Expression of rat endopeptidase-24.18 in COS-1 cells: membrane topology and activity

Pierre-Emmanuel MILHIET,* Denis CORBEIL,* Valérie SIMON,* A. John KENNY,† Philippe CRINE* and Guy BOILEAU*†

*Département de biochimie, Faculté de Médecine, Université de Montréal, C.P. 6128, Succ. A, Montréal, Canada, H3C3J7, and †Department of Biochemistry and Molecular Biology, University of Leeds, Leeds LS2 9JT, U.K.

Endopeptidase-24.18 (E-24.18; EC 3.4.24.18) is a metallo-peptidase of the astacin family and is highly expressed in kidney brush-border membranes of rodents. Rat E-24.18 consists of two disulphide-linked α/β dimers [$(\alpha/\beta)_2$]. In order to investigate the mechanisms of assembly and the importance of each subunit in the enzymic process, the cloned cDNAs for the rat α and β subunits were transiently expressed either alone or together in COS-1 cells. Immunoblotting of cell extracts and spent culture media showed that, when expressed alone, the α subunit is secreted, whereas the β subunit is membrane-bound. In α/β -transfected cells, the α subunit remained membrane-bound, but could be released from the cell surface after papain treatment or after incubation with 10 mM dithiothreitol. Furthermore, mutants of the α subunit in which the putative C-terminal anchor

domain was deleted could still form cell-associated α/β dimers. These results are consistent with a topological model of E-24.18 in which the β subunit is anchored in the plasma membrane and the α subunit is retained at the cell surface through disulphide bridge(s) with the β subunit. Both the α and β recombinant subunits expressed in COS-1 cells showed little azocasein-degrading activity. However, activity of either individual subunits of α/β dimers was increased after mild trypsin digestion, suggesting that in COS-1 cells the enzymes are synthesized as zymogens. Finally, inactivation of the α subunit by site-directed mutagenesis of Glu-157, which is believed to play a role in catalysis, showed that both subunits participate in the enzymic activity of the heterodimer.

INTRODUCTION

Endopeptidase-24.18 (EC 3.4.24.18, Endopeptidase-2, Meprin, E-24.18) is a zinc-metalloectopeptidase present on brush-border membranes (BBM) of rat and mouse kidney proximal tubules [1–4]. The physiological relevance of this enzyme is still unknown, but *in vitro* it can hydrolyse a variety of polypeptides such as bradykinin, lutropin-releasing hormone, insulin B chain and azocasein [5,6]. The HEXXHXXGFXHE sequence in its active site is characteristic of the ‘astacin protein family’, which includes several mammalian ectopeptidases and developmentally implicated regulatory proteins [7,8].

E-24.18 is an oligomeric glycoprotein composed of disulphide-linked subunits. The rat enzyme has been described as a heterotetrameric complex of α and β subunits [$(\alpha/\beta)_2$] which can be dissociated into α/β dimers by using detergent [3,9,10]. Each subunit has a molecular mass of 82 kDa [10,11]. A similar organization has been observed for *N*-benzoyl-L-tyrosyl-*p*-aminobenzoic acid hydrolase (PPH), another proteinase of the astacin family expressed in the human small intestine [12,13]. However, structural organization of the mouse protein is different, since all four subunits are disulphide-linked and the enzyme can exist as homo- or hetero-tetramers composed of 90 kDa α and 110 kDa β subunits [14]. Recent cloning of rat α and β subunits has elucidated the primary structure of E-24.18 [11,15,16]. Both subunits are putative type I membrane proteins and have a similar organization, including a catalytic astacin domain, a MAM domain found in various extracellular molecules

[17], and an EGF (epidermal growth factor)-like region close to the putative transmembrane segment. The primary structure and organization of mouse α and β subunits are also similar to those of rat [18,19].

The multimeric and disulphide-bonded structure of E-24.18 distinguishes it from most other mammalian ectopeptidases, as this organization can only be found in a few membrane-bound enzymes such as Na^+/K^+ -ATPase, γ -glutamyltransferase or farnesyltransferase [20–22]. In the cases documented so far, one subunit allows plasma-membrane anchorage, whereas the other is involved in the enzymic process. We recently reported that the α subunit was not expressed at the cell surface of transfected COS-1 cells [23], but was mostly secreted as a soluble protein in the incubation medium. Cell-associated proteins were probably trapped in the rough endoplasmic reticulum (RER), as they were found to carry endo- β -*N*-acetylglucosaminidase H (endo H)-sensitive carbohydrate side chains. The α subunit secreted by COS-1 cells had little enzymic activity when assayed directly in the azocasein-degradation assay. This activity could, however, be considerably increased after mild trypsin digestion, suggesting that the protein is initially synthesized as a zymogen. In this paper, we have transfected COS-1 cells with the cDNAs for each of the individual subunits of E-24.18 either separately or together, in order to evaluate the contribution of each subunit in the mechanism of membrane anchoring and in the enzymic activity of the protein. Our results suggest that the β subunit is responsible for membrane anchoring of the disulphide-linked α/β dimer. Furthermore, both subunits were found to have latent azocasein-

Abbreviations used: BBM, brush-border membranes; DTT, dithiothreitol; E-24.18, endopeptidase-24.18, EC 3.4.24.18; EGF, epidermal growth factor; endo F, endo- β -*N*-acetylglucosaminidase F; endo H, endo- β -*N*-acetylglucosaminidase H; PPH, *N*-benzoyl-L-tyrosyl-*p*-aminobenzoic acid hydrolase; RER, rough endoplasmic reticulum; TBS, Tris-buffered saline.

† To whom correspondence should be addressed.

degrading activity, which can be revealed after mild trypsin digestion and can contribute to the overall activity of the heterodimer.

MATERIALS AND METHODS

Plasmid construction

The α -subunit expression vector pSVP4- α has been described previously [23], and a similar strategy was used to construct the β -subunit expression vector pSVP4- β . Briefly, the cDNA encoding the full-length β subunit of rat E-24.18 (generously given by Dr. L. B. Hersh, Lexington, KY, U.S.A.) was first subcloned into KS/Bluescript (KS/BS) vector and then digested with restriction endonucleases *KpnI* and *SacI* to generate a 2205 bp fragment. The DNA fragment was blunt-ended with T7 DNA polymerase and then inserted into the *SmaI*-digested pSVP4 vector [24]. The orientation was determined by restriction-nuclease mapping and sequencing. Plasmid pSVP4- α E157V, encoding an inactive form of the α subunit, has been reported previously [23]. C-terminal truncated forms of the α subunit were obtained by PCR mutagenesis by the method described by Ho et al. [25] using *Pfu* DNA polymerase (Stratagene, Palo Alto, CA, U.S.A.). The STOP codons were introduced into the cDNA at the positions corresponding to amino acids 720 (mutant $\alpha\Delta H$) and 673 (mutant $\alpha\Delta EGF$). A 681 bp DNA fragment (*ScaI*–*NdeI*) containing the mutated region was then substituted for the non-mutated region in pSVP4- α . In all cases the 681 bp DNA fragment was sequenced [26] in order to confirm the presence of the mutation and to detect any putative reading mistakes of *Pfu* DNA polymerase.

Transfection of COS-1 cells

Transfection of COS-1 cells [27] has been described in detail previously [28]. Under these conditions 10–15% of the cells are transfected. Medium supplemented with foetal-bovine serum (Gibco, Grand Island, NY, U.S.A.) was replaced 24 h after transfection with synthetic medium containing 1 μ g/ml BSA, 2.5 μ g/ml insulin, 17.5 μ g/ml transferrin, 2 μ g/ml ethanolamine, 100 μ g/ml soybean trypsin inhibitor and 10 μ g/ml aprotinin in Dulbecco's modified Eagle's medium. Synthetic medium was collected 16 h later and concentrated 8-fold by using Centrprep 30 membranes (Amicon, Oakville, Ont., Canada). Transfected cells were scraped in ice-cold PBS and membrane proteins were solubilized with 2% (w/v) n-octyl- β -D-glucopyranoside (octyl-glucoside) in Tris-buffered saline (TBS; 50 mM Tris, pH 7.6, 150 mM NaCl).

Immunoblotting and endoglycosidase digestions

Proteins from cell extracts or concentrated culture media were separated by SDS/PAGE [29] and transferred to nitrocellulose membranes (0.45 μ m pore size) as described previously [30]. The procedure for preparation of BBM was also described by Aubry and co-workers [30]. E-24.18 proteins were then detected by using either polyclonal antibody L5 α , which specifically recognizes the α subunit [23], or polyclonal antibody RRT-151, which recognizes both subunits [10]. The polyclonal antibody L5 α was produced from a fusion protein corresponding to a fragment of 294 residues [23], whereas the purified protein from BBM has been used for immunization [10]. In all cases, biotinylated secondary antibodies were used to reveal antigen–antibody complexes (Vectastain ABC-Immunoperoxidase kit; Vector Laboratories, Burlingame, CA, U.S.A.). Endoglycosidase diges-

tions were performed essentially as described previously [23]. Briefly, cell extracts or concentrated culture media were incubated overnight at 37 °C with 10 m-units of endo H or 0.5 unit of endo- α -N-acetylglucosaminidase F (endo F) according to the manufacturer's protocol. Proteins were then analysed by SDS/PAGE and immunoblotting as described above.

Papain and dithiothreitol (DTT) treatment

Transfected cells were incubated in 4 mM EGTA/TBS for 30 min at 37 °C and harvested by centrifugation at 450 *g* for 10 min. Cell pellets were resuspended in 10 mM Pipes, pH 6.8, and 20 μ g of β -mercaptoethanol-activated papain was added. After a 60 min incubation at 37 °C in an end-over-end shaker, 10 mM iodoacetamide was added to stop the reaction. Treated cells were centrifuged for 20 min in an Eppendorf microcentrifuge (Brinkmann, Mississauga, Ont., Canada). Supernatants and solubilized cell extracts were then analysed by SDS/PAGE and immunoblotting as described above. The procedure was similar for DTT (10 mM) treatment, except that incubations were performed for 30 min in TBS at 25 °C.

Enzyme assay

E-24.18 activity was measured by monitoring the hydrolysis of azocasein to dye-containing peptides that were soluble in 4% (w/v) trichloroacetic acid [31]. Reactions were performed in 0.15 M NaCl/20 mM ethanolamine buffer, pH 9.5 [1]. Briefly, 35 μ l of cell extract or 60 μ l of concentrated culture medium was incubated for 60 min at 37 °C with or without 10 μ g of trypsin in a final volume of 100 μ l. Then 100 μ l of a mixture of protease inhibitors (1 μ M captopril, 1 μ M phosphoramidon, 10 μ M bestatin, 10 μ g/ml aprotinin and 20 μ M leupeptin) was added to the samples and incubated for 20 min under the same conditions. Finally, 900 μ l of buffer containing 11 mg of azocasein was added. After 90 min at 37 °C, a 250 μ l sample was removed and mixed with 1 ml of 4% trichloroacetic acid. Samples were centrifuged in an Eppendorf microcentrifuge for 5 min, and the A_{340} of the supernatant fraction was determined. In all experiments, azocasein degradation could be inhibited with 10 μ M actinonin, a strong inhibitor of E-24.18 (IC_{50} = 35 nM) [32]. Protein concentration was measured by the Bradford technique [33].

RESULTS

Expression of E-24.18 α and β subunits in COS-1 cells

E-24.18 α and β subunits were either expressed independently or co-expressed in COS-1 cells, and the recombinant proteins were detected by immunoblotting using either antibody L5 α , which is specific to the E-24.18 α subunit [23], or antibody RRT-151, which recognizes both subunits [10]. The expression of the α subunit in COS-1 cells has been studied previously [23] and is presented here for comparison purposes. Transfection of COS-1 cells with plasmid pSVP4- α resulted in cell-associated and secreted forms of the α subunit that were detected with both antibody L5 α (Figure 1b, lanes 3 and 4) and antibody RRT-151 (Figure 1a, lanes 3 and 4). The 98 and 82 kDa cell-associated forms were sensitive to digestion with endo H (Figure 1c, lane 2) and probably represented proteins present in the RER. The soluble forms ranged from 90 to 105 kDa and were mostly resistant to digestion with endo H (results not shown).

In extracts of COS-1 cells transfected with plasmid pSVP4- β , the RRT-151 polyclonal antibody mainly detected polypeptides of 105 and 115 kDa, but a faint band of 110 kDa was also

Figure 1 Immunoblotting of α and β subunits expressed in COS-1 cells

Proteins from extracts (C) or culture media (M) of transfected cells were separated by SDS/PAGE (7% acrylamide) under reducing conditions before nitrocellulose transfer and incubation with antibody RRT-151, recognizing both subunits (a), or L5 α , specific to the α subunit (b). Lanes: 1, 2, mock-transfected cells; 3, 4, α -transfected cells; 5, 6, β -transfected cells; 7, 8, α/β -co-expressing cells. (c) shows the results of endoglycosidase digestions performed as described in the Materials and methods section on extracts of proteins from α - (lanes 1–3), β - (lanes 4–6) or α/β - (lanes 7–9) expressing cells. Proteins in lanes 1–3 and 7–9 were revealed with antibody L5 α , and proteins in lanes 4–6 were revealed with antibody RRT-151. (—) corresponds to non-treated samples, and H and F indicate samples digested with endo H or endo F respectively. Dots correspond to the α subunit, and the downward-pointing arrow indicates the endo H-sensitive band in α/β -expressing cells. Positions of size markers are indicated on the left (in kDa).

observed (Figure 1a, lane 5). No significant amounts of the β subunit were detected in the culture medium (Figure 1a, lane 6). The β -subunit-related polypeptides were mostly resistant to digestion with endo H (Figure 1c, lane 5), suggesting that they had travelled through the Golgi apparatus and were present at the plasma membrane. In order to confirm the cell-surface localization, intact β -subunit-expressing cells were incubated with papain. This treatment led to the release of a 85 kDa species in the supernatant, supporting the latter hypothesis (Figure 2b,

Figure 2 Papain and DTT treatment of α/β - and β -transfected COS-1 cells

Intact cells expressing the α and β subunits (a and c) or the β subunit alone (b) were treated (+) or not (—) with papain (a and b) or DTT (c) as mentioned in the Materials and methods section. After centrifugation, the proteins in the pellet (P) or supernatant (S) were separated by SDS/PAGE (7% gel) and detected by immunoblotting with either antibody L5 α (a and c) or RRT-151 (b). Lanes: 1, 2, mock-transfected cells treated with papain; 3, 4, untreated transfected cells; 5, 6, papain-treated cells; 7, 8, supernatants of α/β -transfected cells treated or not with DTT. Positions of size markers are indicated on the left (in kDa).

lane 6). No β subunit could be detected in the supernatant of control cells (Figure 2b, lane 4). It should be noted that the electrophoretic pattern of the β -subunit-related proteins present in the pellet of control and treated cells (Figure 2b, lanes 3 and 5 respectively) is different from that observed with non-incubated cells (Figure 1a, lane 5). This could be due to the experimental conditions used, which may increase degradation of the β subunit. In support of this hypothesis, the β subunit has already been suggested to be less thermostable in papain-solubilized BBM than is the α subunit [11,34]. Endo F treatment of the cell-associated β subunit led to a decrease from a diffuse band of 105/115 kDa to two bands of 85/95 kDa (Figure 1c, lane 6), as expected from the presence of eight N-glycosylation sites predicted by the protein sequence [11].

In extracts of α/β -co-transfected cells, antibody RRT-151 detected five bands of 82, 85, 98, 110 and 115 kDa (Figure 1a, lane 7). Immunoblotting analysis of the same extracts using the α -subunit-specific antibody L5 α (Figure 1b, lane 7) showed that the 82, 85 and 98 kDa polypeptides (indicated by dots) could be attributed to the α subunit. It is noteworthy that the intermediate and more intense 85 kDa band was not observed in cells expressing the α subunit alone, and could result from a different maturation of the α subunit when expressed with the β subunit. The 110 and 115 kDa immunoreactive proteins, detected only with antibody RRT-151, correspond to polypeptides related to the β subunit. An increased expression of the 110 kDa protein was observed in α/β -co-transfected cells as compared with extracts of cells expressing the β subunit alone, also suggesting a different maturation of the β subunit when co-expressed with the α subunit. The β -specific 105 kDa band found in cells expressing the β -subunit is not observed in cells expressing both subunits. This could be explained by a different processing of this species, or by the presence of a more intense α -specific 98 kDa species which would mask the 105 kDa band. In contrast with the observation made previously with α -transfected cells, very small amounts of α subunit were detected in the culture medium of cells transfected with both subunits (Figures 1a and 1b, lane 8).

Figure 3 Oligomerization of α and β subunits in COS-1 cells

Proteins from extracts of transfected cells or from BBM were analysed by SDS/PAGE (5% acrylamide gel) as described in the legend to Figure 1, except that non-reducing conditions were used. Proteins were detected by immunoblotting with either RRT-151 (**a**) or L5 α (**b**) antiserum. Lanes: 1, mock-transfected cells; 2, α -transfected cells; 3, β -transfected cells; 4, α/β -transfected cells; 5, solubilized rat BBM. Positions of size markers are indicated on the left (in kDa).

Moreover, the cell-associated α subunit found in extracts of COS-1 cells expressing both subunits was mostly resistant to digestion by endo H (Figure 1c, lane 8). These results are in agreement with the observation made on the α subunit isolated from BBM [3]. An additional form of the α subunit with a higher molecular mass (98 kDa, indicated by the downward-pointing arrow; Figure 1c, lane 8) was sensitive to endo H digestion. Taken together, these results suggest that the two α -subunit species with the lower molecular mass (82 and 85 kDa) have travelled through the Golgi apparatus, whereas the 98 kDa species was trapped in the RER.

To verify the presence of the α subunit on the cell surface of co-transfected cells, intact cells expressing both subunits were treated with papain. In this case the lower-molecular-mass α -subunit species were released from the cells (Figure 2a, lane 6); in contrast, the 98 kDa band was not, which is consistent with its presence in the RER. The 82 kDa band was only slightly detected in these experimental conditions. A similar treatment of intact cells expressing the α subunit alone failed to release cell-associated α subunits (results not shown). Taken together, these results show that the β subunit has allowed the delivery of the α subunit to the cell surface of co-transfected cells and prevented its secretion into the culture medium.

In rat BBM, E-24.18 is known to be present as disulphide-linked heterodimers [10,11]. In order to study the oligomerization of E-24.18 expressed in COS-1 cells, cell-associated proteins were analysed by SDS/PAGE under non-reducing conditions and immunoblotting (Figure 3). When expressed alone, the α subunit was found both as 190 kDa dimers and as monomers (Figures 3a and 3b, lane 2), whereas the β subunit was found mostly as monomers (Figure 3a, lane 3), although some homodimers of β subunits were occasionally observed with a molecular mass of 235 kDa (results not shown). When both the α and the β subunits were co-expressed in the same transfection experiment, a 210 kDa band was detected in cell extracts (Figures 3a and 3b, lane 4) in addition to the monomers (as mentioned in the Materials and methods section, the difference in staining intensity of monomers and dimers observed with RRT-151 and L5 α antibodies is probably due to differences in their specificity, as they were raised against different antigens). Small amounts of secreted α and α/β dimers could also be made visible by immunoblotting after prolonged staining of the blot (results not shown). Under the same conditions, two bands of 195 and 155 kDa were observed in detergent extracts of rat kidney BBM (Figures 3a and 3b, lane 5). The 195 kDa species observed in BBM extracts most probably corresponds to the 220 kDa α/β dimer found previously under the same conditions in rat BBM [10]. Although we have not analysed thoroughly the structure of the 210 kDa band found in COS-1 cell extracts, its behaviour in SDS/PAGE is consistent with a heterodimeric structure requiring disulphide bridges. Small differences in the mobility of the bands observed in COS-1 cells compared with the bands seen in BBM, as well as the presence of a 155 kDa species in BBM but not in COS-1 cell extracts, might be due to variations in the glycosylation pattern or proteolytic processing.

The results presented above suggest that E-24.18 is a heterodimer consisting of a soluble form of the α subunit linked by disulphide bridges to a membrane-anchored β subunit. To confirm this model, cells transfected with both cDNAs were treated with 10 mM DTT. This treatment released a 85 kDa form of the α subunit, similar to that observed after papain digestion (Figure 2c, lane 8). As a control, the same treatment applied to cells expressing the β subunit alone did not release this subunit (result not shown).

These observations strongly suggest that the putative C-terminal transmembrane domain of the α subunit is not involved in the anchoring of the heterodimer. To test this hypothesis further, we constructed deletion mutants of the α subunit (Figure 4a). In the first one ($\alpha\Delta H$) the cytosolic and putative transmembrane domains of the α subunit were deleted by introduction of a translation stop codon at position 720. In the second mutant ($\alpha\Delta EGF$) the same strategy was used to create a stop codon at position 673, thus deleting the cytosolic, transmembrane and EGF-like domains. When expressed alone in COS-1 cells, these mutants of the α subunits were normally secreted into the culture media as dimers (results not shown). In co-transfection experiments involving the simultaneous expression of shortened α subunits together with the β subunit, a 210 kDa complex with the same electrophoretic mobility as that found in cells transfected with the full-length α subunit was observed (Figure 4b, lanes 1–3). These data demonstrate that the C-terminal region of α subunit including the EGF domain is not needed for membrane anchoring of the polypeptide. Furthermore, this region is probably removed during normal processing of wild-type α subunit in COS-1 cells, as its deletion from the α subunit did not affect the molecular mass of the heterodimer. These results also suggest that the cysteine residue(s) involved in the dimerization are not present in this region.

Figure 4 Schematic illustration of mutated α -subunit proteins and their co-expression with the β subunit

(a) Rat E-24.18 α subunit, encoded by the full-length cDNA, has a modular architecture with a signal peptide, pro-region, astacin-protease domain, MAM (meprin, A5, mw) domain, EGF-like domain, a putative transmembrane domain (TM) and a cytosolic domain (CYT). Key: α , wild-type α -subunit; $\alpha\Delta H$, TGA stop codon was introduced at position 720 [leucine (TTG)]; $\alpha\Delta EGF$, TGA stop codon was introduced at position 673 [arginine (AGA)]. (b) Proteins from extracts of transfected cells were analysed by SDS/PAGE (5%-acrylamide gel) as described in the legend to Figure 1, except that non-reducing conditions were used. Proteins were detected by immunoblotting with L5 α antiserum. Lanes: 1, α/β -transfected cells; 2, $\alpha\Delta H/\beta$ -transfected cells; 3, $\alpha\Delta EGF/\beta$ -transfected cells; 4, α -transfected cells. The apparent molecular mass of the size marker is indicated on the left (in kDa) and the position of α dimers at the right.

Enzymic activity of E-24.18 subunits

Proteolytic activity of recombinant E-24.18 was investigated with azocasein as substrate and was expressed as units/min per mg of protein, one unit corresponding to the degradation of 1.1 μ g of azocasein (Figures 5a and 5b). The enzymic activities detected in transfected COS-1 cell extracts were quite low (less than 5 units/min per mg of protein), compared with that found in BBM (37.6 units/min per mg of protein) (Figure 5a, white boxes). Nevertheless, a significant difference could be observed for β -expressing cells ($P < 0.001$, by Student's *t* test) as compared with mock-transfected cells. However, the rodent E-24.18 has been shown previously to be activated by trypsin [9]; therefore, transfected cells were preincubated for 1 h at 37 °C in the presence of 10 μ g of trypsin before testing for azocasein-degrading activity. In β - and α/β -expressing cells, this treatment resulted in a 15–20-fold increase in activity, which was raised to a level comparable with that found in BBM (Figure 5a, hatched boxes). As observed previously [23], a low azocasein-degrading activity was found in α -expressing cells after trypsin treatment (about 13.2 units/min per mg of protein). In the spent medium, there was no detectable activity before trypsin activation (Figure 5b, white boxes). Trypsin activation, however, revealed a signifi-

Figure 5 Enzymic activity of transfected cells

Proteins from the extracts (a) or the culture medium (b) of transfected cells were assayed for azocasein degradation activity with (▨) or without (□) trypsin activation. Specific activities are expressed as units/min per mg of protein. Solubilized rat BBM were used as a positive control. The results are means of three separate experiments carried out in duplicate, with the S.E.M. indicated by error bars: * $P < 0.005$, ** $P < 0.001$ as compared with mock-transfected cells by Student's *t* test.

cant azocasein-degrading activity in α -transfected-cell spent medium (Figure 5b, hatched boxes). A slight, but not significant, increase in activity was also measured in media from β - and α/β -expressing cells and is consistent with the detection of small amounts of soluble forms in the spent medium by immunoblotting.

To determine the contribution of each subunit in the enzymic activity of a recombinant E-24.18, a mutation was introduced at the position of Glu-157 in the consensus sequence of the astacin domain of the α subunit [15]. When expressed alone in COS-1 cells, the $\alpha E157V$ mutant was normally secreted and formed dimers exactly like the unmutated α subunit; this mutant, however, was devoid of any enzymic activity, even after trypsin treatment [23]. In co-expression experiments, heterodimers containing the $\alpha E157V$ mutant had an azocasein-degrading activity (measured after trypsin treatment) which was 40 % lower than the normal enzyme (from 49 to 30.3 units/min per mg of protein) (Figure 5a). This result suggests that α and β subunits are both involved in catalysis in the assay used for measuring the enzymic activity.

DISCUSSION

The α and β subunits of E-24.18 are putative type I membrane proteins that share significant similarities at the primary-structural level. However, expression of these proteins in COS-1 cells revealed differences in their biosynthesis. As observed previously [23], expression of the α subunit resulted in the secretion of fully glycosylated polypeptides, suggesting that the protein had reached the cell surface through the exocytic pathway. Thus the C-terminal hydrophobic domain initially thought to serve as the membrane anchor was either removed or not used in COS-1 cells. A significant proportion of secreted α subunits formed

disulphide-linked dimers. The α subunits detected in cell extracts carried mostly endo H-sensitive oligosaccharides and were therefore thought to be retained in the RER rather than present at the cell surface. In contrast, β subunits were expressed at the cell surface of transfected COS-1 cells. This assertion is mainly based on the presence in cell extracts of endo H-resistant forms of β subunits which could be released in the supernatant upon treatment of intact cells with papain. Very little dimerization of the β subunit was observed. This behaviour of the rat enzyme is different from that of its mouse homologue, since covalent tetramers of mouse β subunits have been described by Gorbea and co-workers [14].

When α and β subunits were expressed together in COS-1 cells, major changes were observed in the structure and biosynthesis of the α subunit. Very little α subunit was detected in the culture medium, whereas an endo H-resistant 85 kDa polypeptide was found to be cell-associated and could be released into the supernatant by papain treatment. This protein may correspond to the 82 kDa form of the α subunit detected in BBM under similar conditions [3,10,11]. Under non-reducing conditions a protein stained with both L5 α and RRT-151 antibodies was found in cell extracts. The molecular mass of this protein was 210 kDa, which is higher than that of the α_2 homodimers observed in the α -transfected cells. This is consistent with the formation of α/β heterodimers. Although we cannot completely exclude the formation of a complex of two larger forms of α subunits, this appears very unlikely, for two reasons: (i) no forms of the α subunit with a molecular mass higher than 98 kDa have been observed in co-transfected experiments; (ii) the α subunit released from the cell surface using papain or reducing treatment has a molecular mass of about 85 kDa.

The presence of the α subunit at the plasma membrane in α/β -transfected cells could be explained in two ways. First, the presence of β subunits may restore the function of the putative C-terminal anchor domain of the α subunit. Alternatively, the α subunit could be retained at the cell membrane through interactions with the β subunit. This latter hypothesis is supported by the following observations: (i) papain treatment of intact cells released α/β dimers in the supernatant, with a decrease in the molecular mass of the β subunit, but not of the α subunit; (ii) treatment of intact co-transfected cells with 10 mM DTT released the α subunit, but not the β subunit, into the supernatant; (iii) the molecular masses of the α -subunit species released from the cell surface of intact co-transfected cells by papain or DTT treatments are identical; (iv) mutants of the α subunit in which the C-terminal putative anchor domain had been deleted could still form disulphide-linked dimers, which were found at the cell surface when co-expressed with the β subunit. Thus our observations suggest that the C-terminal putative anchor domain of the α subunit is removed by proteolysis in an unidentified compartment of the exocytic pathway. The presence of a consensus furin cleavage site at amino acids 652–655 (RPKR) [23] further supports this hypothesis. The α subunit of E-24.18 would be a new member of a group of membrane-bound proteins known to be converted into soluble forms by proteolytic cleavage of the transmembrane domain [35]. Such proteins include the receptor for transcellular transport of polymeric immunoglobulins [36], transforming growth factor- α [37], the α -amidation enzyme [38], angiotensin-converting enzyme [39,40] and the yeast KEX2 endopeptidase [41].

Maturation of the α and β subunits has not been extensively studied in the present work. However, it appears that both subunits are processed differently whether they are expressed alone or co-expressed in the same cells. Evidence for this is seen in the increased intensity of the α -specific 85 kDa species and of

Figure 6 Topological model of E-24.18 expressed in COS-1 cells

The model of E-24.18 topology is drawn schematically to scale: ■, transmembrane domains; ▨, astacin domains; ▩, putative pro-regions.

the β -specific 110 kDa band in cells expressing the α and β subunits. More work is clearly needed to understand the different maturation steps involved in the biosynthesis of active E-24.18.

The recombinant enzymes expressed in COS-1 cells showed little activity as compared with that of BBM. However, an azocasein-degrading activity could be revealed by mild trypsin digestion of cell extracts and culture media. Both subunits showed similar levels of activity upon trypsin activation, indicating that the astacin domain of each subunit is functional. The observation that the enzyme must be activated by mild trypsin digestion suggests that in COS-1 cells the enzyme is synthesized as an inactive precursor that needs proteolytic activation. Such proteolytic activation of pro-enzymes is known to be necessary for substrate degradation by matrix metallo-proteases [42] or trypsin-like serine proteases [43], and has also been suggested for the members of the astacin family [44]. Three-dimensional structural analysis of astacin and computer-assisted modelling of the α subunit of mouse E-24.18 indicated that the N-terminal residue of the mature protein (Leu-22 for the mouse protein) [19] is completely buried in a water-filled cavity linked to the active site [45]. Activation of the enzyme would require proteolysis of a few N-terminal residues, probably not removed in COS-1 cells. *In vivo*, this N-terminal processing could be achieved by an aminopeptidase which is tightly associated with E-24.18 in mouse kidney [46]. Both subunits appear to contribute to the overall azocasein-degrading activity of the heterodimer measured after trypsin activation, as suggested by the 40% decrease in azocasein-degradation activity observed by introducing a mutation in the active site of the subunit.

In conclusion, our results are consistent with a model in which a soluble α subunit is retained at the cell membrane through disulphide bridge(s) to a type I-anchored β subunit (Figure 6). A similar biosynthetic process has been documented for the Na⁺/K⁺-ATPase, whose β subunit is necessary for the assembly and transport of the sodium pump to the plasma membrane [17]. However, among ectopeptidases, this model has never been observed, and thus represents a new type of processing and anchoring.

We thank Dr. Steve Howell for critical reading of the manuscript and Dr. L. B. Hersh (Lexington, KY, U.S.A.) for generously providing the cDNA coding for the rat β subunit. This work was supported by a grant from the Medical Research Council of Canada to P.C. and G.B. G.B. was the recipient of a Chercheur-Boursier Award from

Le Fonds de la Recherche en Santé du Québec. D.C. was the recipient of a studentship from FCAR.

REFERENCES

- 1 Beynon, R. J., Shannon, J. D. and Bond, J. S. (1981) *Biochem. J.* **199**, 591–598
- 2 Bond, J. S. and Beynon R. J. (1986) *Curr. Top. Cell. Regul.* **28**, 263–290
- 3 Kenny, A. J. and Ingram, J. (1987) *Biochem. J.* **245**, 515–524
- 4 Kenny, A. J., Stephenson, S. L. and Turner, A. J. (1987) In *Research Monographs in Mammalian Ectoenzymes: Cell Surface Peptidases* (Kenny, A. J. and Turner, A. J., ed.), vol. 14, pp. 169–209, Elsevier/North-Holland Biomedical Press, Amsterdam
- 5 Stephenson, S. L. and Kenny, A. J. (1988) *Biochem. J.* **255**, 45–51
- 6 Woltz, R. L., Harris, R. B. and Bond, J. S. (1991) *Biochemistry* **30**, 8488–8493
- 7 Dumermuth, E., Sterchi, E. E., Jiang, W., Woltz, R. L., Bond, J. S., Flannery, A. V. and Beynon, R. J. (1991) *J. Biol. Chem.* **266**, 21381–21385
- 8 Rawlings, N. D. and Barrett, A. J. (1993) *Biochem. J.* **290**, 205–218
- 9 Macadam, G. C., Bond, J. S. and Beynon, R. J. (1990) *Int. J. Biochem.* **22**, 989–996
- 10 Barnes, K., Ingram, J. and Kenny, A. J. (1989) *Biochem. J.* **264**, 335–346
- 11 Johnson, G. D. and Hersh, L. B. (1992) *J. Biol. Chem.* **267**, 13505–13512
- 12 Sterchi, E. E., Naim, H. Y., Lentze, M. J., Hauri, H.-P. and Fransen, J. A. (1988) *Arch. Biochem. Biophys.* **265**, 105–118
- 13 Sterchi, E. E., Naim, H. Y. and Lentze, M. J. (1988) *Arch. Biochem. Biophys.* **265**, 119–127
- 14 Gorbea, C. M., Flannery, A. V. and Bond, J. S. (1991) *Arch. Biochem. Biophys.* **290**, 549–553
- 15 Corbeil, D., Gaudoux, F., Wainwright, S., Ingram, J., Kenny, A. J., Boileau, G. and Crine, P. (1992) *FEBS Lett.* **309**, 203–208
- 16 Johnson, G. D. and Hersh, L. B. (1993) *J. Biol. Chem.* **268**, 17647
- 17 Beckman, G. and Bork, P. (1993) *Trends Biochem. Sci.* **18**, 40–41
- 18 Jiang, W., Gorbea, C. M., Flannery, A. V., Beynon, R. J., Grant, G. A. and Bond, J. S. (1992) *J. Biol. Chem.* **267**, 9185–9193
- 19 Gorbea, C. M., Marchand, P., Jiang, W., Copeland, N. G., Gilbert, D. J., Jenkins, N. A. and Bond, J. S. (1993) *J. Biol. Chem.* **268**, 21035–21043
- 20 Geering, K., Theulax, I., Verrey, F., Hauptle, M. T. and Rossier, B. C. (1989) *Am. J. Physiol.* **257**, C851–C858
- 21 Aretz, W., and Sauber, K. (1990) *Ann. N.Y. Acad. Sci.* **613**, 643–646
- 22 Reiss, Y., Seabra, M. C., Armstrong, S. A., Slaughter, C. A., Goldstein, J. L. and Brown, M. S. (1991) *J. Biol. Chem.* **266**, 10672–10677
- 23 Corbeil, D., Milhiet, P. E., Simon, V., Ingram, J., Kenny, A. J., Boileau, G. and Crine, P. (1993) *FEBS Lett.* **335**, 361–366
- 24 Gomez, S., Boileau, G., Zollinger, L., Nault, C., Rholam, M. and Cohen, P. (1989) *EMBO J.* **8**, 2911–2916
- 25 Ho, S. N., Hunt, H. D., Horton, R. M., Pullen, J. K. and Pease, L. R. (1989) *Gene* **77**, 51–59
- 26 Tabor, S. and Richardson, C. C. (1987) *Proc. Natl. Acad. Sci. U.S.A.* **84**, 4767–4771
- 27 Gluzman, Y. (1981) *Cell* **23**, 175–182
- 28 Noël, G., Zollinger, L., Larivière, N., Nault, C., Crine, P. and Boileau, G. (1987) *J. Biol. Chem.* **262**, 1876–1881
- 29 Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- 30 Aubry, A., Berteloot, A., Beaumont, A., Roques, B. P. and Crine, P. (1987) *Biochem. Cell. Biol.* **65**, 398–404
- 31 Beynon, R. J. and Kay, J. (1978) *Biochem. J.* **173**, 291–298
- 32 Choudry, Y. and Kenny, A. J. (1991) *Biochem. J.* **280**, 57–60
- 33 Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254
- 34 Kounnas, M. Z., Woltz, R. L., Gorbea, C. M. and Bond, J. S. (1991) *J. Biol. Chem.* **266**, 17350–17357
- 35 Ehlers, M. R. W. and Riordan, J. F. (1991) *Biochemistry* **30**, 10065–10074
- 36 Mostov, K. and Blodel, G. (1982) *J. Biol. Chem.* **257**, 11816–11821
- 37 Derynck, R. (1986) *J. Cell. Biochem.* **32**, 293–304
- 38 Beaudry, G. A. and Bertelsen, A. H. (1989) *Biochem. Biophys. Res. Commun.* **163**, 959–966
- 39 Ehlers, M. R. W., Chen, Y.-N. P. and Riordan, J. F. (1991) *Proc. Natl. Acad. Sci. U.S.A.* **88**, 1009–1013
- 40 Wei, L., Alhenc-Gelas, F., Soubrier, F., Michaud, A., Corvol, P. and Clauser, E. (1991) *J. Biol. Chem.* **266**, 5540–5546
- 41 Germain, D., Dumas, F., Vernet, T., Bourbonnais, Y., Thomas, D. Y. and Boileau, G. (1992) *FEBS Lett.* **299**, 283–286
- 42 Woessner, J. F., Jr. (1991) *Eur. J. Biochem.* **214**, 215–231
- 43 Huber, R. and Bode, W. (1978) *Acc. Chem. Res.* **11**, 114–122
- 44 Bode, W., Gomis-Rüth, F. X., Huber, R., Zwilling, R. and Stöcker, W. (1992) *Nature (London)* **358**, 164–167
- 45 Stöcker, W., Gomis-Rüth, F. X., Bode, W. and Zwilling, R. (1993) *Eur. J. Biochem.* **214**, 215–231
- 46 Butler, P. E. and Bond, J. S. (1988) *J. Biol. Chem.* **263**, 13419–13426