non-covalently associated dimers (Fig. 3A), whereas no effect was observed on wild-type PAPP-A dimers (Fig. 3B). In accordance with this observation, the detectable IGFBP-4 proteolytic activity of the immobilized and dissociated PAPP-A mutant was found to be extensively compromised (Fig. 4), whereas the IGFBP-5 proteolytic activity was only moderately reduced (data not shown), corresponding to the expected subunit dissociation. This experiment shows that...
non-covalent dimerization is required and sufficient for LNR functionality, further suggesting that an LNR unit is formed in trans between the subunits of the PAPP-A dimer.

The Pappalysin LNR Modules Differ in Functional Conservation—The PAPP-A homologue, PAPP-A2, which cleaves IGFBP-5, but not IGFBP-4, does not dimerize covalently, although the PAPP-A2 residue corresponding to PAPP-A Cys1130 is also a cysteine (16). However, when analyzed by native PAGE, PAPP-A2 migrated similar to wild-type PAPP-A (Fig. 5A), suggesting that it exists as a non-covalently associated dimer. A weak band, corresponding to monomer, revealed some dissociation of the PAPP-A2 subunits under the experimental conditions employed.

To investigate whether the LNR modules of PAPP-A2 are functionally conserved within the context of the PAPP-A dimer, LNR1–2, LNR3, and LNR1–3 of PAPP-A were replaced with the corresponding PAPP-A2 sequence. All variants were expressed as covalent dimers (Fig. 5B) and displayed PAPP-A wild-type level IGFBP-5 proteolytic activity (Fig. 5C), suggesting that the integrity of the proteolytic domain is maintained intact. When all three LNR modules of PAPP-A2 were inserted into PAPP-A, or when LNR3 alone was replaced by the PAPP-A2 sequence, very little activity against IGFBP-4 could be detected (Fig. 5D, lanes 3 and 4). In contrast, PAPP-A containing PAPP-A2 LNR1–2 showed wild-type level IGFBP-4 proteolytic activity (Fig. 5D, lane 5), demonstrating that full LNR functionality can be obtained with LNR1–2 of PAPP-A2 and LNR3 of PAPP-A. Thus, LNR1–2 are functionally conserved between PAPP-A and PAPP-A2 with respect to IGFBP proteolysis, but LNR3 is not, and this module appears to be critical for LNR unit function.

Identification of Two Regions Required for LNR Functionality—As a strategy to identify other regions that might influence the function of the LNR modules, we took further advantage of the fact that PAPP-A2 cleaves IGFBP-5, but not IGFBP-4, and constructed a set of chimeric PAPP-A/PAPP-A2 proteins with progressively less PAPP-A2 C-terminal sequence (Fig. 6A). All preparations of chimeras were adjusted to contain the same activity against IGFBP-5 prior to analysis of IGFBP-4 cleavage. This experiment identified a region (residues 1064–1098) near the PAPP-A dimerization cysteine (Cys1130), where a shift between no activity against IGFBP-4 and reduced activity occurred (Fig. 6B). Interestingly, PAPP-A/PAPP-A2 chimeras with no IGFBP-4 proteolytic activity were found to migrate as monomers in SDS-PAGE (Fig. 6C, lanes 1–3), whereas chimeras active against IGFBP-4 formed covalent dimers similar to
wild-type PAPP-A (Fig. 6C, lanes 4–6). Further analysis of this region showed that a chimera with this sequence stretch of 35 residues replaced by the PAPP-2 sequence had no activity positions of intact and cleaved IGFBP-4 and -5 are indicated. The results shown are representative of at least three independent experiments.
against IGFBP-4, whereas chimeras with smaller parts of the sequence replaced displayed wild-type level or slightly reduced IGFBP-4 proteolytic activity (Fig. 7A). This indicates that substitution in this region, possibly located near a hypothetical dimerization interface, influences the arrangement of the dimer and therefore in trans interactions of the LNR modules.

Surprisingly, of the initial set of chimeras (Fig. 6, A and B), the chimera PA-P2(1504–1547), which contains LNR3 of PAPP-A, still shows a marked reduction in cleavage of IGFBP-4. Only PA-P2(1529–1547), in which the last 19 C-terminal residues of PAPP-A are replaced with the PAPP-A2 sequence, showed wild-type level activity against IGFBP-4. Construction and analysis of two additional chimeras revealed that the stretch of 24 residues located immediately C-terminal to LNR3 is required for cleavage of IGFBP-4 (Fig. 7B). Thus, in addition to LNR3 itself, this region is important for LNR functionality. Further analysis by single amino acid substitution of charged residues identified three residues C-terminal to LNR3, Arg1529, and Asp1530, as critical for proteolysis of IGFBP-4 by PAPP-A (Fig. 8). At these three positions, substitution with alanine caused a complete loss (Arg1529 and Asp1530) or a substantial reduction (Asp1521) in proteolytic activity against IGFBP-4, but did not affect activity against IGFBP-5. Importantly, no IGFBP-4 proteolytic activity was restored when these mutants were co-expressed with the truncated PAPP-A variant, PA(1–1477) (data not shown), implying a direct role of these C-termi-
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**FIGURE 9. Detection of substrate binding of PAPP-A variants by surface plasmon resonance.** A, complete sensorgram of the interaction between IGFBP-5 and the active site-inactivated PAPP-A variant PA(E483A). c-Myc-tagged IGFBP-5 was injected onto an anti-c-Myc-coated chip (the level of immobilization was 10,000 resonance units), and PA(E483A) was injected during its dissociation phase. Arrows indicate the beginning of injections. B, a similar experiment using IGFBP-5 and PAPP-A variants PA(E483A) (black line), PA(E483A/D1521A) (gray line), PA(E483A/R1529A) (gray line), and PA(E483A/D1530A) (gray line), and PA(E483A/1–1477) (gray line), all at a concentration of 0.15 mg/ml. The portions of the sensorgrams shown correspond to the time from the beginning of injection of PAPP-A variants. C, complete sensorgram of the interaction between IGFBP-4/4IGF-II and PA(E483A), c-Myc-tagged IGFBP-4, IGF-II, and PA(E483A) were injected sequentially onto the anti-c-Myc-coated chip. Arrows indicate the beginning of injections. D, a similar experiment using IGFBP-4/4IGF-II and PAPP-A variants PA(E483A) (black line), PA(E483A/D1521A) (gray line), PA(E483A/R1529A) (gray line), and PA(E483A/D1530A) (gray line), and PA(E483A/1–1477) (gray line), all at a concentration of 0.15 mg/ml. The portions of the sensorgrams shown correspond to the time from the beginning of injection of PAPP-A variants. In all experiments, the background signal was subtracted from recorded signals. The sensorgrams shown are representative of at least four independent experiments.

Proximal residues in permitting the in trans interactions of LNR3, or a direct participation in the binding of IGFBP-4 to PAPP-A. To analyze whether these mutants showed altered substrate binding, IGFBP-4/4IGF-II or IGFBP-5 was immobilized to a BIAcore chip via an antibody. The PAPP-A mutants, further active site-inactivated by means of a Glu to Ala substitution at position 483 against IGFBP-4 (Fig. 4). We therefore also conclude that formation of the Cys1130–Cys1130 intersubunit disulfide bond is not required for LNR functionality (Fig. 2). The Cys1130-mutated PAPP-A forms a non-covalent dimer, which upon dissociation loses its activity against IGFBP-4 (Fig. 4). We therefore also conclude that although formation of the intersubunit disulfide bond is not formed with PA(937–1547), but not with any other of these C-terminal fragments (Fig. 10C and data not shown). In accordance with this observation, only PA(937–1547) was able to rescue the IGFBP-4 proteolytic activity of PA(1–1477) following co-expression (Fig. 10D), supporting our observation that dimerization is important for the in trans interaction of LNR3.

**DISCUSSION**

We here show that the three LNR modules of the metalloprotease PAPP-A determine its specificity by in trans LNR interactions (Fig. 1), most likely by the formation of a functional LNR unit between LNR1–2 and LNR3. Such a functional LNR unit, formed between LNR1–2 and LNR3 from different subunits within the PAPP-A dimer, is required for proteolysis of IGFBP-4, but not IGFBP-5. We also show that formation of the Cys1130–Cys1130 intersubunit disulfide bond is not required for LNR functionality (Fig. 2). The Cys1130-mutated PAPP-A forms a non-covalent dimer, which upon dissociation loses its activity against IGFBP-4 (Fig. 4). We therefore also conclude that although formation of the intersubunit disulfide bond is not...
required, formation of a functional LNR unit depends on dimerization. We further show that PAPP-A2, which cleaves IGFBP-5, but not IGFBP-4, forms dimers (Fig. 5A), although its subunits are not disulfide-linked (16). PAPP-A, in which the LNR1–2 modules were replaced by the corresponding PAPP-A2 sequence, unexpectedly showed wild-type IGFBP-4 activity (Fig. 5D), suggesting that LNR1–2 of PAPP-A2 are able to form a functional unit with LNR3 of PAPP-A. This demonstrates that LNR1 and LNR2 are functionally conserved between PAPP-A and PAPP-A2. However, when LNR3 of PAPP-A was replaced with PAPP-A2 sequence, the activity against IGFBP-4 was almost completely abolished (Fig. 5D).

By the analysis of PAPP-A/PAPP-A2 chimeras (Fig. 6), we further defined a region C-terminal to LNR3, which appears to be required for LNR functionality. We here identified three charged residues (Asp<sup>1521</sup>, Arg<sup>1529</sup>, and Asp<sup>1530</sup>), which are critical for proteolytic activity against IGFBP-4 (Fig. 8). Furthermore, PAPP-A mutants with these residues substituted into alanine were unable to rescue the IGFBP-4 activity of the PAPP-A truncation variant PA(1–1477) in co-expression experiments (data not shown), emphasizing the importance of these residues. Although these charged residues are conserved in PAPP-A2, this region of PAPP-A could not be replaced by the corresponding PAPP-A2 sequence without a substantial loss of IGFBP-4 activity (Fig. 7B). Thus, LNR3 and the C-terminal sequence stretch may be structurally interdependent and both required for formation of the LNR unit. Additionally, by the analysis of the chimeric proteins we identified a region located between residues 1064 and 1098 (Fig. 7A), which appears to be important for PAPP-A dimerization and formation of the Cys<sup>1130</sup>–Cys<sup>1130</sup> intersubunit disulfide.

What is the role of LNR3 in discriminating between the two PAPP-A substrates? The LNR3 module and/or the sequence stretch...
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C-terminal to LNR3 may participate directly in substrate binding to IGFBP-4. It is also possible that an IGFBP-4 substrate binding region may be formed in LNR3 or elsewhere on the trimeric LNR unit, with LNR3 and/or the following sequence stretch being required for its formation. Both of these scenarios are in agreement with the existence in PAPP-A of a classical protease substrate-binding exosite (39). Using surface plasmon resonance analysis, we were able to demonstrate a substrate-proteinase interaction between IGFBP-4/IGF-II and an active site-inactivated variant of PAPP-A, PA(E483A), but not selected PAPP-A variants also mutated in the C-terminal sequence stretch or lacking LNR3 (Fig. 9, C and D). In contrast, all variants analyzed showed binding to IGFBP-5 (Fig. 9, A and B). However, a C-terminal fragment of PAPP-A, PA(1133–1547) (Fig. 10A), was unable to bind IGFBP-4 and also unable to compete with PA(E483A) for substrate binding. This experiment emphasizes the requirement for LNR unit formation, regardless of whether LNR3 and/or the C-terminal sequence stretch participates directly or indirectly in substrate binding. We attempted the expression of a series of C-terminal PAPP-A variants, all containing LNR3 and the sequence stretch C-terminal to LNR3 (Fig. 10, A and B). Although not all variants were expressed, we found that only variant PA(937–1547), which formed a dimer with PA(1–1477) (Fig. 10C), was able to rescue the proteolytic activity of PA(1–1477) against IGFBP-4 (Fig. 10D). This experiment suggests that LNR3 cannot access LNR1–2 to form a functional LNR unit unless dimerization occurs, and it further supports, in an independent manner, that such dimerization requires the sequence stretch between residues 1064 and 1098, contained within PA(937–1547).

Compared with the Notch receptor family where three copies of the LNR module invariably are arranged consecutively, an interaction in trans of LNR1–2 and LNR3 within the PAPP-A dimer appears to compensate for the interrupted arrangement of the LNR modules in the pappalysins. Although the spatial interrelationship of the three Notch LNR modules is yet unknown, it is tempting to speculate that a similar trimeric LNR unit is formed in both protein families. Accordingly, a high degree of conservation of the short linker sequence between the Notch LNR modules of different species as well as gain-of-function mutations located in these, has suggested that the Notch LNR modules structurally depend on each other (40). This is supported by the finding that several hydrophobic residues are affected when a single LNR module was examined by NMR in the presence of a neighboring LNR module (41), indicating the existence of hydrophobic interactions between the three LNR modules. Furthermore, it has been suggested that residues from adjacent Notch LNR modules might contribute to the coordination of calcium ions (41), and calcium depletion has been found to cause activation and subunit dissociation of the Notch receptor (25). PAPP-A IGFBP-4 proteolytic activity likewise depended on calcium ions (29), indicating that calcium plays an analogous stabilizing role for the LNR modules of both proteins. This is further supported by the finding that the individual substitution of conserved acidic residues within each of the LNR modules, thought to coordinate calcium ions, causes the loss of activity against IGFBP-4 without any change in activity against IGFBP-5 (29).

Recently, the structure of ulilysin, an archaeal protein that shares sequence similarity with PAPP-A, but only encompasses the proteolytic domain, was solved (42). Ulilysin does not contain LNR modules and displays broad substrate specificity, as it cleaves several extracellular matrix proteins and all the IGFBPs, except for IGFBP-1, at several sites. This indicates that the additional modules and domains of PAPP-A function to control the specificity of the proteolytic domain. Another example of a metzincin with changed substrate specificity upon removal of domains is ADAMTS-4, in which the deletion of a C-terminal spacer domain resulted in broader substrate specificity. However, the isolated proteolytic domain of ADAMTS-4 displayed reduced proteolytic activity (43). It is tempting to speculate that association of LNR3 with LNR1–2 may function to control substrate access to the active site. Such an alternative model is speculative, but would be in accordance with models of LNR function within the Notch receptor, where the modules are thought to play a role in restraining the proteolytic cleavage of the receptors by the metzincin proteinase ADAM17/TACE (21, 23, 26). Further investigation is necessary to delineate details of the mechanism by which the proposed LNR unit regulates PAPP-A substrate specificity.

In conclusion, our data suggest that a specificity determining LNR unit is formed in trans by an association of LNR1–2 and LNR3 from different subunits of the PAPP-A dimer, and that it depends on PAPP-A dimerization. Identified charged residues located C-terminal to LNR3 appear to be required for the function of such a LNR unit. Our findings further suggest that the pappalysin and Notch LNR modules have a similar intermodular relationship.

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