Proteolytic processing of the α -subunit of rat endopeptidase-24.18 by furin

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Endopeptidase-24.18 (EC 3.4.24.18; meprin) is a multisubunit metalloproteinase of the astacin family. It is found in brush-border membranes of rodent kidney and human intestine. The membrane-bound enzyme is composed of α/β dimers. Molecular cloning has shown that both subunits have a similar structural domain organization. Soluble α_2 dimers have also been observed *in vivo* and in transfected cells. The structures of all known α -subunits contain, upstream from the transmembrane domain, the sequence RXXR, which corresponds to the RXX/RR consensus sequence for specific cleavage by furin. In order to investigate the involvement of this putative cleavage site in the secretion process of endopeptidase-24.18 α -subunit, we expressed in COS-1 cells rat α -subunits in which residues R⁶⁵⁵ or S⁶⁵⁶ (within the sequence R⁶⁵²PKRS⁶⁵⁶) were mutated to valine or leucine respectively. In contrast to the wild-type protein, the

α R655V and α S656L mutants were not secreted in the culture medium. Moreover, when cells expressing the α -subunit were infected with a furin-encoding vaccinia virus, immunoblotting showed a shift of the major cell-associated form of endopeptidase-24.18 α -subunit from 98 kDa to 85 kDa and an increase in the amounts of secreted α -subunit. This shift in molecular mass was not observed with the mutant α -subunits. As observed for the 98 kDa species, the 85 kDa cell-associated protein was sensitive to endoglycosidase H treatment, suggesting that the proteolytic cleavage occurred in the endoplasmic reticulum or in an early Golgi compartment. Similar experiments using PACE4 and PC5 instead of furin showed that these enzymes were not able to generate the 85 kDa species. We conclude that furin is most probably the cellular enzyme involved in the proteolysis resulting in secretion of rat endopeptidase-24.18 α -subunit.

INTRODUCTION

Endopeptidase-24.18 (EC 3.4.24.18, meprin, endopeptidase-2, PABA-peptide hydrolase, E-24.18) is a zinc-metalloproteinase expressed in the brush-border membranes of adult rodent kidney [1,2] and human intestine [3,4]. E-24.18 is a member of the astacin family of proteases which includes several developmentally regulated proteins. Consistent with the role of these molecules in development, E-24.18 was recently observed in several regions of the embryonic rat brain [5]. The proteins of the astacin family are characterized by the presence of the consensus sequence HEXXHXXGFXHE (X is any amino acid) in their active site [6]. *In vitro*, E-24.18 can hydrolyse a variety of peptides including bradykinin, substance P and luteinizing hormone-releasing hormone [7–9]. More recently E-24.18 has been shown to be able to degrade several proteins of the extracellular matrix including laminin, type-IV collagen and fibronectin [10].

E-24.18 is one of two known mammalian ectopeptidases possessing a heteromeric structure [2,11]. The functional unit of rat and mouse E-24.18 seems to be disulphide-linked α/β dimers which can then associate through non-covalent interactions to form tetramers, $(\alpha/\beta)_2$ [12–14]. In the case of the mouse E-24.18, the existence of α_2 dimers non-covalently associated with α/β dimers to form tetramers has been postulated [14]. The rat α - and β -subunits have been cloned [12,15] and predicted to be anchored in the membrane by a putative C-terminal hydrophobic domain. However, when expressed in heterologous COS-1 cells [16] or human kidney 293 cells [17], the α -subunit was found to be mostly secreted into the culture medium and no mature protein was detected at the cell surface. Similar results were reported with a mouse/human hybrid α -subunit expressed in COS-1 cells

[18]. Expression of both α - and β -subunits in the same cells resulted, however, in retention of the α -subunit at the cell surface through disulphide linkage(s) to the membrane-anchored β -subunit [17,19]. It was then suggested that the solubilization of the α -subunit observed in α -subunit-expressing cells resulted from the proteolytic cleavage of the putative transmembrane domain. Consistent with this hypothesis, Marchand et al. [14] have recently shown that in mouse kidney brush-border membranes the α -subunit contains neither the putative C-terminal transmembrane domain nor the epidermal growth factor (EGF)-like domain (see Figure 1) predicted from the α -subunit cDNA structure. Thus the proteolytic cleavage responsible for the secretion of the α -subunit should occur at a site located upstream from the EGF-like domain (Figure 1). We have observed previously the presence of the sequence RPKR located 16 amino acid residues upstream from the EGF-like domain of rat E-24.18 α -subunit [16]. This sequence conforms to the furin consensus cleavage site (RXX/RR) [20] and is present in all E-24.18 α -subunits characterized so far (Figure 1). We proposed then that it could be the site of proteolysis resulting in the secretion of the α -subunit [16]. Furin is an ubiquitously expressed serine protease with a subtilisin-like catalytic domain which cleaves a wide range of precursor proteins in the exocytic pathway [21,22].

In the present study we have examined the involvement of this consensus furin site in the solubilization of the α -subunit of rat E-24.18. Our results show that mutation of the RPKR furin site prevents solubilization of the E-24.18 α -subunit and that furin is most probably the cellular enzyme performing the proteolysis. Furthermore, we suggest that the cleavage occurs either in the endoplasmic reticulum or in an early Golgi compartment.

Abbreviations used: E-24.18, endopeptidase-24.18 (EC 3.4.24.18); EGF, epidermal growth factor; endo F, endo- α -N-acetylglucosaminidase F; endo H, endo- β -N-acetylglucosaminidase H; MAM, protein domain found in Mekrin, A5 protein and receptor protein tyrosine phosphatase Mu (μ); POMC, pro-opiomelanocortin; PPH, N-benzoyl-L-tyrosyl-p-aminobenzoic acid hydrolase; RER, rough endoplasmic reticulum; TBS, Tris-buffered saline.

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Figure 1 Schematic illustration of α - and β -subunits of E-24.18

α - and β -subunits of rat E-24.18 have a modular architecture with, from the N- to the C-terminus, a signal peptide, a pro-region, an astacin-like catalytic domain, a MAM domain (see the Abbreviations footnote), an EGF-like domain, a transmembrane domain (TM) and a cytosolic domain (CYT). The region of the α -subunit containing the furin site (boxed) and the sequence alignment between species are presented.

MATERIALS AND METHODS

Site-directed mutagenesis and plasmid construction

The α -subunit expression vector pSVP4- α has been described previously [16,19]. For site-directed mutagenesis a 1153 bp *Bam*HI–*Hind*III DNA fragment from the α -subunit of rat E-24.18 was subcloned in M13mp18 RF DNA. This fragment contains the region encoding the furin site. Site-directed mutagenesis was performed on single-stranded recombinant M13 DNA according to the method described by Taylor et al. [23] using a Sculptor kit purchased from Amersham Inc. Oligodeoxyribonucleotides for mutagenesis were synthesized by the phosphoramidite procedure using a Pharmacia Gene Assembler. Recombinant M13 phages carrying the mutations were screened directly by sequencing [24]. A 951 bp *Xmn*I–*Sca*I DNA fragment containing the mutation was substituted for the wild-type fragment in pSVP4- α . The presence of the mutations was confirmed by double-stranded DNA sequencing [25] on the resulting plasmids.

Transfection of COS-1 cells

Transfection of COS-1 cells has been described in detail previously [26]. Medium supplemented with fetal 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 using Centrprep 30 membranes (Amicon, Ont., Canada). Transfected cells were scraped in ice-cold PBS (137 mM NaCl, 2.7 mM KCl, 7.3 mM Na₂HPO₄, 1.8 mM KH₂PO₄, pH 7.4), centrifuged and membrane proteins were solubilized using 2% (w/v) *N*-octyl β -D-glyco-

pyranoside (octyl glucoside) in Tris-buffered saline (TBS; 50 mM Tris/HCl, 150 mM NaCl, pH 7.4).

Co-expression of E-24.18 α -subunit with furin, PACE4 or PC5

The construction of the vaccinia recombinant viruses expressing the human furin, human PACE4 or mouse pro-opiomelanocortin (POMC) have been described previously [27,28]. Construction of vaccinia recombinant virus expressing mouse PC5 was performed essentially as described for the other viruses and is reported in Benjannet et al. [29]. Both PACE4 and PC5 were shown to be active in the processing of pro7B2 [29]. The infection of transfected COS-1 cells (see above) was performed at a multiplicity of infection of 1 plaque-forming unit/cell, 30 h after transfection. Cells were first washed with PBS and infected during 20 min at room temperature with viral particles diluted in the same buffer. Cells were then washed with PBS and incubated for 17 h in the synthetic medium described above. Harvesting was performed as described above for transfected cells.

Immunoblotting procedure and enzymic assay

Proteins from cell extracts or concentrated culture media were separated by SDS/PAGE [30] and transferred to nitrocellulose membranes (0.45 μ m pore size) as described previously [31]. E-24.18 proteins were then detected using the polyclonal antibody L5 α which specifically recognizes the α -subunit [16]. Biotinylated immunoglobulins (Ig) against rabbit IgG were used to reveal antigen–antibody complexes (Vectastain ABC-Immunoperoxidase kit; Vector Laboratories, Burlingame, CA, U.S.A.). E-24.18 activity was measured by monitoring the hydrolysis of azocasein into dye-containing peptides that were soluble in 4% (w/v) trichloroacetic acid [32], exactly as described previously [19]. Specific activities are expressed as units/min per mg of

protein, one unit corresponding to the degradation of 1.1 μ g of azocasein. Protein concentrations were measured using the Bradford technique [33].

Endoglycosidase digestions

Proteins recovered from octyl glucoside solubilization of COS-1 cells or from concentrated media were treated with 10 m-units of endo- β -*N*-acetylglucosaminidase H (endo H) or 0.5 unit of endo- α -*N*-acetylglucosaminidase F (endo F) (Boehringer Mannheim) for 18 h at 37 °C according to the manufacturer's protocol. Control samples were incubated in the same conditions but without enzyme. Proteins were analysed by immunoblotting as described above.

Computer analysis of protein sequences

For all computer-assisted analysis of protein sequences the PC GENE software package was used. Sequence alignment was performed using the Dayhoff MDM-78 matrix method.

RESULTS

Mutation of the furin site

The α - and β -subunits of rat E-24.18 display the same domain organization: this includes the catalytic astacin-like, MAM (see Abbreviations footnote) [34], EGF-like, transmembrane and cytosolic domains (Figure 1). The protein sequence is also well conserved between both subunits (39% identity and 52.5% similarity) except for a segment located immediately upstream of the EGF-like domain. A stretch of 55 amino acid residues is found in the α -subunit but is absent in the β -subunit. The putative furin cleavage site is located in this region of the α -subunit and is conserved among the different species already cloned (boxed in Figure 1; RPKR in the rat and RQKR in the human and mouse species).

The presence of a furin cleavage site in the α -subunit and its absence in the β -subunit could explain the solubilization of the former and the membrane-bound nature of the latter. We consequently proceeded to modify the consensus furin site of the α -subunit of rat E-24.18 by site-directed mutagenesis. Arg in the P₁ position has been shown to be strictly necessary for furin cleavage [35]. Thus Arg⁶⁵⁵ located in P₁ of the putative furin-cleavage site of the α -subunit (Figure 2a) was replaced by a valine (α R655V mutant). Mutation of the serine residue in P'₁ to give an aliphatic amino acid considerably reduced cleavage by furin, albeit to a lesser extent than mutations at the P₁ site [35]. Thus, in another mutant, the serine in P'₁ was replaced by a leucine (α S656L mutant).

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

The non-mutated and mutated α -subunits of rat E-24.18 were expressed by transfecting the corresponding cDNAs in COS-1 cells. As previously observed [16,19] the cell-associated non-mutated α -subunit migrated mostly as a 98 kDa band (Figure 2b, lane 2). Faint bands of 82 and 85 kDa were also observed by immunoblotting. The secreted immunoreactive species found in spent culture medium migrated as a broad band of approximately 100 kDa (Figure 2c, lane 2).

In extracts of COS-1 cells expressing α R655V and α S656L mutants, a major band with a mobility identical to that of the

Figure 2 Involvement of the furin site in α -subunit secretion

(a) The P₁ (Arg) and P'₁ (Ser) residues of the furin motif (underlined) were replaced by Val and Leu respectively (α R655V and α S656L mutants). (b) and (c) Proteins from cell extracts (b) or media (c) of transfected cells were analysed by SDS/PAGE (7.5% acrylamide gel) under reducing conditions before nitrocellulose transfer and incubation with L5 α polyclonal antibody. Lane 1, mock-transfected cells; lane 2, α -transfected cells (α WT); lane 3, α R655V-expressing cells; lane 4, α S656L-expressing cells. Positions of molecular mass markers are indicated on the left.

major band observed for non-mutated α -subunit was detected (98 kDa band: Figure 2b, lanes 3 and 4). No immunoreactive material of α R655V or α S656L mutants could be detected in the spent culture medium (Figure 2c, lanes 3 and 4). These results indicate that mutations in the consensus furin cleavage site interfere with the normal processing pathway of the α -subunit and prevent secretion of the protein.

Enzymic activity of non-mutated and mutated E-24.18 α -subunits

It cannot be ruled out at this point that the mutations introduced in the E-24.18 α -subunit interfered with proper folding of the protein, resulting in the retention of the enzyme in the rough endoplasmic reticulum (RER). To answer this question we determined the enzymic activity of the cell-associated forms of the wild-type and mutant α -subunits. As previously observed for the α - and β -subunits of E-24.18 [17,19], under standard conditions (without trypsin treatment) no substrate degradation could be detected for either the wild-type enzyme or the mutants (Table 1). However, preincubation in the presence of trypsin resulted in the activation of the proteins. Under these conditions both mutants showed enzymic activities similar to that of the wild-type α -subunit (Table 1), suggesting that the mutations introduced at the furin site do not interfere with the normal folding of the enzyme.

Table 1 Enzymic activity of α R655V and α S656L mutants

Cell-associated proteins from transfected COS-1 cells were assayed for azocasein degradation with or without trypsin activation (10 μ g/ml) in 0.15 M NaCl/20 mM ethanolamine, pH 9.5, as described in the Materials and methods section. Specific activities are expressed as units/min per mg of protein. The results are the means of three separate experiments carried out in duplicate and the standard errors are indicated.

Cell extracts	Enzymic activities (units/min per mg of protein)	
	– Trypsin	+ Trypsin
Mock	0.10 $\pm$ 0.10	0.00 $\pm$ 0.21
α WT	1.31 $\pm$ 0.11	6.35 $\pm$ 0.42
α R655V	1.43 $\pm$ 0.16	10.80 $\pm$ 0.65
α S656L	0.90 $\pm$ 0.11	9.69 $\pm$ 0.72

Figure 3 Co-expression of E-24.18 α -subunit with furin

Proteins from cell extracts (a) or media (b) of transfected and subsequently infected cells (see the Materials and methods section) were analysed by SDS/PAGE and immunoblotting as described in Figure 2. Lane 1, mock-transfected cells infected with the furin-expressing virus; lane 2, α WT-transfected cells infected with POMC-expressing virus; lanes 3, 4 and 5, α WT-, α R655V- and α S656L-transfected cells infected with the furin-expressing virus, respectively. Positions of molecular mass markers are indicated on the left.

Overexpression of furin in COS-1 cells expressing non-mutated or mutated E-24.18 α -subunits

In order to investigate further the role of furin in the solubilization of E-24.18 α -subunit, cultures of COS-1 cells transfected with vector pSVP4- α containing the cDNA for the wild-type α -subunit or vectors encoding the α R655V or α S656L mutants were subsequently infected with a recombinant vaccinia virus encoding the furin cDNA. A POMC-expressing recombinant vaccinia virus was used as a control. Immunoblots from pSVP4- α -transfected COS-1 cells, infected with the POMC-expressing virus, showed a pattern similar to that previously observed in simple transfections. The cell-associated and secreted proteins were mainly expressed as 98 kDa and 100 kDa bands respectively (Figures 3a and 3b, lane 2). However, when pSVP4- α -transfected cells were infected with the furin-expressing virus the cell-associated proteins shifted from 98 kDa to 85 kDa (Figure 3a, lane 3). This difference of 13 kDa between the 98 and 85 kDa species is consistent with cleavage within the furin site, which would remove a peptide of 10602 Da. In the spent culture medium, an increase in the intensity of the 100 kDa band was observed (Figure 3b, lane 3). When the α R655V and α S656L mutants were co-expressed with furin, no intracellular processing was observed (Figure 3a, lanes 4 and 5 respectively). No protein related to the α -subunit could be observed in the culture medium

Figure 4 Endoglycosidase treatment of non-mutated and mutated α -subunits co-expressed with furin

Proteins from cell extracts (a) or media (b) of transfected and subsequently infected cells were treated with either endo H or endo F as described in the Materials and methods section, before analysis by SDS/PAGE and immunoblotting as described in the legend to Figure 2. Lanes 1–3, mock-transfected cells infected with furin-expressing virus; lanes 4–6, α WT-transfected cells infected with POMC-expressing virus; lanes 7–9, α WT-transfected cells infected with a furin-expressing virus; lanes 10–12, α R655V-transfected cells infected with a furin-expressing virus; lanes 13–15, α S656L-transfected cells infected with a furin-expressing virus. The samples were either untreated (–) or digested with endo H (H) or endo F (F). Positions of molecular mass markers are indicated on the left.

of cells expressing the α R655V mutant (Figure 3b, lane 4). For the α S656L mutant, however, small amounts of immunoreactive protein could be detected in the culture medium (Figure 3b, lane 5). This result is consistent with the previous observation that the S₁ subsite appears to tolerate less changes than the S₁' subsite and that an aliphatic amino acid residue at the P₁' position is not well tolerated by furin [35,36].

Taken together, these results support the hypothesis that exogenous furin expressed in cells previously transfected with an α -subunit expression vector is capable of processing the recombinant α -subunit. Cleavage most probably occurs at the predicted consensus site, as mutations introduced at this site interfere with the processing of the recombinant α -subunit. These results are also consistent with the hypothesis that cleavage by furin must occur to allow secretion of the protein.

Cellular localization of the proteolytic event

In order to obtain information on the intracellular compartment where cleavage of the rat E-24.18 α -subunit polypeptide occurs, intracellular and secreted proteins from α -subunit-expressing COS-1 cells infected with vaccinia viruses were treated with endo H and endo F, and analysed by immunoblotting with the α -subunit-specific antibody L5 α . Cell-associated 98 kDa wild-type α -subunits were sensitive to digestion by endo H and endo F (Figure 4a, lanes 4 to 6), as observed previously in the absence of infection by vaccinia virus [19]. This indicates that these proteins had not reached the Golgi apparatus where complex oligosaccharides responsible for resistance to endo H digestion are acquired. When pSVP4- α -transfected COS-1 cells were infected with the furin-expressing virus, the 85 kDa immunoreactive material resulting from the processing of the 98 kDa precursor was also sensitive to endo H treatment (Figure 4a, lanes 7 to 9). The cell-associated 98 kDa band corresponding to α -subunit mutants, α R655V and α S656L, was also sensitive to

Figure 5 Co-expression of E-24.18 α -subunit with PACE4 or PC5

Proteins from cell extracts of transfected and subsequently infected cells (see the Materials and methods section) were analysed by SDS/PAGE and immunoblotting as described in Figure 2. Lane 1, mock-transfected cells infected with the furin-expressing virus; lanes 2, 3, 4 and 5 show α WT-transfected cells subsequently infected with POMC-, furin-, PACE4- and PC5-expressing viruses, respectively. Positions of molecular mass markers are indicated on the left.

endo H digestion (Figure 4a, lanes 10 to 12 and lanes 13 to 15). Proteins recovered in the culture medium of COS-1 cells transfected with pSVP4- α and infected with either POMC- or furin-expressing vaccinia virus were mostly resistant to endo H digestion (Figure 4b, lanes 4 to 6 and 7 to 9, respectively), suggesting that these soluble species have been secreted through the exocytic pathway. These results suggest that the full-length enzyme precursor, probably anchored as a type-I membrane protein, is retained in an early compartment of the exocytic pathway. Thus cleavage at the furin site appears to occur in the RER or an early Golgi compartment.

Cleavage of E-24.18 α -subunit by other furin-like enzymes

At least two convertases, other than furin, that process protein precursors in the constitutive exocytic pathway have been characterized: PACE4 [37] and PC5 [38]. In order to test the possibility that the furin-like enzymes PACE4 or PC5 may solubilize the E-24.18 α -subunit, COS-1 cells transfected with pSVP4- α were infected with recombinant vaccinia viruses carrying the furin, PACE4 or PC5 cDNAs. As shown in Figure 5, furin can process the 98 kDa intracellular species into the 85 kDa form (lane 3) but PACE4 (lane 4) or PC5 (lane 5) cannot.

DISCUSSION

We observed previously in the α -subunit of E-24.18 the conservation between species of the sequence RXKR, which corresponds to a consensus furin cleavage site upstream from the EGF-like domain. Interestingly, the presence of proline residues in P_6 or P_3 of the cleavage site was also observed by Matthews et al. [39] in a survey of furin substrate specificity using substrate phage display. Furthermore, computer analysis of the protein sequence around this putative furin cleavage site revealed a high propensity for a β -turn structure immediately upstream. Such structures are required for efficient cleavage by furin [40]. These considerations prompted us to analyse the role of this sequence in the secretion of the α -subunit observed in different systems [16,18]. The results presented in this paper indicate that the proteolytic events resulting in the secretion of rat E-24.18 α -subunit occur at the RPKR⁶⁵⁵ furin cleavage site and that furin itself is most probably the intracellular protease involved in the process. These conclusions are supported by several observations summarized below.

In COS-1 cells transfected with an expression vector encoding wild-type E-24.18 α -subunit, the major species observed is the 98 kDa endo H-sensitive form. Carbonate extraction of isolated intracellular membranes showed that this species was membrane-

associated (results not shown). Thus we believe that the 98 kDa species corresponds to the full-length E-24.18 α -subunit. Infection of COS-1 cells expressing E-24.18 α -subunit with a vaccinia virus carrying the human furin cDNA resulted in the processing of the 98 kDa species into a 85 kDa form, which now became the predominant species. This form has a mobility similar to that of the species observed at low levels in the absence of exogenous furin expression. The small amount of the 85 kDa form observed in cells expressing the α -subunit alone as compared with the amount observed in cells infected with the virus carrying the furin cDNA is consistent with the low level of endogenous furin expressed in COS-1 cells [41]. Alternatively, we can also argue that after processing, the α -subunit is very rapidly transported to the cell surface for secretion. This in turn would result in very low steady-state levels of processed forms in intracellular compartments.

The decrease of 13 kDa observed between the 98 and 85 kDa forms is consistent with cleavage at the furin site and could be blocked by mutations in positions P_1 or P'_1 of the predicted furin cleavage site. Similar mutations have been shown previously to block completely (P_1) or considerably slow down (P'_1) cleavage by furin [35]. These mutations also abolished or considerably diminished the secretion of the enzyme in simple transfection experiments. Overexpression of recombinant furin in transfected COS-1 cells expressing these mutants could not restore normal levels of secretion. Taken together, these results suggest that the endogenous processing of the major intracellular precursor species of the α -subunit also proceeds by cleavage at the furin site and that the processed form is rapidly transported to the cell surface. If cleavage at this site is prevented by mutation, unprocessed forms never reach the cell surface and remain in the RER where they are most probably degraded.

It is interesting to note that the 82 kDa form observed in cells expressing rat E-24.18 wild-type α -subunit is also present in cells expressing the mutant α -subunits. This observation suggests that a second proteolytic cleavage site is present upstream from the furin site. However, in cells expressing only the α -subunit, cleavage at this site is a minor event that does not involve the action of furin since the level of the 82 kDa species is not increased by the expression of exogenous furin.

Other furin-like enzymes, such as PACE4 and PC5 or PC6B (results not shown) [40], did not promote processing of the intracellular 98 kDa species into the 85 kDa form. This ruled out the possibility that these other known enzymes of the exocytic pathway could be involved in the processing of the α -subunit.

Processing of protein precursors by furin has been described essentially as an event occurring in the *trans* Golgi network [43]. Furthermore, it has been shown by Rehemtulla et al. [44] that a furin mutant with the Lys-Asp-Glu-Leu (KDEL) RER retention motif fused to its C-terminus was unable to process the von Willebrand factor in COS-1 cells. In COS-1 cells expressing rat E-24.18 α -subunit we have observed the intracellular accumulation of the 98 kDa species with very little 85 kDa form. The 98 kDa form was sensitive to endo H digestion and is most probably accumulated in the RER, since for secretory and plasma membrane proteins exit from the RER is the rate-limiting step of their intracellular transport [45]. The presence of the major intracellular form of E-24.18 α -subunit in the RER is also consistent with indirect immunofluorescence studies performed on α -subunit-expressing COS-1 cells with an α -subunit-specific polyclonal antibody, which showed a diffuse perinuclear fluorescence characteristic of RER labelling [16]. It is interesting to note that the 85 kDa species accumulated after infection of COS-1 cells with the vaccinia virus carrying the human furin cDNA is also sensitive to endo H digestion. Taken together these

results suggest that furin can be active in the RER. Activity of furin in the RER is not inconsistent with the maturation process of the enzyme. Activation of furin requires cleavage of the pro-region. This activation occurs in the RER through an auto-proteolytic event [46]. Moreover, a truncated mutant of Kex2p to which the yeast RER retention signal His-Asp-Glu-Leu had been attached to the C-terminus retained activity [47]. The discrepancy between the results presented in this paper and the observation of Rehemtulla et al. [44] is not clear. However, as discussed by these authors, one cannot rule out the possibility that the interaction of the mutant furin with the KDEL receptor resulted in loss of enzymic activity or sequestration of the enzyme in an unfavourable intracellular compartment. Alternatively, the high levels of expression obtained with the vaccinia virus system used in this study may have led to processing of E-24.18 α -subunit in the RER, even if furin normally shows little activity in this compartment.

The presence of at least three molecular species suggests an elaborate biosynthetic pathway for rat E-24.18 α -subunit. The temporal and spatial steps involved in this pathway are still unknown. However, the observation that the 85 kDa protein accumulated in the RER and the absence of endo H-resistant α -subunit in mutant α R655V suggest that cleavage at the furin site is essential for exit from the RER. It also indicates that the cytosolic and/or transmembrane domains of the α -subunit have features mediating the retention of this protein in the RER. We are presently investigating this last point.

Several proteins, including the α -subunit of E-24.18, the integrin α -chain [48] and the ovule zona pellucida proteins ZP2 and ZP3 [49], have predicted type-I membrane protein structure. However, the mature forms of these proteins are not anchored in the plasma membrane and the presence of consensus furin cleavage sites upstream from the putative transmembrane domain lead to speculation about the role of furin in the solubilization of these polypeptides. In the present study we have shown convincingly for the first time that furin can transform a predicted integral membrane protein into a soluble one. Interestingly, the functional unit of all these substrates is multimeric.

Soluble forms of E-24.18 have been detected *in vivo* in the human intestine [3,4] and in mouse and rat urine [50]. Moreover, a urinary form of mouse E-24.18 which only contains the α -subunit has been reported [51]. Therefore, the investigation of the secretion of the α -subunit in transfected cells may help us to understand the physiological mechanisms underlying this process.

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REFERENCES

- Beynon, R. J., Shannon, J. D. and Bond, J. S. (1981) *Biochem. J.* **199**, 591–598.
- Kenny, A. J., Stephenson, S. L. and Turner, A. J. (1987) in *Research Monographs in Mammalian Ecto-enzymes: Cell Surface Peptidases* (Kenny, A. J. and Turner, A. J., eds.), vol. 14, pp. 169–209. Elsevier/North Holland Biomedical Press, Amsterdam.
- Sterchi, E. E., Naim, H. Y., Lentze, M. J., Hauri, H.-P. and Fransen, J. A. (1988) *Arch. Biochem. Biophys.* **265**, 105–118.
- Sterchi, E. E., Naim, H. Y. and Lentze, M. J. (1988) *Arch. Biochem. Biophys.* **265**, 119–127.
- Spencer-Dene, B., Thorogood, P., Nair, S., Kenny, A. J., Harris, M. and Henderson, B. (1994) *Development* **120**, 3213–3226.
- Rawlings, N. D. and Barrett, A. J. (1993) *Biochem. J.* **290**, 205–218.
- Kenny, A. J. and Ingram, J. (1987) *Biochem. J.* **245**, 515–524.
- Stephenson, S. L. and Kenny, A. J. (1988) *Biochem. J.* **255**, 45–51.
- Wolz, R. L., Harris, R. B. and Bond, J. S. (1991) *Biochemistry* **30**, 8488–8493.
- Kaushal, G. P., Walker, P. D. and Shah, S. V. (1994) *J. Cell Biol.* **126**, 1319–1327.
- LaVallie, E. R., Rehemtulla, A., Racie, L. A., DiBlasio, E. A., Ferenz, C., Grant, K. L., Light, A. and McCoy, J. M. (1993) *J. Biol. Chem.* **268**, 23311–23317.
- Johnson, G. D. and Hersh, L. B. (1993) *J. Biol. Chem.* **268**, 17647.
- Barnes, K., Ingram, J. and Kenny, A. J. (1989) *Biochem. J.* **264**, 335–346.
- Marchand, P., Tang, J. and Bond, J. S. (1994) *J. Biol. Chem.* **269**, 15388–15393.
- Corbeil, D., Gaudoux, F., Wainwright, S., Ingram, J., Kenny, A. J., Boileau, G. and Crine, P. (1992) *FEBS Lett.* **309**, 203–208.
- Corbeil, D., Milhiet, P. E., Simon, V., Ingram, J., Kenny, A. J., Boileau, G. and Crine, P. (1993) *FEBS Lett.* **335**, 361–366.
- Johnson, G. D. and Hersh, L. B. (1994) *J. Biol. Chem.* **269**, 7682–7688.
- Dumermuth, E., Eldering, J. A., Grünberg, J., Jiang, W. and Sterchi, E. E. (1993) *FEBS Lett.* **335**, 367–375.
- Milhiet, P. E., Corbeil, D., Simon, V., Kenny, A. J., Crine, P. and Boileau, G. (1994) *Biochem. J.* **300**, 37–43.
- Hosaka, M., Nagahama, M., Kim, W., Watanabe, T., Hatsuzawa, K., Ikemizu, J., Murakami, K. and Nakayama, K. (1991) *J. Biol. Chem.* **266**, 12127–12130.
- Schalken, J. A., Roebroek, A. J. M., Oomen, P. P. C. A., Wagenaar, J. C., Debuyne, F. M. J., Bloemers, H. P. J. and Van de Ven, W. J. M. (1987) *J. Clin. Invest.* **80**, 1545–1549.
- Barr, P. J., Mason, O. B., Landsberg, K. E., Wong, P. A., Kiefer, M. C. and Brake, A. J. (1991) *DNA Cell Biol.* **10**, 319–329.
- Taylor, J. W., Ott, J. and Eckstein, F. (1985) *Nucleic Acids Res.* **13**, 8764–8785.
- Sanger, F., Nicklen, S. and Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A.* **74**, 5463–5467.
- Tabor, S. and Richardson, C. C. (1987) *Proc. Natl. Acad. Sci. U.S.A.* **84**, 4767–4771.
- Noël, G., Zollinger, L., Larivière, N., Nault, C., Crine, P. and Boileau, G. (1987) *J. Biol. Chem.* **262**, 1876–1881.
- Benjannet, S., Rondeau, N., Day, R., Chrétien, M. and Seidah, N. G. (1991) *Proc. Natl. Acad. Sci. U.S.A.* **88**, 3564–3568.
- Benjannet, S., Rondeau, N., Paquet, L., Boudreault, A., Lazure, C. and Seidah, N. G. (1993) *Biochem. J.* **294**, 735–743.
- Benjannet, S., Savaria, D., Chrétien, M. and Seidah, N. G. (1995) *J. Neurochem.* **64**, 2303–2311.
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685.
- Aubry, A., Berteloot, A., Beaumont, A., Roques, B. P. and Crine, P. (1987) *Biochem. Cell Biol.* **65**, 398–404.
- Beynon, R. J. and Kay, J. (1978) *Biochem. J.* **173**, 291–298.
- Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254.
- Beckman, G. and Bork, P. (1993) *Trends Biochem. Sci.* **18**, 40–41.
- Watanabe, T., Nakagawa, T., Ikemizu, J., Nagahama, M., Murakami, K. and Nakayama, K. (1992) *J. Biol. Chem.* **267**, 8270–8274.
- Benjannet, S., Reudelhuber, T., Mercure, C., Rondeau, N., Chrétien, M. and Seidah, N. G. (1992) *J. Biol. Chem.* **267**, 11417–11423.
- Liu, Y.-C., Kawagishi, M., Mikayama, T., Inagaki, Y., Takeuchi, T. and Ohashi, H. (1993) *Proc. Natl. Acad. Sci., U.S.A.* **90**, 8957–8961.
- Lusson, J., Vieau, D., Hamelin, J., Day, R., Chrétien, M. and Seidah, N. G. (1993) *Proc. Natl. Acad. Sci. U.S.A.* **90**, 6691–6695.
- Matthews, D. J., Goodman, L. J., Gorman, C. M. and Wells, J. A. (1994) *Protein Sci.* **3**, 1197–1205.
- Kawaoka, Y. and Webster, R. G. (1988) *Proc. Natl. Acad. Sci. U.S.A.* **85**, 325–328.
- Yanagita, M., Nakayama, K. and Takeuchi, T. (1992) *FEBS Lett.* **311**, 55–59.
- Nakagawa, T., Murakami, K. and Nakayama, K. (1993) *FEBS Lett.* **327**, 165–171.
- Molloy, S. S., Thomas, L., VanSlyke, J. K., Stenberg, P. E. and Thomas, G. (1994) *EMBO J.* **13**, 18–33.
- Rehemtulla, A., Dorner, A. J. and Kaufman, R. J. (1992) *Proc. Natl. Acad. Sci. U.S.A.* **89**, 8235–8239.
- Klausner, R. D. (1989) *Cell* **57**, 703–706.
- Leduc, R., Molloy, S. S., Thorne, B. A. and Thomas, G. (1992) *J. Biol. Chem.* **267**, 14304–14308.
- Chaudhuri, B., Latham, S. E. M., Helliwell, S. B. and Seeboth, P. (1992) *Biochem. Biophys. Res. Commun.* **183**, 212–219.
- Lenter, M. and Vestweber, D. (1994) *J. Biol. Chem.* **269**, 12263–12268.
- Yurewicz, E. C., Hibler, D., Fontenot, G. K., Sacco, A. G. and Harris, J. (1993) *Biochim. Biophys. Acta* **1174**, 211–214.
- Flannery, A. V., Dalzell, G. N., Stephen, A. G. and Beynon, R. J. (1990) *Biochem. Soc. Trans.* **18**, 1023–1024.
- Gorbea, C. M., Beynon, R. J. and Bond, J. S. (1995) in *Mammalian Brush Border Membrane Proteins* (Lentze, M., Naim, H. Y. and Grand, R. J., eds.), pp. 89–97. Thieme Medical Publishers, New York.