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Rat endopeptidase-24.18 α subunit is secreted into the culture medium as a zymogen when expressed by COS-1 cells

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Endopeptidase-24.18 (EC 3.4.24.18, E-24.18) is an oligomeric Zn-ectoenzyme. The α and β subunits have been cloned from both rat and mouse kidneys. The primary structure of these subunits revealed that they both contain the consensus Zn binding site and that they are members of the astacin family. Analysis of the hydropathy plot also suggested that they are anchored by a C-terminal hydrophobic domain. In order to verify the mode of anchoring of the rat E-24.18 α subunit and to test the functionality of the astacin-like domain in the α subunit when expressed alone, COS-1 cells were transfected with a cloned cDNA for rat α subunit. Despite the presence of its putative transmembrane domain, the α subunit was not anchored in the plasma membrane but rather secreted as a dimer into the culture medium. When the enzymatic activity of the secreted recombinant protein was tested in the azocasein degradation assay, the α subunit was found to be inactive. Activity could, however, be revealed after mild trypsin digestion. This activity was abolished by replacing the Glu-157 in the active site by Val. Taken together our results suggest that the α subunit of Endopeptidase-24.18 contains a latent astacin-like Zn metallopeptidase activity which could be secreted as a soluble enzyme by kidney and intestine.

Meprin; Astacin; Ectoenzyme; Zymogen

1. INTRODUCTION

Endopeptidase-24.18 (EC 3.4.24.18., meprin, Endopeptidase-2, PABA-peptide hydrolase, E-24.18) is an oligomeric metallopeptidase present on the brush border membranes of rodent kidney proximal tubules [1] and in human intestine [2,3]. This peptidase hydrolyses in vitro a variety of extended peptides [1,4,5], but its physiological function remains unknown. Although E-24.18 was predominantly identified as a membrane-bound ectoenzyme with a protease-sensitive stalk domain, it has also been found as a soluble form that retains proteolytic activity in rodent urine [6].

E-24.18 is composed of two subunits (α and β) and displays a multimeric organization of disulphide-linked subunits. The rat kidney enzyme has a tetrameric structure resulting from the non-covalent association of disulphide-linked α and β subunits, $(\alpha\beta)_2$ [7]. On the other hand, three covalent tetrameric forms have been reported for mouse kidney enzyme: α_4, $\alpha_2\beta_2$ and β_4 [8]. The homotetrameric form of β subunit was shown to be the latent metallopeptidase meprin B [9].

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Abbreviations: E-24.18, endopeptidase-24.18; RER, rough endoplasmic reticulum; PNGase F, peptide *N*-glycosidase F; endo H, endo-β-*N*-acetylglucosaminidase H; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; BBM, brush border membrane.

Recently, the primary structures of the α and β subunits of rat and mouse E-24.18 have been deduced from the cDNA clones [7,10–12]. Both subunits have a similar organization and contain an astacin-like protease domain which is characterized by the HExxHxxGFxHE consensus Zn binding sequence [13,14]. The rat E-24.18 α subunit is originally synthesized as a preproenzyme of 748 amino acids and the mature form starts at Asn^{67}, implying the cleavage of the pro-region upstream of the astacin domain [10]. This protein contains two hydrophobic segments located at the N-terminus and close to the C-terminus of the protein [10], respectively. The N-terminal hydrophobic segment is most likely the signal peptide whereas the 20-amino acid C-terminal hydrophobic segment might anchor the protein into the plasma membrane.

In this paper we examined the anchoring of the α subunit in the plasma membrane by expression of the recombinant enzyme in transfected COS-1 cells. This system was also used to directly test the functionality of the astacin-like domain in the catalytic activity of the enzyme.

2. EXPERIMENTAL

2.1. *Plasmids construction*

The full-length cDNA coding for the α subunit of rat E-24.18 was subcloned into the eukaryotic expression vector pSVP4 [15] which contains the SV40 early promotor/enhancer region. This plasmid, designated pSVP4-α was used for expression of the wild-type α subunit

cDNA in COS-1 cells. Conversion of the Glu^{157} codon (GAG) to Val (GTG) was accomplished by oligonucleotide-directed mutagenesis using a M13 subclone previously described (clone 6.2) [10], according to the method of Taylor et al. [16]. The presence of the mutation was confirmed by DNA sequencing [17]. The mutated cDNA was substituted for the wild-type cDNA fragment into pSVP4-α vector.

2.2. *Production of antibody against MBP-α subunit fusion protein*

A bacterial fusion protein of the E-24.18 α subunit was generated by inserting in frame the *Eco*RI cDNA fragment of clone 6.2 into a pMAL-c vector (New England Biolabs Ltd., Canada) downstream from the *malE* gene encoding maltose-binding protein (MBP). The expression plasmid, called pMAL-α, was used to transform competent HB101 *E. coli*. The MBP-α subunit fusion protein was overexpressed and purified according to the manufacturer's protocol (ProFusion Kit, New England Biolabs Ltd., Canada). A polyclonal antibody L5α was generated in a New Zealand white rabbit with MBP-α subunit fusion protein.

2.3. *Transfection of COS-1 cells*

COS-1 cells [18] were transfected using the standard calcium-phosphate coprecipitation procedure [19]. The day following transfection, the serum-containing medium was changed for synthetic medium [20]. Spent medium was recovered 16 hours later and concentrated 8-fold by ultrafiltration using Centricon PM30 membranes. Transfected cells were harvested by scraping with a rubber policeman in ice cold Tris-buffered saline (TBS) (3 ml/Petri dish), pelleted by centrifugation and lysed with 2% (w/v) *N*-octyl-β-D-glucopyranoside (octylglucoside) in TBS.

2.4. *Western blotting*

Proteins from solubilized cells or concentrated culture media were resolved on SDS-PAGE [21] and transferred to nitrocellulose membranes (Schleicher and Schuell, Keene, USA; pore size 0.45 μm) using previously published procedures [22]. After transfer, the membranes were preincubated in TTBS solution (0.2% Tween-20 in TBS) for 30 min and a 1:300 dilution of the L5α antiserum in TTBS was then added and incubated for 30 min at room temperature. After 3 washes in TTBS, a 1:500 dilution in TTBS of a biotinylated second antibody directed against rabbit immunoglobulins was added and incubated in the same conditions. The complexes were visualized with a Vectastain ABC-Immunoperoxidase kit (Vector Laboratories, Burlingame, CA) according to the manufacturer's instructions. Endoglycosidase digestions prior to immunoblotting were performed as described previously [23]. The relative amount of the different molecular species was evaluated by densitometric scanning using ultroscan XL Enhanced Laser Densitometer (LKB Bromma).

2.5. *Immunofluorescence microscopy*

COS-1 cells, grown on plastic coverslips placed in tissue culture wells, were transfected with pSVP4-α vector as described above. Two days after transfection, immunofluorescent staining was performed essentially as described [24] using a polyclonal antibody L5α and an FITC-conjugated goat anti-rabbit IgG (Dimension Laboratories Inc. Canada), except that the non-specific binding sites were blocked with 0,1% (w/v) bovine serum albumin PBS solution.

2.6. *Enzyme assay*

E-24.18 activity was measured using azocasein as substrate by monitoring at 340 nm the release of dye-containing peptides that were soluble in 4% (w/v) trichloroacetic acid (TCA). Reactions were performed in 0.15 M NaCl/20 mM ethanolamine (pH 9.5) buffer [25]. Briefly, 35 μl of solubilized COS-1 cells or 60 μl of 8-fold concentrated culture medium were incubated for 60 min at 37°C with or without 10 μg trypsin in a final volume of 100 μl. A mixture (100 μl) of different protease inhibitors (1 μM captopril, 1 μM phosphoramidon, 10 μM bestatin, 10 μg/ml aprotinin and 20 μM leupeptin) was then added to the samples and incubated for 20 min in the same conditions. Finally, 900 μl of solution containing 11 mg of azocasein was added. After 90 min of incubation at 37°C, 250 μl aliquots were precipitated in 1 ml TCA. The samples were centrifuged for 5 min and the absorbance of the supernatant was determined at 340 nm. Specific activities are expressed as units/mg protein/min, one unit corresponding to the degradation of 1.1 μg azocasein. Protein concentrations were estimated by the method of Bradford [26].

3. RESULTS

3.1. *Production and characterization of a E-24.18 α subunit-specific antibody*

A rabbit was immunized with an *E. coli*-derived fusion protein containing a portion of E-24.18 α subunit (see Section 2). Specificity of the antibody, named L5α, was verified by immunoblotting using detergent solubilized rat kidney membrane proteins. As shown in Fig. 1 lane 1, a single immunoreactive band with an apparent molecular weight (M_r) of 82 kDa was detected. This is in agreement with the M_r of the α subunit previously reported [7]. The antibody did not recognize the β subunit of rat E-24.18 (Milhiet et al., submitted).

3.2. *Expression of E-24.18 α subunit in COS-1 cells*

COS-1 cells were transfected with pSVP4-α plasmid and expression of the α subunit was monitored using SDS-PAGE and immunoblotting with the L5α polyclonal antibody. In the supernatant of octyl-glucoside solubilised cell membranes (cell extracts), E-24.18 α subunit was present as two distinct species with apparent M_r of 98,000 and 82,000 (Fig. 1, lane 4). The 82 kDa band represented only a small fraction of the cell-associated α subunit. To our surprise, analysis of proteins secreted in the culture medium revealed significant

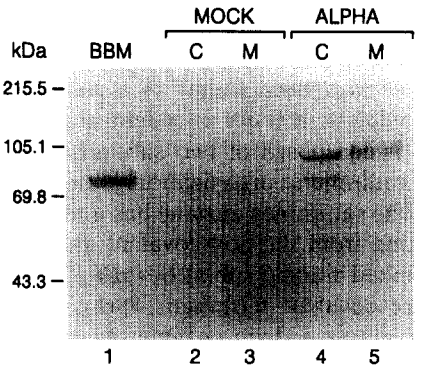

Fig. 1. Immunoblot analysis of rat E-24.18 α subunit expressed in COS-1 cells. Proteins corresponding to one-fifth of a Petri dish of transfected cells were either recovered by octylglucoside solubilization of the cell pellet (C) or by concentration of the culture medium (M). The samples were analyzed by SDS-PAGE on 7.5% gel followed by immunoblotting with antiserum L5α. Lane 1: rat kidney BBM preparation (30 μg); lanes 2,3: mock-transfected cells; lanes 4,5: α-transfected cells. The position of apparent molecular mass of the prestained proteins (myosin, 215.5 kDa; phosphorylase B, 105.1 kDa; bovine serum albumine, 69.8 kDa; ovalbumin, 43.3 kDa) (all obtained from Gibco) is indicated at the left.

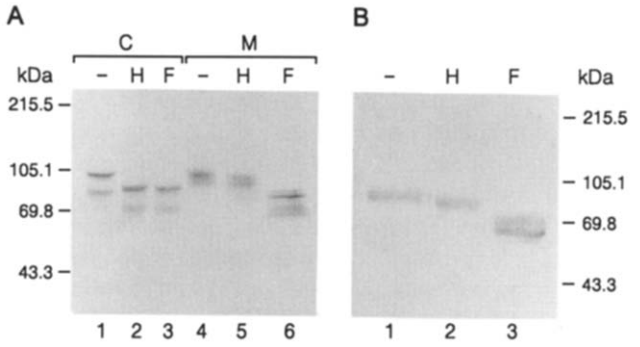

Fig. 2. Endoglycosidase treatment of the E-24.18 α subunit. Proteins samples were treated with either endo H or PNGase F as described in Section 2, before analysis by SDS-PAGE as described in the legend [illegible] culture medium (M) of transfected COS-1 cells. Panel B: rat kidney BBM preparations (30 μg). The samples were either untreated (−), [illegible] digested with endo H (H) or PNGase F (F). Position of size markers are indicated on both sides in kDa.

amounts of α subunit immunoreactive material which appeared as a broad band with M_r between 90,000 and 105,000 (Fig. 1, lane 5). These secreted molecules represented about 55–60% of the total amount of immunoreactive material present in both cell extracts and spent culture medium. No staining was observed in the cell extracts or culture medium of mock-tranfected cells (Fig. 1, lanes 2 and 3, respectively). The different forms of the protein recovered in the cell extracts were next submitted to endoglycosidase digestions. The two forms of the cell-associated α subunit were sensitive to both endo H and PNGase F (Fig. 2A, lanes 2 and 3) which reduced their apparent M_r by about 12 kDa. This result is consistent with the presence of 6 *N*-glycosylation sites predicted by the cDNA-derived sequence. In contrast, the 'soluble forms' of the α subunit were mostly resistant to endo H digestion (Fig. 2A, lane 5). Treatment with PNGase F converted the α subunit into four lower M_r forms, from 83 to 67 kDa (Fig. 2A, lane 6). A similar pattern of sensitivity to glycosydases was observed with the BBM-derived α subunit (Fig. 2B, lanes 2 and 3).

3.3. *Cellular localization of E-24.18 α subunits*

The localization of the cell-associated E-24.18 α subunit in transfected cells was next studied by indirect immunofluorescence microscopy with the L5α polyclonal antibody. There was no staining at the cell surface of intact cells (Fig. 3B). However, when cells were permeabilized with Triton X-100 before incubation with the antiserum, a diffuse cytoplasmic fluorescence was observed around the nucleus consistent with a RER labelling (Fig. 3D). No staining was observed in mock-transfected cells (not shown). Thus both the endo H sensitivity of the cell-associated α subunits (see Fig. 2A, lane 2) and their cellular localization suggest that the cell-associated α subunits are present in the RER and not at the cell surface.

3.4. *Oligomerization of E-24.18 α subunit*

The multimeric organization of E-24.18 is unique among endopeptidases. The fact that the mouse α subunit has been found as homotetramers and the high degree of identity between rat and mouse α subunits, prompted us to investigate whether the rat α subunit alone could spontaneously form dimers. Both the cell extract and culture media of COS-1 cells expressing the α subunit were analyzed under non-reducing conditions by SDS-PAGE and immunoblotting. Under those conditions, most of the α subunit immunoreactive material detected in cellular extracts migrated as triple band with a M_r around 190,000 whereas the intensity of the bands corresponding to the two monomeric species (82 and 98 kDa) (Fig. 4 lane 5) had greatly diminished. Thus these high molecular weight forms are thought to result from the various combinations of the two monomeric species (82/82, 82/98, 98/98 kDa). In the spent culture medium only two bands were detected with mobilities corresponding to the lower molecular weight species found in cell extracts. The M_r of these dimers were found to be slighly lower than that of the α/β dimer present in rat BBM (lane 1). In addition the monomeric forms representing less than 10% of the secreted molecules were found (lane 5). In COS-1 cells, these rat E-24.18 α subunits therefore appear to be mostly secreted as dimers.

3.5. *Enzymatic activity of E-24.18 α subunit*

The rat α and β subunits contain both an astacin-like protease domain. It is not known if the catalytic activity

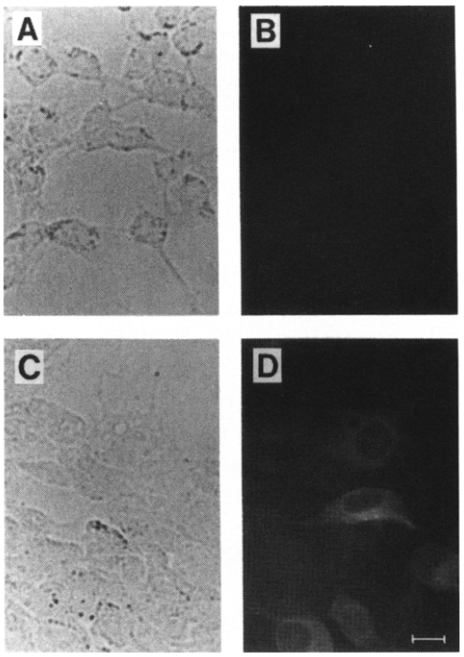

Fig. 3. Immunofluorescent staining of COS-1 cells expressing E-24.18 α subunit. Transfected COS-1 cells were grown on coverslips and processed for indirect immunofluorescence as described in Section 2. Panel B: intact cells. Panel D: Triton X-100 permeabilized cells. Panels A and C show phase contrast images of the cells. Bar = 10 μm.

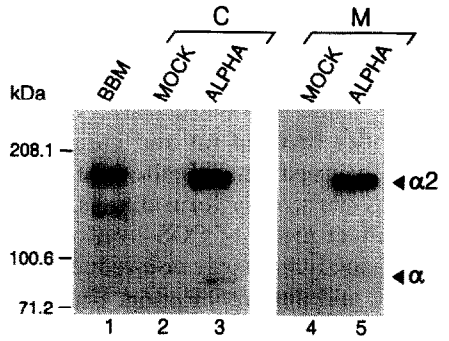

Fig. 4. Oligomerization of α subunit in COS-1 cells. Proteins were recovered from the cells (C) or culture media (M) as described in the legend of Fig. 1 and analyzed by SDS-PAGE on 5% gel in non-reducing conditions prior the immunoblotting. Lane 1: rat kidney BBM preparation (30 μg); lane 2,4: mock-transfected cells; lane 3,5: α-transfected cells. The solid triangles represent monomers and dimers of E-24.18 α subunit. Molecular weight standards in kDa are indicated at the left.

depends on one or both subunits. We therefore assayed the proteolytic activity of the recombinant α subunit using azocasein as substrate. Enzymatic activity was expressed as units/mg protein/min. When assayed directly, no activity was detected in the cell extract or culture medium of transfected cells (Fig. 5A,B). However, as mouse E-24.18 (meprin A) has been shown to be activated by trypsin treatment when azocasein and insulin B chain were used as substrates [28], we decided to preincubate our samples for 1 hour at 37°C with 10 μg trypsin. After inhibition of trypsin by aprotinin, the samples were then tested for their azocasein degradation activity. In these experimental conditions, a significant proteolytic activity was detected in the samples recovered from the transfected cells (Fig. 5A,B, hatched boxes), whereas no enzymatic activity was observed in control mock-transfected cells. It is noteworthy that cell-associated forms of the α subunit are active despite their lack of fully processed oligosaccharides. To ensure that the proteolytic activity observed was due to the astacin-like domain of the recombinant enzyme, we replaced the catalytic glutamic acid-157 found in the consensus sequence (HExxH) by a valine residue (mutant αE157V). Although the secretion level of the mutated enzyme was not affected by the mutation (data not shown), no significant activity was detected in the cellular extract or concentrated medium of the αE157V-transfected cells after the trypsin treatment, demonstrating the functional implication of the astacin-like domain.

4. DISCUSSION

Molecular cloning of rat and mouse α subunit cDNAs predicts that in both species the α subunit is a membrane-bound protein anchored by its C-terminus [10,11]. We therefore decided first to verify the anchoring of the recombinant α subunit at the cell surface after transfection in COS-1 cells. Our results show that 55–60% of the total α subunit synthesized by COS-1 cells is recovered in the secretion medium as Endo H resistant glycoproteins whereas the rest of the molecules are not located at the cell surface but rather appear to be trapped in the rough endoplasmic reticulum as Endo H sensitive forms. This suggests that the material recovered in the secretion medium has been secreted through the exocytic pathway and does not result from leakage of damaged cells. Thus in COS-1 cells at least, the α subunit does not appear to be a membrane-bound enzyme of the cell surface. The solubilization mechanism of the α subunit molecule is unknown. Although we cannot rule out the possibility that the putative transmembrane domain is not functional, the molecular weight (about 67,000) of the faster migrating band found after PNGase F digestion of the α subunit secreted by COS-1 cells (Fig. 2A lane 6) is significantly smaller than the M_r predicted from the cDNA-derived sequence (77,873) suggesting a proteolytic cleavage of the protein. Although this might be a phenomenon restricted to the COS-1 cell system, several observations lead us to believe that the same mechanism occurs in epithelial cells of the kidney and intestine: (i) when expressed in Madin–Darby canine kidney cells, the α sub-

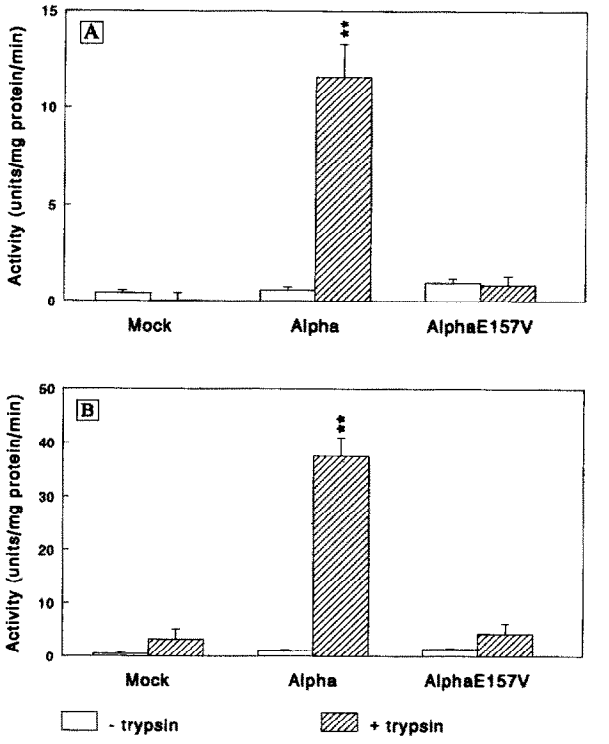

Fig. 5. Enzymatic activity of transfected cells. Cell-associated (A) and soluble (B) proteins from transfected COS-1 cells were assayed for azocasein degradation with or without trypsin activation as described in Section 2. The results are the means of three separate experiments carried out in duplicate. The standard error (S.E.) is indicated by error bars. (**$P < 0.005$, as compared to mock-transfected cells using the Student's t-test).

unit has also been found to be secreted as a soluble protein (Corbeil et al., unpublished results); (ii) in the BBM of mouse or rat kidneys where it is expressed as a heterodimer in association with the β subunit, the molecular weight of the α subunit does not change after solubilization of the activity by papain treatment [7,8], suggesting anchoring of a truncated α subunit through disulphide bonding with the β subunit rather than by its own putative membrane-spanning domain; (iii) the molecular weight of the deglycosylated α subunit obtained after solubilization of E-24.18 from rat kidney BBM is the same as that of the deglycosylated protein secreted by COS-1 cells (Fig. 2B, lane 2) and very close to that reported for mouse and human α subunits [3,9]. Two potential dibasic cleavage sites were found in the carboxyl terminal region of the α subunit; one of them was perfectly conserved between the rat and mouse α subunit. The RPKR sequence (residues 653 to 656) corresponds to the consensus RXK/RR motif involved in precursor cleavage catalysed by furin [29]. As furin has already been proposed to act in the RER-Golgi complex [30], intracellular cleavage of the C-terminal region by furin could explain the secretion of the α subunit. Confirmation of the potential furin cleavage site by site-directed mutagenesis is now in progress in our laboratory. Finally, the fact that the inactive mutant αE157V is secreted with the same efficiency shows that the secretion mechanism does not implicate an autocatalytic process as reported for another cell-surface glycoprotein [31].

Our results clearly show that the rat α subunit alone, like the mouse protein, was able to form disulphide-bound multimers. However, only the dimeric form α_2 was observed. This contrasts with the α_4 structure reported in vivo for the mouse protein. This different behaviour is surprising given that all the cysteine residues were perfectly conserved between the two species. In particular, both contain EGF-like domain that could potentially mediate protein–protein interactions [27]. However, no disulphide-linked tetramers were observed in vivo in the rat. Similarly, biogenesis of human E-24.18 is well documented and it has been shown that this protein forms only dimers [2]. From these data, it appears that the covalent tetrameric forms of the α subunit of E-24.18 are specific for the mouse enzyme. Thus, it is possible that structural features specific to the mouse protein or some factors found only in mouse epithelial cells allowed tetramer formation in this species. These different oligomerization processes between rodent species may account for distinct enzymatic properties [1] and for the expression of the homotetramers at the plasma membrane of the mouse kidney. In the rat, α/β dimers represent the E-24.18 membrane-bound forms found at the plasma membrane of the kidney cells [7]; α_2 dimers could however constitute the E-24.18 activity secreted into rat urine. Consistent with this latter hypothesis, we have recently observed by immunoblotting the presence of the α subunit into the rat urine (Simon, V. and Corbeil, D., unpublished results). The physiological function of this enzyme is unknown but it was postulated to be implicated in the processing of urinary peptides that in part determine urinary odors [6].

When the catalytic properties of the recombinant rat α subunit were investigated using azocasein as substrate, it was found that the α subunit is initially synthesized in COS-1 cells as a zymogen. There is evidence that activity of mouse meprin A towards small peptides does not require trypsin activation [28]. The activity of our recombinant α subunit towards low molecular weight substrates is presently under investigation. The in vivo mechanism for the enzyme activation is not known. Sequence alignment of all members of the astacin family showed a basic residue (arginine or lysine) or a cluster of basic residues located at the junction between the pro-region and the astacin-like domain [31]. Cleavage at this site would generate a protein with a NH_2-terminal peptide sequence identical to that found by amino acid sequencing of the mature form of the α subunit [10]. Therefore, it is reasonable to suggest that a trypsin-like processing enzyme may be involved in the activation of the enzyme. Taken together our results suggest the presence of two sets of proteolytic events occuring both at the N- and C-termini of the α subunit. Some of these processing steps might be cell-specific and account for part of the heterogeneity of the immunoreactive material found in cell extract and medium of transfected cells. In addition some post-translational Golgi-associated modifications such as *O*-linked glycosylation and tyrosine sulfation might also occur on the α subunit and contribute to its heterogeneity. Solving the exact nature of all the forms of α subunit will require further work using sequence specific antibodies combined with peptide mapping and microsequencing experiments. Finally, it is clear that the astacin-like domain is directly involved in the enzymatic activity of the rat E-24.18 α subunit, since the substitution of the first glutamic acid (E-157) in the consensus Zn-binding site of the astacin-like domain by a valine residue essentially abolished any azocasein-degrading activity.

In summary, our results show that the α subunit protein is secreted as a soluble form despite the presence of a putative transmembrane domain in its primary structure. Furthermore, our results provide conclusive evidence that the astacin-like domain is enzymatically active in the rat E-24.18 α subunit although the enzyme is initially synthetized as a zymogen. Further studies are in progress in our laboratory to establish more completely the specificity of this enzyme.

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