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The Lin12-Notch Repeats of Pregnancy-associated Plasma Protein-A Bind Calcium and Determine Its Proteolytic Specificity*

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The Lin12-Notch repeat (LNR) module of about 35 residues is a hallmark of the Notch receptor family. Three copies, arranged in tandem, are invariably present in the extracellular portion of the Notch receptors. Although their function is unknown, genetic and biochemical data indicate that the LNR modules participate in the regulation of ligand-induced proteolytic cleavage of the Notch receptor, a prerequisite to intramembrane cleavage and Notch signaling. Outside the Notch receptor family, the LNR module is present only in the metalloproteinase pregnancy-associated plasma protein-A (PAPP-A) and its homologue PAPP-A2, which also contain three copies. Curiously, LNR modules 1 and 2 are present within the proteolytic domain of PAPP-A/A2, but LNR3 is separated from LNR2 by more than 1000 amino acids. The growth factor antagonists insulin-like growth factor-binding protein (IGFBP)-4 and -5 are both substrates of PAPP-A. We provide here evidence that the PAPP-A LNR modules function together to determine the proteolytic specificity of PAPP-A. Analysis of C-terminally truncated PAPP-A mutants followed by the analysis of LNR deletion mutants demonstrated that each of the three PAPP-A LNR modules is strictly required for proteolytic activity against IGFBP-4 but not for proteolytic activity against IGFBP-5. Individual substitution of conserved LNR residues predicted to participate in calcium coordination caused elimination (D341A, D356A, D389A, D1484A, D1499A, and D1502A) or a significant reduction (D359A and E392A) of IGFBP-4 proteolysis, whereas IGFBP-5 proteolysis was unaffected. The activity of the latter mutants against IGFBP-4 could be partially rescued by calcium, and the addition of the calcium-binding protein calbindin D9k to wild-type PAPP-A eliminated activity against IGFBP-4 but not against IGFBP-5, demonstrating that the PAPP-A LNR modules bind calcium ions. We propose a model in which LNR3 is spatially localized in proximity to LNR1 and -2, forming a single functional unit.

The insulin-like growth factors (IGF-I and -II)[1] have potent anabolic and mitogenic effects mediated primarily through the type 1 IGF receptor and are important during fetal and postnatal development (1, 2). Their biological activities are modulated by a family of six IGF-binding proteins (IGFBPs) to which the IGFs bind with high affinities and then become unable to interact with the receptor (3). Proteolytic cleavage in the central region of the IGFBPs is an important means by which the biological activities of the IGFs are regulated. The cleavage products have insignificant affinities for the IGFs, and proteolysis thus causes dissociation of bioactive IGF (4).

The metalloproteinase pregnancy-associated plasma protein-A (PAPP-A, pappalysin-1, EC 3.4.24.79) cleaves IGFBP-4 (5) and -5 (6) dependent and independent, respectively, of IGF. In humans, birth weight correlates with the maternal level of PAPP-A (7), and PAPP-A-null mice are proportional dwarfs (8), strikingly similar in phenotype to IGF-II-null mice. These findings emphasize the role of PAPP-A in fetal development, but PAPP-A is ubiquitously expressed in human tissues (9). Recent data suggest the involvement of PAPP-A in ovarian follicular development (10), human implantation (11), wound healing (12), and atherosclerosis (13).

PAPP-A belongs to the metzincin superfamily of metalloproteinases but is distinct from other members, such as the matrix metalloproteinases (MMPs) and the ADAMs (14, 15). The secreted protein is a disulfide-linked 400-kDa homodimer of 1547 residue subunits. Its proteolytic domain of ~300 residues is located in the N-terminal half of the subunit, and five complement control protein modules (CCP1–5), which mediate cell surface adhesion (16, 17), are located near the C terminus (Fig. 1*A*). Other tentatively defined domains are present N-terminal to the proteolytic domain and between the proteolytic domain and the CCP modules. Additionally, two Lin12-Notch repeats (LNRs) are inserted in the proteolytic domain (Fig. 1, *A* and *B*), and a third LNR module is located C-terminal to CCP5 (Fig. 1*A*) (18, 19). The domain organization of PAPP-A is shared with PAPP-A2, the only other close relative of PAPP-A (14, 20).

Members of the Notch receptor family are large, type I membrane proteins comprising an extracellular (EC) subunit and a predominantly intracellular, membrane-spanning (TM-IC) subunit, to which the EC subunit is noncovalently bound (Fig. 1*D*). The EC subunit has a large but variable number of EGF-

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[1] The abbreviations used are: IGF, insulin-like growth factor; IGFBP, insulin-like growth factor-binding protein; PAPP-A, pregnancy-associated plasma protein-A; MMP, matrix metalloproteinase; ADAM, a disintegrin and metalloprotease; CCP, complement control protein; LNR, Lin12-Notch repeat; EC, extracellular; TM-IC, transmembrane intracellular; d-, deleted (as in dLNR1); ELISA, enzyme-linked immunosorbent assay; MBP, major basic protein.

like repeats and always three uninterrupted LNR modules. The intracellular portion of the TM-IC subunit contains sequence elements, including adjacent ankyrin repeats, which interact directly with transcription factors (21). During maturation of the receptor, the EC and TM-IC subunits result from cleavage at S1 (Fig. 1*D*) by a furin-like convertase (22).

Notch signaling determines cell fates in invertebrates and vertebrates by controlling how cells respond to developmental signals (23). The ligands of Notch receptors are extracellular domains of specific surface molecules expressed on adjacent cells. Upon ligand binding, the Notch receptor becomes susceptible to proteolytic cleavage at S2 (Fig. 1*D*). As a consequence of this, it undergoes intramembrane proteolysis at S3, resulting in release of the intracellular domain of the TM-IC subunit and thus activation of the Notch transcriptional response (23, 24).

The LNR modules are thought to be important for keeping the Notch receptor in a resting state prior to ligand binding. Receptors in which the LNR modules are mutated (25) or deleted (26–28) are constitutively cleaved at S2 and thus are active. Recently, an isolated LNR module from human Notch1 was shown to bind calcium (29, 30), and calcium depletion has been found to cause activation and subunit dissociation (28).

Outside the family of Notch receptors, the LNR module is not known to occur in any other proteins, except for PAPP-A and PAPP-A2 (31). Curiously, although the number of modules is conserved between Notch and PAPP-A, the *en bloc* occurrence in the Notch receptors is not. We have studied the function of the LNR modules of PAPP-A, and we present here data demonstrating that they bind calcium and determine the proteolytic specificity of PAPP-A.

EXPERIMENTAL PROCEDURES

Plasmid Construction—Mutants of the 1547-residue human PAPP-A polypeptide were obtained using three pBluescript II SK+ vectors, each containing PAPP-A cDNA corresponding to residues 1–407 (pB1–407),[2] 408–988 (pB408–988), or 989–1547 (pB989–1547) (14). Mutations were introduced by overlap extension PCR (32) using *Pfu* DNA polymerase (Promega), and the mutated cDNAs were swapped into the HindIII/BspEI, BspEI/KpnI, or KpnI/XbaI sites of pPA-BspEI (14), a modified version of the PAPP-A wild-type construct pcDNA3.1-PAPP-A (33) containing a BspEI site silently introduced at residue 407. Outer primers, derived from pBluescript II SK+, were 5′-TAATACGACTCACTATAGGG-3′ and 5′-AATTAACCCTCACTAAAGGG-3′. All constructs were verified by sequence analysis. At least two independent clones of each construct were used for further analysis. Plasmid pB1–407 was used for the construction of mutants d[Cys^{334}–Cys^{360}], d[Ala^{367}–Cys^{393}], d[Cys^{334}–Cys^{393}], D341A, D356A, D359A, N374A, D389A, and E392A. Plasmid pB408–988 was used for mutants Trp^{586}stop, Glu^{853}stop, and His^{951}stop. Plasmid pB989–1547 was used for mutants Cys^{1130}stop, Cys^{1478}stop, Cys^{1504}stop, d[Cys^{1478}–Cys^{1503}], D1484A, D1499A, and D1502A.

Tissue Culture and Transfection—Human embryonic kidney 293T cells (293tsA1609neo) (34) 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 were transfected 18 h later by calcium phosphate co-precipitation using 10 μg of plasmid DNA (35). Plasmid DNA constructs used were wild-type PAPP-A or PAPP-A mutants as detailed above and cDNA encoding wild-type IGFBP-4 (14) or -5 (20). After 48 h, the medium was harvested, cleared by centrifugation, and replaced by serum-free medium (CD 293, Invitrogen) for another 48 h to facilitate Western blotting or purification (6).

Enzyme-linked Immunosorbent Assay (ELISA)—The levels of recombinant PAPP-A in culture supernatants were measured by a standard sandwich ELISA. PAPP-A polyclonal antibodies, anti-PAPP-A/proMBP (36), were used for capture, and a PAPP-A monoclonal antibody (mAb 234-5) (37) followed by peroxidase-conjugated anti-mouse IgG (P260, DAKO) was used for detection. The epitope of this monoclonal antibody is intact in all PAPP-A mutants of this study.[3] PAPP-A/proMBP purified from pregnancy serum (36) was used to establish standard curves.

Western Blotting—For immunovisualization of PAPP-A or PAPP-A mutants, protein contained in culture media (~10 μg of PAPP-A/ml) was separated by nonreducing SDS-PAGE (10–20% Tris-glycine gels) and blotted onto a polyvinylidene difluoride membrane (Millipore). The blots were blocked with 2% Tween 20, equilibrated in TST (50 mM Tris-HCl, 500 mM NaCl, 0.1% Tween 20, pH 9.0), and then incubated overnight at room temperature with primary antibody, polyclonal antidenatured PAPP-A/proMBP (36), diluted in TST containing 1% fetal bovine serum. Blots were washed in TST, further incubated for 1 h at room temperature with peroxidase-conjugated secondary antibody (P217, DAKO) diluted in TST, and then washed again with TST. The blots were developed using enhanced chemiluminescence (ECL, Amersham Biosciences), and images were captured with a Kodak Image Station 1000 (Eastman Kodak Co.). Molecular weights were estimated using a prestained marker, Precision Plus protein standard (Bio-Rad).

Measurement of Proteolytic Activity—The proteolytic activity of wild-type PAPP-A and PAPP-A mutants was assessed by incubating appropriately diluted culture medium with purified, ^{125}I-labeled natural substrates IGFBP-4 and -5. After incubation, the reaction mixtures were separated by nonreducing 10–20% SDS-PAGE, and cleavage was visualized by autoradiography. Briefly, reactions (total volume of 20 μl) were carried out in STC (100 mM sodium chloride, 50 mM Tris-HCl, 1 mM $CaCl_2$, pH 7.5) at an enzyme:substrate molar ratio of 1:200 (0.03 nM enzyme, 6 nM substrate). The reaction mixtures contained 0.2 ng of PAPP-A (1 μl of PAPP-A or PAPP-A mutant containing supernatant diluted about 50× in STC according to the level of expression measured by ELISA) and 40.000 cpm of the binding proteins (~4 ng contained in 2 μl of 50 mM Tris-HCl, pH 7.5). Reactions were carried out in the presence (IGFBP-4) or absence (IGFBP-5) of 20 nM IGF-II (Bachem) diluted in STC from a stock solution of 20 μM in 1 mM HCl, and the reverse experiments were carried out as controls. Following incubation at 37 °C, reactions were stopped by the addition of 10 mM EDTA and mixed with denaturing sample buffer for gel electrophoresis. For the comparison of wild-type PAPP-A with PAPP-A mutants, conditions were chosen that resulted in ~50% cleavage of the IGFBP substrate (2 h of incubation). For quantitative measurements (14), the degree of cleavage in each reaction was determined by measuring band intensities with a PhosphorImager (Amersham Biosciences). The activities of mutants were expressed as the amount of product generated relative to product generated by wild-type PAPP-A at <50% cleavage. Assays in which the effect of Ca^{2+} was investigated were carried out in distilled (3×) water. Some assays were carried out in the presence of EDTA, EGTA, or calcium-free bovine calbindin D9k (38). As judged by SDS-PAGE and compositional amino acid analysis, the preparation of calbindin was >95% pure.

RESULTS

The C-terminal Domain of PAPP-A Is Required for Proteolysis of IGFBP-4 but Not for Proteolysis of IGFBP-5—To investigate the role of PAPP-A domains outside the proteolytic domain, PAPP-A truncation mutants (Fig. 1*C*) were expressed in 293T cells. The sites of truncation were chosen in accordance with the known pattern of disulfide bonds (19) to avoid disulfide-based polymerization. Mutants truncated immediately after the proteolytic domain (PA1–585) or immediately after M1 (PA1–852) did not express at measurable levels, but mutants truncated at or after residue 950 expressed at the level of wild-type PAPP-A (Fig. 2*A*).

None of the expressed mutants were capable of cleaving IGFBP-4 under conditions where wild-type PAPP-A cleaved >50% of the substrate (Fig. 2*B*). However, the truncated proteins all showed wild type-like activity against IGFBP-5, indicating that the core of the proteolytic domain in the truncated proteins is intact and not compromised by incomplete folding.

Each of the Three LNR Modules of PAPP-A Is Critical for Proteolysis of IGFBP-4—Our findings suggest that residues of the 70-residue C-terminal domain, including LNR3, participate in the regulation of PAPP-A proteolytic activity, although these residues are separated from the proteolytic domain by ~900

[2] The numbering of the mature PAPP-A polypeptide (18) is used in this paper. Glu^{1} of mature PAPP-A is at position 81 of preproPAPP-A (Swiss-Prot accession number Q13219).

[3] V. Rodacker and C. Oxvig, unpublished observation.

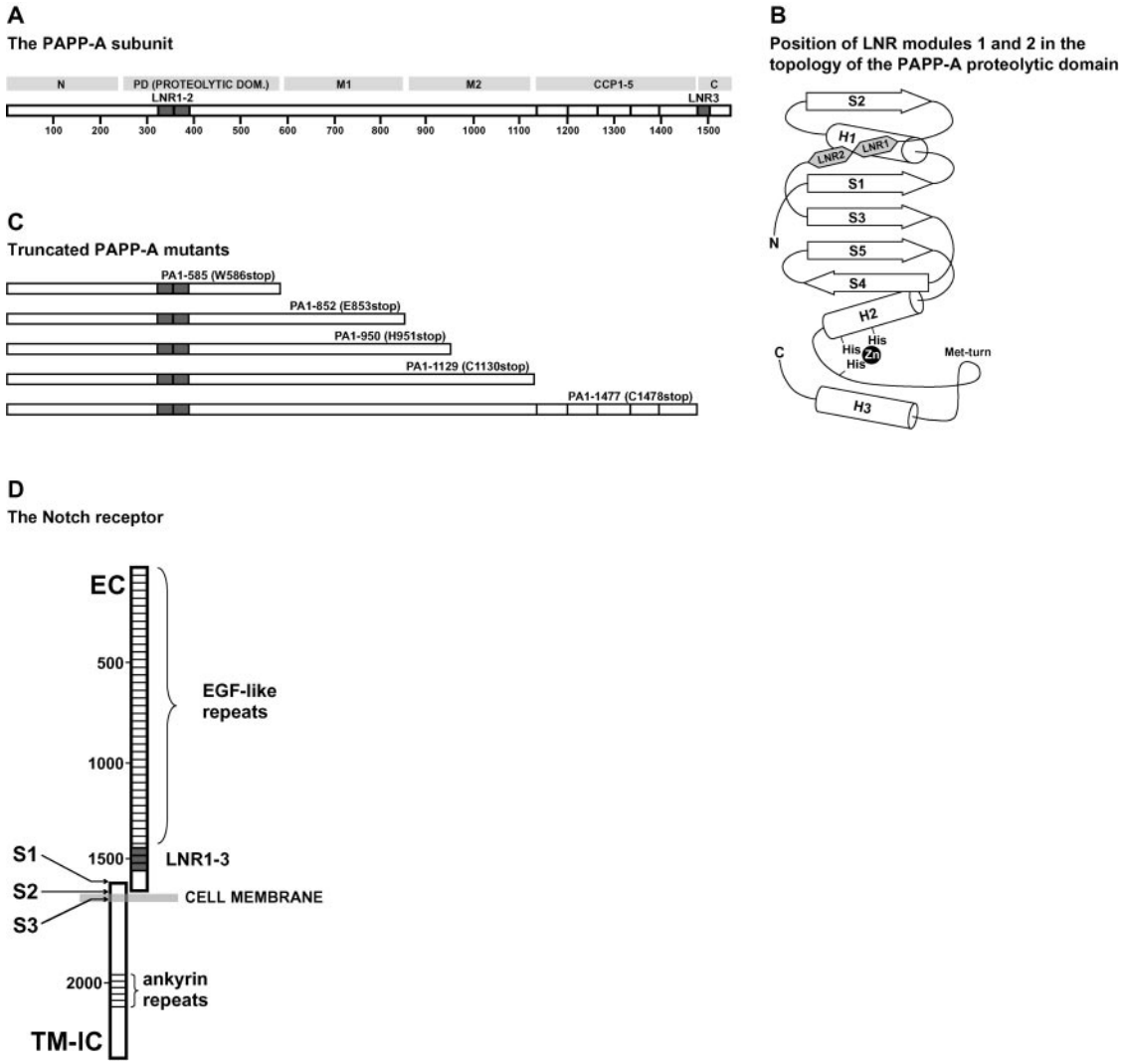

FIG. 1. **Schematic diagram of the PAPP-A subunit, topology of its proteolytic domain, diagram of truncated PAPP-A variants, and schematic diagram of the Notch receptor.** *A*, the following sequence elements of the 1547-residue PAPP-A subunit are indicated by *gray bars*: a putative N-terminal domain (*N*), a proteolytic domain (*PD*), two hypothetical central domains (*M1* and *M2*) defined on the basis of disulfide structure (19), a sequence stretch containing five CCP modules (*CCP1–5*), and a C-terminal domain (*C*) (14, 19). The neighboring LNR1 and LNR2 modules within the proteolytic domain and the isolated LNR3 module of the C-terminal domain are indicated by *shading*. *B*, topological diagram showing secondary structure elements common to members of the metzincin superfamily (adapted from Stöcker *et al.* (39)). In addition to β-strands (*S1–S5*) and α-helices (*H1–H3*), the predicted position in the structure of LNR modules 1 and 2 (14) is shown by *shaded hexagons*. The active site zinc ion is coordinated by three histidine residues of the elongated zinc binding sequence. *C*, the initial set of PAPP-A mutants, each C-terminally truncated, is depicted by *open bars*. *D*, a large, highly variable number of EGF-like repeats and always three LNR modules are present in the extracellular subunit of the Notch receptor. The EC subunit is noncovalently bound to the transmembrane intracellular subunit (28), which in addition to ankyrin repeats contains other sequence elements important for interactions with the transcription apparatus (21). During maturation, the receptor is cleaved at S1 by a furin-like convertase. Binding of ligand to the EC subunit (outside the LNR region) induces proteolytic cleavage at S2. This, in turn, allows intramembrane proteolysis at S3, by which the intracellular portion of TM-IC is released (22). The diagram is drawn to scale and corresponds to human Notch1 (GenBank™/EBI accession number P46531) of 2536 residues.

residues in the primary structure. We then expressed a series of LNR deletion mutants and a mutant truncated immediately after LNR3 (PA1–1503). As the disulfide connectivity within the C-terminal domain is not completely resolved (19), we cannot exclude the existence of a disulfide bond between LNR3 and the remaining C-terminal 44 residues. However, all mutants of this series expressed at the level of wild-type PAPP-A and migrated in SDS-PAGE as dimers, close to the wild-type protein at 400 kDa (Fig. 3*A*).

All of the mutants lacked detectable activity against IGFBP-4 but were active against IGFBP-5 (Fig. 3*B*). This immediately suggests that LNR1 and LNR2 are also critical for the regulation of proteolytic activity against IGFBP-4. Compared with wild-type PAPP-A, the IGFBP-5 proteolytic activity of some mutants, in particular dLNR1, was partially impaired. This may be because of a general conformational effect of deleting only one of the two modules inserted between putative β-strands 2 and 3 of the proteolytic domain (14). In accordance with this interpretation, dLNR1–2, in which the entire inserted sequence is removed, shows less reduction in activity against IGFBP-5 than both dLNR1 and dLNR2.

Mutants dLNR3 and PA1–1503 both lacked activity against IGFBP-4 and thus did not allow a functional discrimination between LNR3 and the remaining C-terminal 44 residues; both sequence elements may be important for the proteolysis of IGFBP-4. Alternatively, the folding and/or correct disulfide bond formation of LNR3 may depend on the remaining residues, as indicated above.

Mutation of LNR Residues Predicted to Bind Calcium—The above results prompted us to hypothesize that PAPP-A LNR1,

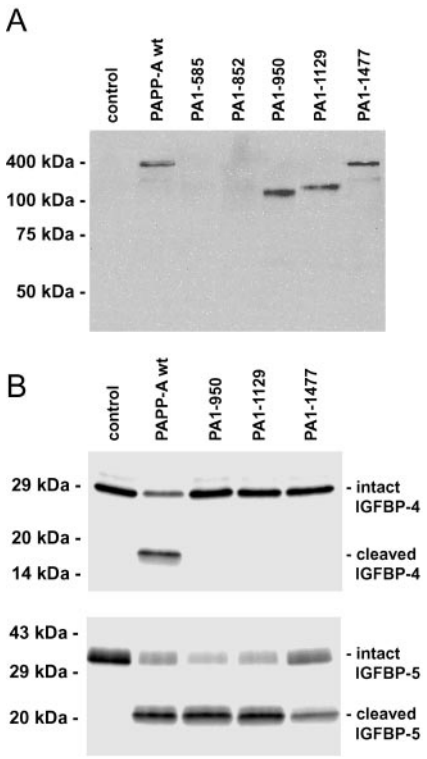

FIG. 2. **The C-terminal region of PAPP-A is required for proteolysis of IGFBP-4 but not for proteolysis of IGFBP-5.** *A*, Western blot of wild-type and truncated PAPP-A mutants. Culture media from transfected 293T cells were separated by nonreducing 10–20% SDS-PAGE and analyzed using antibodies against PAPP-A. Cells were transfected with empty vector (*control*), cDNA encoding wild-type PAPP-A (*PAPP-A wt*), and cDNA encoding PAPP-A mutants PA1–585 (*W586stop*), PA1–852 (*E853stop*), PA1–950 (*H951stop*), PA1–1129 (*C1130stop*), and PA1–1477 (*C1478stop*) as indicated. Mutants PA1–585 and PA1–852 were undetectable in ELISA. *B*, cleavage analysis of IGFBP-4 (*upper gel*) and IGFBP-5 (*lower gel*), both natural substrates of PAPP-A. Wild-type PAPP-A or PAPP-A mutants were incubated (2 h at 37 °C) with ^{125}I-labeled substrate (1:200). Intact substrate (approximately 30 and 35 kDa, respectively) and co-migrating cleavage products (approximately 15 and 20 kDa, respectively) were separated by SDS-PAGE and visualized by autoradiography. The positions of intact and cleaved IGFBP-4 and -5 are indicated.

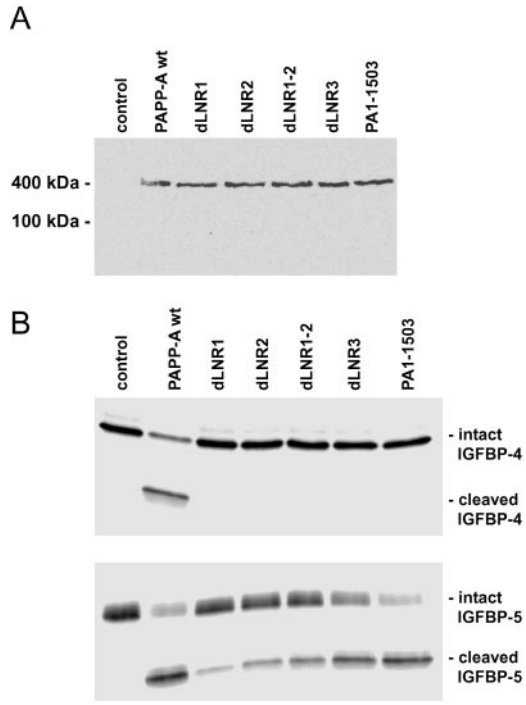

FIG. 3. **IGFBP-4 proteolytic activity of PAPP-A depends on each of LNR modules 1, 2, and 3.** *A*, PAPP-A Western blot of culture medium from 293T cells transfected with empty vector (*control*), cDNA encoding wild-type PAPP-A (*PAPP-A wt*), and cDNA encoding PAPP-A mutants dLNR1 (d[Cys^{334}–Cys^{360}]), dLNR2 (d[Ala^{367}–Cys^{393}]), dLNR1–2 (d[Cys^{334}–Cys^{393}]), dLNR3 (d[Cys^{1478}–Cys^{1503}]), and PA1–1503 (Cys^{1504}stop) as indicated. *B*, proteolytic activity against IGFBP-4 (*upper gel*) and IGFBP-5 (*lower gel*) assessed using ^{125}I-labeled substrates followed by autoradiography (see Fig. 2 for further details).

-2, and -3 function in a manner similar to one another, possibly forming a single functional unit, although the three modules are not located next to each other in the sequence.

An alignment of the PAPP-A LNR modules with LNR modules from a representative member of the Notch receptor family, human Notch1, revealed that in addition to cysteines, some residues with acidic or amide side chains appeared to be highly conserved between the two groups of LNRs (Fig. 4*A*). Within the entire Notch family, three positions in particular are conserved (marked with *bullets* in Fig. 4*A*) (29, 30). By the analysis of *in vitro* folding (29) and by NMR studies (30), residues in these three positions recently have been shown to be positioned favorably for the coordination of a calcium ion in an isolated recombinant LNR1 module derived from human Notch1.

To test whether the corresponding residues in each of the three PAPP-A LNR modules are critical for its function, we expressed and analyzed mutants with single residues substituted to alanine. All of the nine mutants exhibited unaffected proteolysis of IGFBP-5 (Fig. 4*B*), consistent with the activity of truncation and deletion mutants against this substrate. However, six of the mutants showed only residual activity against IGFBP-4 (Fig. 4*B*), indicating that the substituted residues may indeed function as ligands for metal ions and that metal ion binding is required for proteolysis of IGFBP-4 but not for proteolysis of IGFBP-5. Each of the three residues in LNR3 are critical for the proteolysis of IGFBP-4, and only one of the nine residues, Asn^{374} of LNR2, did not affect activity against IGFBP-4 when substituted to alanine (Fig. 4*B*).

Importantly, all LNR modules of PAPP-A contain at least one potential metal ion ligand that is critical for proteolysis of IGFBP-4 but not for proteolysis of IGFBP-5. This supports our interpretation that the IGFBP-5 proteolytic activity of LNR1 and -2 deletion mutants is impaired because the conformation of the proteolytic domain in these mutants is slightly distorted, not because the LNR1–2 region is directly involved in the proteolysis of IGFBP-5.

Mutants that lacked activity against IGFBP-4 were also inactive in the absence of IGF. Furthermore, like wild-type PAPP-A (6), cleavage of IGFBP-5 by these mutants was slightly reduced in the presence of IGF (data not shown).

PAPP-A Cleavage of IGFBP-4, but Not IGFBP-5, Requires Ca^{2+}—To examine the influence of calcium ions on the proteolytic activity of PAPP-A, a calcium titration experiment was carried out. Buffers and dilutions of reagents were prepared using distilled (3×) water. In the absence of added Ca^{2+}, PAPP-A did cleave IGFBP-4, but at 0.1 mM Ca^{2+} we observed a 1.4-fold increase in proteolytic activity (Fig. 5). Between 0.1 and 1 mM, the activity was further increased slightly, but activity was decreased at higher concentrations of Ca^{2+}. The apparent maximum at 1 mM Ca^{2+} may reflect Ca^{2+}-LNR interactions but may also be caused by Ca^{2+} binding to other hypothetical Ca^{2+} binding sites of the proteolytic domain, as know from other metzincins (39). This experiment indicates that PAPP-A has calcium binding site(s) associated with proteolysis of IGFBP-4 that are at least partly saturated when no Ca^{2+} is actively added and therefore appears to bind calcium with relatively high affinity. A similar experiment showed that

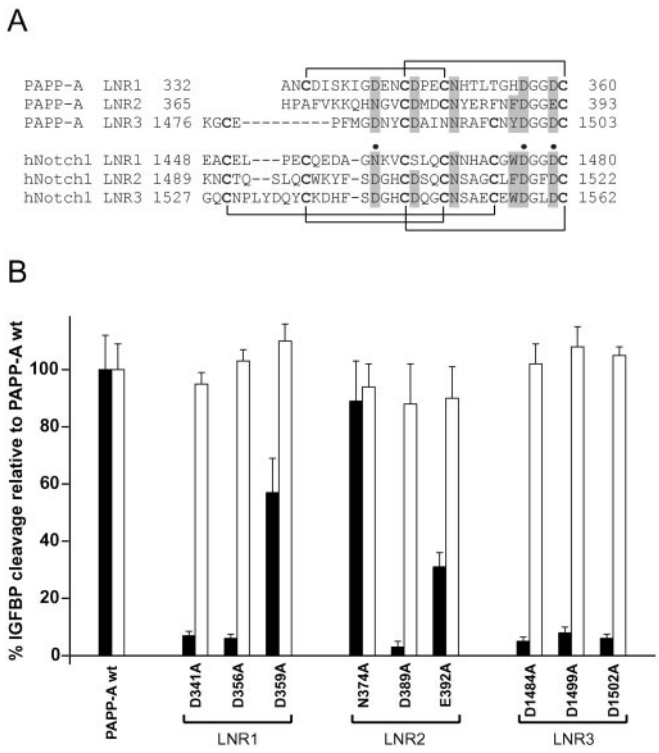

FIG. 4. **Proteolytic activity of single-residue mutants of PAPP-A LNR1, -2, and -3.** *A*, sequence alignment of PAPP-A and Notch LNR modules. LNR1–3 of human Notch1 (*hNotch1*) (GenBank™/EBI accession number P46531) and LNR1–3 of human PAPP-A are aligned. Cysteine residues are shown in *boldface*, and highly conserved residues within the family of Notch receptors and PAPP-A are *shaded*. Conserved residues, which are ligands for a calcium ion in LNR1 of human Notch1 (29, 30), are indicated with *bullets*. Known or predicted disulfide pairing of PAPP-A cysteine residues (19) and the disulfide pairing of the recombinant LNR1 module of human Notch1 (29) are indicated with *lines*. *B*, LNR residues predicted to participate in calcium coordination were individually substituted into alanine (Asp^{341}, Asp^{356}, and Asp^{359} of LNR1; Asn^{374}, Asp^{389}, and Glu^{392} of LNR2; and Asp^{1484}, Asp^{1499}, and Asp^{1502} of LNR3 as indicated). The ability of the mutated proteins to cleave IGFBP-4 (*filled columns*) and IGFBP-5 (*open columns*) is expressed relative to the activity of wild-type PAPP-A (*PAPP-A wt*). Results are the average of three independent measurements. *Vertical lines* indicate standard deviations.

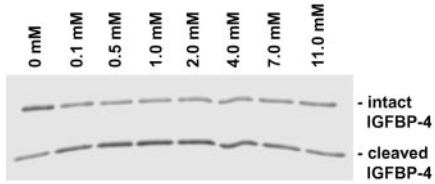

FIG. 5. **Activity of PAPP-A as a function of Ca^{2+}.** Cleavage of IGFBP-4 was carried out in a reaction to which no Ca^{2+} was added (0 mM Ca^{2+}) and in buffers with the indicated amounts of Ca^{2+} added. The positions of intact and cleaved IGFBP-4 are indicated. From 0 to 0.1 mM Ca^{2+} the degree of cleavage is increased 1.4-fold.

proteolysis of IGFBP-5 is not influenced significantly by changes in the concentration of calcium ions (data not shown).

In reaction buffers with no added Ca^{2+}, we were unable to selectively remove the traces of calcium ions by co-incubation with EGTA. Both EGTA and EDTA inhibited PAPP-A activity against IGFBP-4 and -5, possibly by chelating the active site zinc ion of PAPP-A. After treatment with chelating agents, the activity against both IGFBP-4 and -5 could be restored by the addition of excess calcium (data not shown), demonstrating that IGFBP-4 proteolytic activity of PAPP-A can be reversibly abolished by the removal of calcium.

However, the addition of the calcium-binding protein calbindin D9k selectively inhibited proteolysis of IGFBP-4 with almost no effect on the proteolysis of IGFBP-5 (Fig. 6). Each molecule of calbindin D9k specifically and cooperatively binds two calcium ions with high affinities (K_{d1} = 0.5 μM and K_{d2} = 0.3 μM) (40). As proteolysis of IGFBP-4 is practically abrogated at 3 μM calbindin (Fig. 6), the concentration of Ca^{2+} in reactions with no actively added Ca^{2+} is probably less than 6 μM. We conclude that PAPP-A possesses at least one high affinity calcium ion binding site that must be occupied for proteolysis of IGFBP-4 to occur.

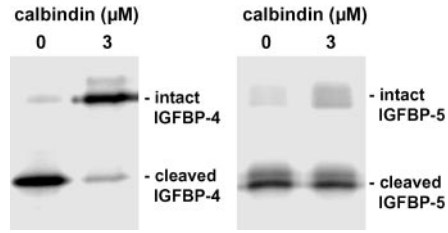

FIG. 6. **Specific sequestering of Ca^{2+} strongly inhibits PAPP-A cleavage of IGFBP-4 but not IGFBP-5.** Cleavage of IGFBP-4 and IGFBP-5 was carried out in the absence or presence of calbindin D9k (3.0 μM). The positions of intact and cleaved IGFBP-4 and -5 are indicated. One molecule of calbindin D9k cooperatively binds two calcium ions (K_{d1} = 0.5 μM and K_{d2} = 0.3 μM) (40).

LNRs of PAPP-A Bind Ca^{2+}—The above experiments demonstrate that Ca^{2+} is required for PAPP-A activity against IGFBP-4, not that Ca^{2+} is actually bound by the PAPP-A LNRs. We reasoned that if the PAPP-A LNR modules form calcium binding sites, it might be possible to rescue the activity of single-residue LNR mutants with compromised, but not abrogated, ability to cleave IGFBP-4 by increasing the concentration of calcium ions.

Using wild-type PAPP-A, we observed a 1.4-fold increase in cleavage from 0 to 2 mM Ca^{2+} (Fig. 5). In a similar experiment with mutant D359A of LNR1 and mutant E392A of LNR2, we observed that both of these mutants gained more activity than wild-type PAPP-A, increasing the concentration of Ca^{2+} from 0 to 2 mM (Fig. 7). None of the three single-residue mutants of LNR3 allowed a similar analysis, as they all lacked the ability to cleave IGFBP-4. These experiments strongly suggest that residues of the PAPP-A LNR modules participate in the coordination of calcium ions.

DISCUSSION

Three copies of the LNR module, arranged in tandem, are invariably present in the extracellular portion of Notch receptors (Fig. 1*D*). Outside the family of Notch receptors, the LNR module is present only in the metalloproteinase PAPP-A and its homologue PAPP-A2. Like the Notch receptors, these two proteins contain three LNR modules. However, modules 1 and 2 are present within their proteolytic domains, and the third LNR module is present outside this domain, separated by more than 1000 amino acids (Fig. 1*A*).

We have found that the LNR modules determine the proteolytic activity of PAPP-A. Analysis of C-terminally truncated PAPP-A mutants (Fig. 2) followed by the analysis of LNR deletion mutants (Fig. 3) demonstrated that each of the three PAPP-A LNR modules is strictly required for proteolytic activity against IGFBP-4. In contrast, all mutants cleaved IGFBP-5, most of them at the level of wild-type PAPP-A. LNR1 and -2 deletion mutants showed decreased activity against IGFBP-5, likely because the proteolytic domain is destabilized in these mutants. Further analysis by less invasive single-residue substitution supported this interpretation.

The LNR module does not contain any sequence motif known to be involved in metal ion binding. However, alignment shows

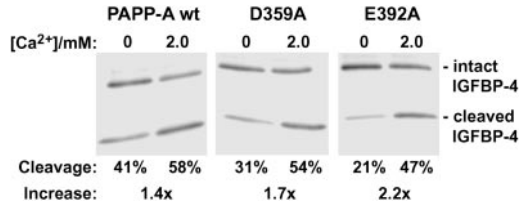

FIG. 7. **The activity of mutants with decreased activity can be partially rescued by increasing the concentration of calcium.** The activity of wild-type PAPP-A (*PAPP-A wt*) was compared with the activity of mutants D359A and E392A at 0 and 2 mM Ca^{2+} (indicated *above* each *lane*). This experiment was performed several times with similar results; a representative gel is shown. The degree of cleavage in each reaction and the increase in cleavage from 0 to 2 mM Ca^{2+} are indicated *below* the *lanes*.

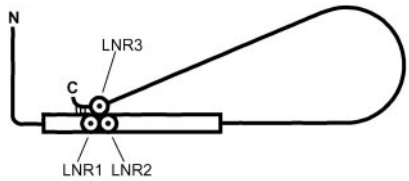

FIG. 8. **Hypothetical model of the spatial arrangement of PAPP-A LNR1–3.** In the primary structure of PAPP-A, LNR3 is located near the C terminus, separated from LNR2 by more than 1000 residues (Fig. 1*A*). In this model, LNR3 is located spatially in proximity to LNR1–2. The structure of the individual LNR modules may depend on a calcium ion, or calcium may be required for their association. In a variant of this model, PAPP-A LNR3 of one subunit associates with LNR1–2 of another subunit in an antiparallel manner, as PAPP-A is a dimeric protein. The termini (*N* and *C*) of the PAPP-A polypeptide are indicated. The proteolytic domain is represented by the *open bar*, the LNR modules by *circles*, and putative calcium ions by *dots*. *Vertical lines* indicate a possible interaction between the C-terminal domain and LNR or the proteolytic domain.

that three conserved residues, recently found to function in the coordination of a calcium ion in an isolated Notch1 LNR1 module (30), are also present in each of the PAPP-A LNRs (Fig. 4*A*). We hypothesized that individual mutation of these residues into alanine removes one calcium ligand, thereby weakening or eliminating a calcium binding site. Of the nine single-residue PAPP-A mutants, six lacked activity toward IGFBP-4, two showed significantly reduced activity, and one showed unaltered activity. In contrast, none of the nine mutants exhibited compromised activity against IGFBP-5 (Fig. 4*B*).

There are no long range disulfide bonds in the PAPP-A subunit, which brings LNR3 into the proximity of LNR1 and -2 (19). However, because all three LNR modules function together with the proteolytic domain, LNR3 is probably spatially localized close to this domain. It is tempting to speculate that the three LNR modules together form a single functional unit (Fig. 8). In such a model, the structure of the individual LNR modules may depend on a calcium ion. Alternatively, calcium may be required for their association. Although we cannot distinguish between these two possibilities, we note that mutant PA1–1503, which is truncated C-terminally to LNR3, is inactive against IGFBP-4 (Fig. 3). Hence, the C-terminal 44 residues of PAPP-A (1504–1547) may be essential for the correct localization of LNR3, or they may be required for the correct folding of this module.

A functional unit composed of LNR1–3 may interact in an obligate manner with the binding of one substrate, IGFBP-4, but not the other, IGFBP-5. Substrate binding to sites located outside the proteolytic domain is known from the MMPs (41); fibronectin type II modules are present within the proteolytic domains of MMP-2 and -9, and a neighboring hemopexin domain is present C-terminal to the proteolytic domain of most MMPs. Such exosites favor the binding and cleavage of substrate and are believed to function in the preparation of substrates such as collagen (41). Alternatively, however, as IGFBP-4 and -5 are very similar substrates, an LNR unit may function to regulate the specificity of the proteolytic domain.

Proteolytic cleavage of IGFBP-4 is increased when the concentration of Ca^{2+} is raised 0.1 mM above the background level of 3× distilled water (Fig. 5). Furthermore, in the presence of the calcium-binding protein calbindin D9k, proteolysis of IGFBP-4, but not IGFBP-5, was abrogated (Fig. 6). Together with the results from the analysis of mutants, these data suggest that PAPP-A is active against IGFBP-4 when the LNR modules are saturated with Ca^{2+} but is inactive against this substrate when Ca^{2+} is lost. However, to provide more direct evidence of Ca^{2+} binding by the PAPP-A LNRs, we analyzed the dependence on Ca^{2+} of LNR mutants D359A and E392A, showing decreased rather than abrogated activity against IGFBP-4. The activity of these mutants can be partially rescued by increased calcium (Fig. 7), which we take as evidence that Ca^{2+} is actually bound by the PAPP-A LNR module. In the NMR structure of the isolated Notch1 LNR1 module, the coordination sphere of the Ca^{2+} ion is not fully occupied. We suggested that in the intact receptor, additional residues might participate in stabilizing interactions within or between the LNR modules (30). Because mutation in each of the three LNR modules of PAPP-A abrogates LNR functionality, the structural integrity of each module likely depends on all three modules and may involve residues from one LNR module acting as a ligand of Ca^{2+} in another module.

A recent study revealed that the Notch receptor acts as a sensor during vertebrate left-right determination, amplifying small differences in extracellular calcium into differential gene expression (42), but whether the LNRs are involved in the sensory mechanism is not known. The isolated LNR1 module of Notch1 binds Ca^{2+} with a K_d of 40 μM (30), and our data indicate that the PAPP-A LNRs, in the context of an intact structure, have similar affinities. Unless modulated *in vivo*, affinities of this magnitude are unlikely to be effective in sensing differences in concentrations around 50× higher than K_d. However, the decrease in proteolysis of IGFBP-4 at increased concentrations of Ca^{2+} (Fig. 5) may be physiologically relevant in specialized tissues such as bone, in which PAPP-A is believed to be important (8, 43).

Finally, our finding that the LNRs are involved in regulating the proteolytic specificity of PAPP-A may hold clues to the function of the LNR modules in the Notch receptors. In fact genetic and biochemical data indicate that the LNRs regulate ligand-induced proteolytic cleavage at the S2 site of the Notch receptor (25–28), but details of how this might occur are not known. The present data directly link the LNRs with proteolysis and may allow a common theme to be defined that can be further explored in both systems. Interestingly, Kuzbanian (Kuz) (44, 45) and TNF-α-converting enzyme (TACE or ADAM 17) (46, 47), both known to function as S2 proteinases, belong to the metzincin superfamily of metalloproteinases (39), as does PAPP-A (14). Thus, they all have proteolytic domains with very similar folds, and it is tempting to hypothesize that the LNR of PAPP-A and the LNR of the Notch receptor interact with the core proteolytic domains in a similar manner, thus regulating specificity in both cases.

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Enzyme Catalysis and Regulation:

The Lin12-Notch Repeats of Pregnancy-associated Plasma Protein-A Bind Calcium and Determine Its Proteolytic Specificity

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