The C-terminal Domain of Aminopeptidase A Is an Intramolecular Chaperone Required for the Correct Folding, Cell Surface Expression, and Activity of This Monozinc Aminopeptidase*

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Aminopeptidase A (APA, EC 3.4.11.7) is a type II integral membrane glycoprotein responsible for the conversion of angiotensin II to angiotensin III in the brain. Previous site-directed mutagenesis studies and the recent molecular modeling of the APA zinc metallopeptidase domain have shown that all the amino acids involved in catalysis are located between residues 200 and 500. The APA ectodomain is cleaved in the kidney into an N-terminal fragment corresponding to the zinc metallopeptidase domain, and a C-terminal fragment of unknown function. We investigated the function of this C-terminal domain, by expressing truncated APAs in Chinese hamster ovary and AtT-20 cells. Deletion of the C-terminal domain abolished the maturation and enzymatic activity of the N-terminal domain, which was retained in the endoplasmic reticulum as an unfolded protein bound to calnexin. Expression in trans of the C-terminal domain resulted in association of the N- and C-terminal domains soon after biosynthesis, allowing folding rescue, maturation, cell surface expression, and activity of the N-terminal zinc metallopeptidase domain. We also show that the C-terminal domain is not required for the catalytic activity of APA but is essential for its activation. Moreover, we show that the C-terminal domains of aminopeptidase N (EC 3.4.11.2, APN) also promotes maturation and cell surface expression of the N-terminal domain of APN, suggesting a common role of the C-terminal domain in the monozinc aminopeptidase family. Our data provide the first demonstration that the C-terminal domain of an eukaryotic exopeptidase acts as an intramolecular chaperone.

Aminopeptidase A (APA, 1 EC 3.4.11.7) is a homodimeric membrane-bound zinc metalloproteinase that is activated by calcium and specifically cleaves the N-terminal glutamyl or aspartyl residue from peptide substrates such as angiotensin II (Ang II) and cholecystokinin-8 (1, 2) in vitro. APA is present in many tissues, particularly in the renal and intestinal brush border epithelial cells and in the vascular endothelium (3). APA has been identified in several brain nuclei involved in the control of body fluid homeostasis and cardiovascular functions, together with other components of the brain renin-angiotensin system (4). Studies using specific and selective APA inhibitors (5) have demonstrated that, in vivo, APA is responsible for the conversion of brain Ang II to angiotensin III (Ang III) (6) and that brain Ang III, and not Ang II as established in the periphery, exerts a tonic stimulatory action in the central control of blood pressure in spontaneously hypertensive rats (7). Therefore, the inhibition of central but not peripheral APA with specific and selective inhibitors leads to a large decrease in arterial blood pressure (7), suggesting that central APA might be an interesting candidate target for the treatment of hypertension (8). Molecular cloning of mouse, rat, and human APA (9–12) predicts a type II integral membrane protein of 945 residues composed of a short 17-residue N-terminal cytoplasmic tail, a 22-residue transmembrane domain, and an extracellular domain containing the active site, including the consensus sequence HEXXH...E found in the zinc metalloprotease family, the -zincinins (13). Site-directed mutagenesis studies, based on alignment of the APA sequence with the sequences of other monozinc aminopeptidases, were initially used to probe the organization of the APA active site (14–19). We used the functional data collected in these studies and the recently resolved x-ray crystal structure of leukotriene A4 hydrolase/aminopeptidase (EC 3.3.2.6) (20), a bifunctional zinc metalloenzyme, to construct a three-dimensional model of the mouse APA extracellular domain from residues 79 to 559, corresponding to the zinc metallopeptidase domain (21). According to this model, the zinc metallopeptidase domain is folded into a flat triangle composed of an N-terminal subdomain consisting mostly of -sheets, a globular catalytic subdomain, and a C-terminal helical subdomain. The active site is located at the interface of the N- and C-terminal subdomains and contains all the residues involved in zinc binding, substrate binding, and catalysis previously characterized by site-directed mutagenesis.

It has been suggested that APA has two distinct extracellular domains in vivo. First, proteolytic fragmentation studies of purified pig APA (22) showed that this protein exists in vivo as two polypeptides of 107 and 45 kDa (22). N-terminal sequencing showed that the 107-kDa fragment was the N-terminal extracellular domain of APA from residues 43 to 602, corresponding to the active site-containing zinc metallopeptidase domain we have modeled, whereas the 45-kDa fragment corresponded to the C-terminal domain of APA, from residues 603 to 942. Second, structural studies based on electron microscopy of purified pig intestinal APN incorporated into lipid membranes also suggested the existence of a C-terminal globular domain different from the zinc metallopeptidase domain (23). Finally, in the rat hippocampus, a short variant of APA produced by...
alternative splicing and lacking the C-terminal domain has been cloned. This protein displayed no APA activity when produced in COS-1 cells (24). Similarly, in the absence of the C-terminal domain, the N-terminal zinc metalloproteinase domain of mouse APA, when produced alone in COS-1 cells, was an inactive enzyme, which remained blocked in the endoplasmic reticulum (25). This suggests that the C-terminal domain of APA may be involved in the correct folding of APA. We tested this hypothesis by constructing two truncated forms of recombinant mouse APA, one corresponding to the N-terminal metalloproteinase domain from the first residue 598, tagged at its N terminus with the FLAG epitope (FLAG-N-APA), and the other corresponding to the C-terminal domain, from residues 595 to 945, tagged at its N terminus with the HA epitope (HA-C-APA). These proteins were expressed in CHO and AtT-20 cells, either separately or in trans, for studies of the influence of the C-terminal domain of APA on the maturation, subcellular localization, folding, and activity of the N-terminal metalloproteinase domain. To determine if there is a common role for the C-terminal domain of monozinc aminopeptidases, we have investigated the role of the C-terminal domain of APN. For this purpose, we produced the FLAG-tagged N-terminal domain of APN from residues 1 to 606 (FLAG-N-APN) and the HA-tagged C-terminal domain of APN from residues 607 to 966 (HA-C-APN) and studied if the role of the C-terminal domain of APN was similar to that of the C-terminal domain of APA.

**EXPERIMENTAL PROCEDURES**

**Materials**—Restriction endonucleases and DNA-modifying enzymes were obtained from New England Biolabs Inc. (Hitchin, England) and were used according to the manufacturer’s instructions. The Expand high-fidelity Taq polymerase PCR system was purchased from Roche Applied Science (Mannheim, Germany). The liposomal transfection reagent LipofectAMINE 2000, the pSecTag2 vector, pcDNA3 vector, pcDNA3.1-His vector, and the monoclonal anti-Xpress antibody (anti-Xpress mAb) were purchased from Invitrogen. The polyclonal anti-HA antibody (anti-HA Ab) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and the monoclonal anti-HA antibody (clone 12CA5, anti-HA mAb) was purchased from Roche Applied Science. The monoclonal M2 anti-FLAG antibody (anti-FLAG mAb) and the polyclonal anti-calnexin antibody (anti-calnexin Ab) were purchased from Sigma, and the monoclonal anti-protein disulfide isomerase antibody (anti-PDI mAb) was purchased from Immunochemistry (Heidelberg, Germany). The synthetic substrate 1-γ-glutamyl-β-naphthylamide (GluNA) was purchased from Bachem (Bundereff, Switzerland).

**Plasmid Constructs**—The subcloning of the cDNA encoding the mouse APA (9) and tagged at its N terminus with a polyhistidine tail (His6-WT-APA) has been described elsewhere (18). FLAG-WT-APA and FLAG-N-APA were constructed in pcDNA3 by PCR, using the mouse APA cDNA library (Clontech Laboratories, Inc.) using a forward primer encoding residues 1 of APN (primer E: ATG GCC AAG GGG TTC TAC) and a reverse primer encoding residues 596–598 of APA, replacing residue 599 by a stop codon and introducing an upstream HindIII restriction site was used for both constructs (primer A: T CAT CAA GCTT ATG AAG GAC TAC GAC GAC GAC GAG GCC ATG AAC TTT GCA GAG GAA GAG). A reverse primer encoding the last six residues of APA and introducing a downstream HindIII restriction site was used for FLAG-WT-APA (primer B: ATC TGC AGA ATT CTA ACT GCT AGC GAG GCC ATG AAC TTT GCA GAG GAA GAG). The reverse primer encoding the first seven residues of APN and introducing an upstream HindIII restriction site was used (primer G: TC ATC AAG CTT ATG AAG GAC TAC GAC GAC GAT CAC GAC GAC GCC ATG AAG GGG TTC TAC ATT). The reverse primer used for this construct was primer B.’ C. Cloning from the mouse brain Marathon-Ready cDNA library (Clontech Laboratories, Inc.) using a forward primer encoding residues 1–7 of APN (primer E: ATG GCC AAG GGG TTC TAC ATT) and a reverse primer encoding residues 601–606 of APN, replacing residue 607 by a stop codon, and introducing a downstream EcoRI restriction site (primer F: ATC TGC AGA ATT CTA GGG TTC TAC AGC GAC ATC CAG CCA). The resulting PCR product was used as a template to construct FLAG-N-APN in pcDNA 3 by PCR. A forward primer encoding the FLAG epitope, the first seven residues of APN and introducing an upstream HindIII restriction site was used (primer G: TC ATC AAG CTT ATG AAG GAC TAC GAC GAC GAT CAC GAC GAC GCC ATG AAG GGG TTC TAC ATT). The reverse primer was primer F. C-APN was cloned from the mouse brain Marathon-Ready cDNA library using a forward primer encoding the residues 607–966 of APN (primer H: CAG AGT GCA AAG TTT CAG ACA), and a reverse primer encoding the residues 961–966 of APN and introducing a downstream EcoRI restriction site (primer I: TAC TGC AGA ATT CTA GCT GGT TCT TGT GAA). The resulting PCR product was used as a template to construct HA-C-APN in pSecTag2 by PCR. A forward primer encoding the C-terminal TA peptide of APN, residues 607–614 of APN and introducing an upstream SfiI restriction site was used (primer J: GCG GCC CAG CCG TCA TAC GCT GAT GCT CAA TCT GCT CAG AGT GCA AAG TTT CAG ACA). The reverse primer was primer I. The absence of nonspecific mutations was confirmed by automated sequencing on an Applied Biosystems 377 DNA Sequencer with dye deoxy-terminator chemistry.

**Cell Culture, Transfection of CHO-K1 and AtT-20 Cells, and Purification of Recombinant His6-WT-APA and His6-N-APA CHO-K1 (American Type Culture Collection, Manassas, VA) cells were maintained in Ham’s F-12 medium (Invitrogen) supplemented with 7% fetal bovine serum (Invitrogen), 0.5 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin from Roche Applied Science (Mannheim, Germany). The synthetic substrate 1-γ-glutamyl-β-naphthylamide (GluNA) was purchased from Bachem (Bundereff, Switzerland).

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buffer, pH 6.8, and 1 μl of Triton X-100 was added to prevent the denaturation of glycosidases by SDS. The samples were then incubated with or without peptide N-glycosidase F (PNGase F) (Roche Applied Science) (5 milliliters at 37 °C for 18 h), or endoglycosidase H (Endo H) (Roche Applied Science) (1 unit at 37 °C for 18 h). The reaction was stopped by adding Laemmli buffer, and the samples were subjected to SDS-PAGE in 5%, 7.5%, or 10% acrylamide gels, which were then dried by incubation for 1 h in lysis buffer. For immunoprecipitation, lysates were incubated with 1 μg of anti-HA Ab or anti-calnexin Ab, and 10% v/v protein A-Sepharose for 2–3 h. The beads were washed once with lysis buffer, then three times with PBS and once with 20 mM Tris-HCl buffer, pH 6.8, resolved by 7.5% SDS-PAGE, and subjected to Western blotting using an anti-FLAG mAb.

**Immune depletion of HA-C-APA**—300,000 CHO cells were transiently transfected with FLAG-N-APA and HA-C-APA. The cells were grown for 36 h and then extracted by incubation in lysis buffer overnight. Lysates were then immunoprecipitated by incubation with 1 μg of anti-HA mAb, and 10% v/v protein A-Sepharose for 4 h. Supernatants were separated from the bead complex and subjected to two more rounds of immunoprecipitation. Clearing of the samples from HA-C-APA was monitored by immunoblotting, with an anti-HA mAb, the beads and the supernatants at each round of immunoprecipitation. The FLAG-N-APA levels in each sample were compared by anti-FLAG mAb (1:1000) immunoblotting of the samples, for equivalent amounts of protein. Immunoreactive material was detected with an alkaline phosphatase-coupled anti-mouse antibody, using ATTOPHOS as the chemiluminescent substrate. Chemiluminescence was measured using a phosphorimaging device (Bio-Rad).

**Enzyme Assay of Purified APA**—Purified APA was used to transfect CHO cells, and the expression of FLAG-WT-APA and FLAG-N-APA by treating immunoprecipitates with Student’s unpaired t test. Differences were considered significant if p was <0.05.

**RESULTS AND DISCUSSION**

APA is composed of a cytoplasmic domain, a transmembrane domain, and an extracellular domain divided into two parts: an N-terminal zinc metallopeptidase domain up to residue 598, including the active site of the enzyme, and a C-terminal domain corresponding to the last 347 amino acid residues of the extracellular domain of APA. We investigated the impact of the C-terminal domain of APA on the generation of a correctly membrane-bound active APA enzyme.

To this end, we generated several cDNA constructs (Fig. 1) encoding either the entire mouse APA or one of the individual domains, which we then expressed in CHO cells. We assessed the folding, trafficking, subcellular localization, and enzymatic activity of each of the corresponding recombinant proteins. One construct corresponded to the entire sequence of the mouse wild-type APA (WT-APA), tagged at its N terminus either with the FLAG epitope (FLAG-WT-APA), or with a polyhistidine (His6) tail (His6-WT-APA) to allow purification of the enzyme. A second construct (N-APA) included the mouse APA sequence from residues 1 to 598 (which corresponds to Asn-602 of pig APA) containing the cytoplasmic domain, the transmembrane domain functioning as a signal peptide, the N-terminal zinc metallopeptidase domain, tagged at its N terminus either with the FLAG epitope (FLAG-N-APA), or with the polyhistidine tail (His6-N-APA). A third construct was also generated, corresponding to the C-terminal domain of APA from residues 596 to 945 (C-APA). Because the C-terminal domain does not possess APA activity, it mediates the folding, trafficking into the ER, the cleavable signal sequence of mouse Igα (32) was fused to the mouse APA C-terminal domain sequence allowing ER translocation and secretion of the fusion protein. This third construct was tagged at its N terminus with the HA epitope (HA-C-APA). The cDNA constructs encoding FLAG-WT-APA, FLAG-N-APA, and HA-C-APA were used to transfect CHO cells, and the expression of the corresponding recombinant proteins was studied.

**Comparison of the Expression of the N-terminal Monozinc Metallopeptidase Domain of APA with That of WT-APA**—We investigated whether deletion of the C-terminal domain of APA affected the maturation of the N-terminal domain, by performing metabolic labeling and pulse-chase experiments with FLAG-WT-APA and FLAG-N-APA. For FLAG-WT-APA, we detected a single immunoprecipitated band with an apparent molecular mass of 140 kDa after 30 min of pulse and a second band of 160 kDa after 90 min of chase (Fig. 2A). In contrast, for FLAG-N-APA, we detected only an 85-kDa immunoprecipitated band, even after 5 h of chase (Fig. 2A). In addition, the intensity of the band corresponding to FLAG-N-APA decreased after 3 h of chase, probably due to degradation, because the protein was not secreted (Fig. 2C).

We further investigated the pattern of processing of FLAG-WT-APA and FLAG-N-APA by treating immunoprecipitates with PNGase F or Endo H. The treatment of glycoproteins with PNGase F results in the removal of all N-linked oligosaccharide side chains and was used as a positive control for the deglycosylation of recombinant APAs. Treatment with Endo H removes immature, but not medial Golgi-processed N-linked oli-
gosaccharide side chains. The treatment of FLAG-WT-APA with PNGase F led to the disappearance of both the 160- and 140-kDa forms of the protein, resulting in a single band 110 kDa in size. FLAG-N-APA was also sensitive to this treatment and yielded a 75-kDa band. For wild-type APA, the 140-kDa form was Endo H-resistant and shifted to 110 kDa upon treatment (Fig. 2B). For FLAG-N-APA, the 85-kDa form was Endo H-sensitive and shifted to 75 kDa upon treatment (Fig. 2B). Thus, the high molecular mass form of APA corresponded to the mature glycosylated complex sorting from the Golgi apparatus. In contrast, the 85-kDa form of FLAG-N-APA corresponded to an immature protein that was not transported to the Golgi apparatus. We then investigated the subcellular localization of FLAG-WT-APA and FLAG-N-APA by performing double-labeling immunofluorescence experiments with a rabbit polyclonal anti-(rat APA) antibody and a monoclonal antibody raised against the endogenous protein disulfide isomerase (PDI), a chaperone protein resident in the ER.

Confocal microscopy analysis of CHO cells expressing either FLAG-WT-APA or FLAG-N-APA showed that WT-APA was located at the plasma membrane, whereas FLAG-N-APA and PDI were co-localized in the ER (Fig. 2D). This retention in the ER, in contrast to the membrane location of WT-APA, is consistent with the incorrect maturation of the truncated form of APA, deleted of its C-terminal domain.

We investigated the basis of this impaired maturation and transport block by comparing the folding patterns of FLAG-WT-APA and FLAG-N-APA in a trypsin sensitivity assay. FLAG-WT-APA was trypsin-resistant even after 90 min of treatment, demonstrating that the correct folding of native WT-APA prevents its degradation by trypsin (Fig. 3A). Changes in the sensitivity of APA to trypsin may therefore reflect changes in folding pattern. Consistent with this hypothesis, the unfolded APA mutant Ala-220 (21) was totally degraded after 15 min of trypsin treatment (not shown). FLAG-N-APA was entirely degraded by 15 min of trypsin treatment, whereas FLAG-WT-APA was resistant to trypsin, suggesting that FLAG-N-APA is retained in the ER as an unfolded protein (Fig. 3A).

Analysis of the functional role of a protein may also provide information about its folding status. Unlike FLAG-WT-APA, FLAG-N-APA was completely devoid of specific enzymatic activity (Fig. 3B), consistent with the incorrect folding of this protein.

The biosynthetic labeling, confocal microscopy, and trypsin assay data together demonstrate that expression of the N-terminal zinc metallopeptidase domain of APA alone in CHO cells results in the production of an unfolded, transport-incompetent protein that has no enzyme activity, is rapidly degraded, and is unable to pass the ER quality control (for review see Ref. 33). These findings are consistent with those of Ofner et al. (25) and shed light on the potentially critical role of the C-terminal domain of APA in facilitating the folding, trafficking, and plasma membrane expression of APA.

Expression of a Secreted Form of C-APA—We then analyzed the biosynthetic and structural features of C-APA expressed alone, to determine the effect of the C-terminal domain of APA on the folding and transport behavior of the N-terminal zinc metallopeptidase domain of APA. C-APA does not possess a signal sequence for translocation into the ER. We therefore generated a construct encoding the entire C-APA sequence, tagged at its N terminus with the HA epitope (HA-C-APA), fused to the cleavable Igx leader sequence (LS) (HA-C-APA).

We transfected CHO cells with this construct. Following metabolic labeling, immunoprecipitation with an anti-HA mAb, and endoglycosidase digestion analysis, we detected in the cell lysate, after a 30-min pulse, a single Endo H-sensitive immunoprecipitated band with an apparent molecular mass of 45 kDa (Fig. 4, A and B). After 1 h of chase, two larger Endo H-resistant species with apparent molecular masses of 50 and 55 kDa were detected in the culture medium. Thus, the C-terminal domain of APA, unlike the N-terminal domain of APA, exited from the ER, was processed by the Golgi apparatus and was secreted into the culture medium as a mature protein. This secreted protein was resistant to trypsin digestion, because this treatment gave less than 50% degradation after 90 min (Fig. 4C). Consistently, confocal microscopy analysis of CHO cells transfected with the HA-C-APA construct showed C-APA expression, as detected with an anti-HA Ab, in vesicles probably corresponding to secretory vesicles (Fig. 4D). These data suggest that the C-terminal domain of APA was correctly folded and secreted independently of the N-terminal domain.
Expression in Trans of the C-terminal Domain of APA Rescues the Functions of the N-terminal Zinc Metallopeptidase Domain—A growing number of membrane and secretory proteins have been shown to have pro-domains at their N-terminal ends that undergo post-translational proteolytic cleavage in the late secretory pathway after the acquisition of transport competence. These domains, known as intramolecular chaperones, are responsible for the correct folding of their cognate catalytic domain. The presence of such intramolecular chaperone domains, or pro-domains, has been reported in enzymes such as subtilisin (34) and \( \alpha \)-lytic protease (35). Studies with these bacterial enzymes have shown that deletion of the N-terminal propeptides results in inactive enzymes maintained in an unfolded state. Results from the in vitro refolding of bacterial subtilisin (36) and in vivo studies with several proteases, such as bacterial \( \alpha \)-lytic protease (37), subtilisin (38), thermolysin (39), \textit{Saccharomyces cerevisiae} proteinase A (40), and Kex2p (41), have all indicated that pro-domains can activate the protease domain in trans. The presence of functional pro-domains in the eukaryotic membrane type 1 matrix metalloproteinase and in mammalian endoproteases of the subtilisin family has also been reported (42–44). Recently, an intramolecular chaperone domain located at the C terminus of the intestinal enzyme, sucrase-isomaltase has been described. The sucrase domain is autonomous and folds independently (45). However, to our knowledge, no such intramolecular chaperone domain has ever been reported in the monozinc aminopeptidase family.

We investigated whether the correct folding of the N-terminal zinc metallopeptidase domain of APA required the presence of the C-terminal domain of APA, functioning as an intramolecular chaperone via a direct interaction between the two domains. To this end, we co-expressed the FLAG-N-APA and HA-C-APA constructs in CHO cells and assessed the matura-

**Fig. 2.** FLAG-N-APA is not matured and is rapidly degraded within the cell. A, CHO cells stably expressing wild-type and truncated FLAG-tagged APAs were labeled for 30 min with \(^{35}\)S)methionine/cysteine and subjected to chase periods with serum-free medium for various periods of time. Proteins were immunoprecipitated from cell lysates with an anti-FLAG mAb, and resolved by SDS-PAGE. B, after 5 h of \(^{35}\)S)methionine/cysteine labeling, proteins were immunoprecipitated from cell lysates with an anti-FLAG mAb and treated with PNGase F or Endo H. Samples were subjected to SDS-PAGE. C, cells were incubated overnight in Opti-MEM medium, and secreted proteins were precipitated with trichloroacetic acid. Cell lysate proteins and secreted proteins were resolved by SDS-PAGE and immunoblotted with an anti-FLAG mAb. D, CHO cells stably expressing FLAG-WT-APA or FLAG-N-APA were fixed in cold methanol. APA co-immunolocalization was performed using polyclonal rabbit anti-(rat APA) serum and monoclonal anti-protein disulfide isomerase (PDI) antibody. FLAG-WT-APA (blue) was present at the plasma membrane, whereas FLAG-N-APA (blue) was co-localized in the ER with PDI (red). Immunolabeled cells were analyzed by confocal microscopy. Bar = 20 \( \mu \)m.
was accumulated in cell process, probably corresponding to secretory vesicles (see Fig. 7A). This demonstrates that the pattern of expression of N-APA and C-APA does not depend on cell type. In cells expressing both the N- and C-terminal domains of APA, the membrane localization of the N-APA suggested that, in the presence of the C-terminal domain of APA, the N-terminal zinc metallopeptidase domain of APA displayed a similar pattern of trafficking to WT-APA. Furthermore, the presence of both proteins at the plasma membrane suggests that they may be associated.

Interaction between the N- and C-terminal Domains—We investigated whether N-APA and C-APA interacted with each other by means of co-immunoprecipitation experiments. Cell lysates from CHO cells expressing FLAG-N-APA, either alone or together with HA-C-APA, were subjected to immunoprecipitation with antibodies recognizing HA-C-APA (anti-HA mAb) but not FLAG-N-APA. Then, the presence in the immunoprecipitate of FLAG-N-APA was detected with an anti-FLAG mAb. FLAG-N-APA was detected only in immunoprecipitates from cells co-expressing both domains, as the 85-kDa and 100-kDa forms (Fig. 6A). Thus, in CHO cells co-expressing the N- and C-terminal domains of APA, C-APA is associated with both the immature and the mature form of N-APA, because these two forms of N-APA are co-immunoprecipitated with C-APA. This suggests that the N- and C-terminal domains of APA interact shortly after their biosynthesis.

In addition, metabolic labeling of CHO cells co-transfected with the FLAG-N-APA and the HA-C-APA constructs, followed by immunoprecipitation of cell lysates and culture media with an anti-HA mAb, showed that the immature form of HA-C-APA was interacting with the immature 85-kDa form of N-APA after 30 min of pulse, in agreement with the association between both proteins shortly after their biosynthesis. HA-C-APA was present in only very small amounts in the medium after 3 h (Fig. 6B). In contrast, HA-C-APA was found as a mature protein in the cell lysate, interacting with the mature form of FLAG-N-APA, as shown by the presence of a 100-kDa band (Fig. 6B), even after 6 h of incubation (not shown). Thus, the co-expression of both domains in CHO cells induced retention of the mature form of C-APA at the plasma membrane. This finding is consistent with the observed change in the transport behavior of C-APA in the presence of N-APA resulting from a specific interaction between the two proteins.

Finally, the purification by metal affinity chromatography of His$_6$-N-APA (Fig. 6C) from CHO cells expressing both HA-C-APA and His$_8$-N-APA led to the detection in the eluate of both N-APA and C-APA, identified with the anti-Xpress and anti-HA antibodies as 100-kDa and 55-kDa proteins, respectively. This finding confirms the close association between the two proteins.

Enzymatic Activity of the N-APA-C-APA Complex—We investigated whether the membrane-bound N-APA-C-APA complex had recovered the enzymatic properties of WT-APA. Using the purified preparation from CHO cells co-transfected with His$_6$-N-APA and HA-C-APA, as described above, we showed that this complex had similar enzymatic characteristics to His$_6$-WT-APA (Table 1). His$_8$-N-APA had kinetic parameters and calcium activation patterns similar to those of His$_8$-WT-APA. We also assessed the ability of a specific and selective inhibitor of APA, glutamate phosphonate (a transition state pseudo-analog), to inhibit His$_6$-WT-APA and the His$_8$-N-APA-HA-C-APA complex. This inhibitor had similar inhibitory potencies toward both enzymes. In conclusion, the expression of the C-terminal domain in trans led to the N- and C-terminal domains of APA becoming associated soon after biosynthesis, restoring the folding, processing, plasma membrane expression, and activity of
the N-terminal zinc metallopeptidase domain. These data strongly suggested that the C-terminal domain acted as an intramolecular chaperone.

**Involvement of the C-terminal Domain of APA in Activation but Not in Enzymatic Activity of APA**—An important feature of intramolecular chaperones is their involvement in the folding, but not in the activity of the enzyme. We investigated whether the C-terminal domain was required for activity or only for activation of the N-terminal domain of APA by immunodepleting HA-C-APA from the lysates of CHO cells co-expressing
FLAG-N-APA and HA-C-APA, by incubating cell lysates three times for 4 h each on an anti-HA column and measuring APA enzymatic activity (Fig. 6D). We found that the immunodepletion of co-transfected CHO cell extracts did not affect the activity of the N-terminal domain after complete dissociation from the C-terminal domain, as shown by comparison with the original extracts containing N-APA/H18528C-APA complexes. The disappearance of the C-terminal domains was assessed by anti-HA immunoblotting of the samples (not shown) and beads (Fig. 6D). We cannot completely rule out the possibility that the lysate still contains some C-terminal domain that cannot be detected by Western blot analysis but that could be sufficient for activating the N-terminal domain. It should, however, be emphasized that to dismiss this possibility we have performed three rounds of immunodepletion and that the level of the N-terminal domains remains constant after the first one. Thus, the C-terminal domain was required only for APA activation, acting as an intramolecular chaperone ensuring the correct folding of the N-terminal zinc metallopeptidase domain, but not for the enzymatic activity.

Interaction of the N-terminal Zinc Metallopeptidase Domain of APA and Calnexin—Given that the N-terminal monozinc metallopeptidase domain expressed alone is retained in the ER, we examined its interaction with calnexin, a molecular chaperone involved in the proper folding of proteins exiting the from culture medium and cell lysates with an anti-HA mAb and, then were resolved by SDS-PAGE. C, His6-N-APA co-expressed with HA-C-APA was purified on a Talon column. Immunoblotting of the purification eluate was performed with the anti-Xpress mAb and anti-HA Ab. D, CHO cells were co-transfected with the FLAG-N-APA and HA-C-APA constructs. Cell lysates were subjected to three rounds of incubation with anti-HA mAb immobilized on protein A-Sepharose. The presence of HA-C-APA in the immunoprecipitates was monitored by immunoblotting, using an anti-HA mAb. The APA activity of FLAG-N-APA was compared in native cell lysates (S0), or cell lysates resulting from one (S1), two (S2), or three (S3) incubations. Activity measurements were performed on equivalent quantities of FLAG-N-APA and in the presence or absence of the APA inhibitor glutamate phosphonate, to confirm that hydrolysis was indeed due to APA activity. Activity is expressed as a percentage of APA activity in S0. Results are representative of four separate experiments.
ER (47). Expression of FLAG-N-APA alone in AtT-20 cells, followed by immunoprecipitation with an anti-calnexin Ab, resulted in the detection of FLAG-N-APA with an apparent molecular mass of 85 kDa (Fig. 7C). This band corresponded to the immature form of FLAG N-APA, which remained in the ER and interacted with the ER chaperone calnexin.

When AtT-20 cells were co-transfected with FLAG-N-APA and HA-C-APA, the immunoprecipitation of cell lysates with an anti-calnexin Ab followed by immunoblotting with an anti-FLAG mAb did not result in the detection of FLAG-N-APA. Cell lysates were also subjected to anti-calnexin immunoblotting and anti-FLAG immunoprecipitation followed by anti-FLAG immunoblotting, to check the amount of each protein. C, AtT-20 cells were transiently transfected with the FLAG-N-APA construct, either alone or with HA-C-APA. Proteins were immunoprecipitated from cell lysates with anti-calnexin Ab. Immunoprecipitates were resolved by SDS-PAGE and subjected to immunoblotting with anti-FLAG mAb. Cell lysates were also subjected to anti-calnexin immunoblotting and anti-FLAG immunoprecipitation followed by anti-FLAG immunoblotting, to check the amount of each protein. D, AtT-20 cells were transiently transfected with the FLAG-N-APA construct, either alone or with HA-C-APA and were labeled for 30 min with [35S]methionine/cysteine and subjected to a 2-h chase period with Opti-MEM medium. Cell lysates were divided into two equal aliquots for immunoprecipitation with anti-FLAG mAb or anti-calnexin Ab. The immunoprecipitates were analyzed by SDS-PAGE and phosphorimaging.

The C-terminal domain of APA acts as an intramolecular chaperone. Is the Role of the C-terminal Domain of APA Extendable to Other Monozinc Aminopeptidases?—We studied if the role of intramolecular chaperone of the C-terminal domain of APA could be extended to other membrane-bound monozinc aminopeptidases. To this end, we generated constructs corresponding to the FLAG-tagged N-terminal domain of APN from residues 1 to 606 and to the HA-tagged C-terminal domain of APN from residues 607 to 966. We studied the maturation of FLAG-N-APN, HA-C-APN expressed alone, and FLAG-N-APN co-expressed with HA-C-APN. For this purpose, we performed pulse-chase experiments on transiently transfected CHO cells.

C-terminal domain of APA allows correct folding of the N-terminal zinc metallopeptidase domain of APA via an association of the two domains. This confirms that the C-terminal domain of APA acts as an intramolecular chaperone.
expressed with the C-terminal domain, the N-terminal domain, immunoprecipitated with an anti-FLAG mAb, was detected as an 85-kDa form after a 30-min pulse. A second band, 100 kDa in size was detected after 60 min of chase (Fig. 8C). Thus, co-expression of these two proteins resulted in the production of a high molecular mass form of 100 kDa of FLAG-N-APN.

Confocal microscopy analysis of AtT-20 cells (Fig. 8D) or CHO cells (not shown) expressing FLAG-N-APN showed the retention of this protein in the ER. In AtT-20 cells (Fig. 8D) or in CHO cells (not shown), HA-C-APN expressed alone was present in vesicles and accumulated in the cell process. In cells expressing both the N- and C-terminal domains of APN, both domains were often co-localized at the plasma membrane.

Concluding Remarks—The C-terminal domain of APA fulfills all the requirements for an intramolecular chaperone. We show here that activation can take place in trans in eukaryotic cells for two members of the monozinc aminopeptidase family. These data provide, to our knowledge, the first evidence for the presence of an intramolecular chaperone domain in a mammalian exopeptidase. Furthermore, all the endoproteases for which intramolecular chaperone activity has been reported, whether cytosolic or membrane-associ- ated, share a common structure, with an N-terminal pro- domain. The recent expression in trans of the C-terminal domain of sucrase-isomaltase provided evidence that sucrase acts as an intramolecular chaperone for this enzyme (45). The data presented here further demonstrate that the C-terminal domain of APA acts as an intramolecular chaperone that is absolutely required for APA activation, but not involved in the enzymatic activity of APA.

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C-terminal Domain of APA Acts as Intramolecular Chaperone

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