Identification of the cysteine residues implicated in the formation of α_2 and α/β dimers of rat meprin

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Meprin (endopeptidase-24.18; EC 3.4.24.18) is a multisubunit zinc-metallopeptidase found in the brush-border membranes of rodent kidney and human intestine. The α and β subunits of meprin are disulphide-linked to form either soluble α_2 homodimers or membrane-associated α/β heterodimers. The aim of the present study was to identify the cysteine residue(s) implicated in the formation of α_2 and α/β dimers and to investigate the effects of dimerization on intracellular transport and processing of the α subunit. Three cysteine residue candidates for the formation of disulphide bonds in the α subunit were selected by hydrophobic cluster analysis. These residues, located at positions 309, 560 and 562, were mutated to serine residues. When the resulting α subunit mutants were expressed alone in COS-1 cells, the αC560S and αC562S mutants were found to be secreted as α_2 homodimers whereas the αC309S mutant was found as monomers in the culture medium. In double-transfection experiments with the wild-type β subunit, the αC560S and αC562S mutants behaved exactly as the wild-type α subunit and formed membrane-bound α/β heterodimers. In contrast, the αC309S mutant was not retained at the cell surface but rather secreted as monomers in the culture medium, as was found in the simple transfection experiment. These results show that, despite the normal expression level and folding of the protein in a transport-competent form, the αC309S mutant is unable to form α_2 homodimers or α/β heterodimers. This suggests that Cys^{309} is the unique residue of the α subunit implicated in the α_2 and α/β dimerizations. Hydrophobic cluster analysis of the α and β subunit sequences predicts that Cys^{309} is similar to Cys^{306} of the β subunit. We mutated the latter residue to a serine and expressed the βC306S mutant and the wild-type α subunit in the same COS-1 cells. No β_2 or α/β dimers were observed on immunoblotting, showing that Cys^{306} of the β subunit is required for the formation of intermolecular disulphide bonds both in β_2 homodimers and in α/β heterodimers. Taken together, these results suggest that the α/β heterodimeric form of meprin is held together by a single disulphide bond linking Cys^{309} in the α subunit to Cys^{306} in the β subunit.

INTRODUCTION

Meprin (endopeptidase-24.18; endopeptidase-2; PABA-peptide hydrolase; E-24.18; EC 3.4.24.18) is a zinc-metallopeptidase expressed in the brush-border membranes of rodent kidney [1,2] and human intestine and kidney [3–5]. The active site of meprin shares the consensus sequence HEXXHXXGFXHE (where X is any amino acid) with all known members of the astacin family, which includes several eukaryotic regulatory proteins implicated in development [6,7] and a prokaryotic metallopeptidase named flavastacin [8]. Meprin can hydrolyse *in vitro* a variety of peptides including bradykinin, substance P and luteinizing hormone-releasing hormone [9–11]. Although the function of meprin remains unknown, Kaushal et al. [12] have recently demonstrated that it is the major enzyme in the renal cortex capable of degrading components of the extracellular matrix *in vitro*. This suggests a potential role for meprin in renal pathophysiology [13]. Moreover, recent studies have established that meprin is present in discrete locations in the rat embryo during a period of active craniofacial morphogenesis [14]. Meprin might thus be involved in the control of cell growth and differentiation during mammalian embryonic development.

Rat and mouse meprins seem to be constituted of disulphide-linked α/β dimers associating through non-covalent interactions to form tetramers $(\alpha/\beta)_2$ [15–17]. The existence of α_2 dimers non-covalently associated with α/β dimers to form tetramers has also been postulated [17]. The primary structures of the α and β subunits of meprin [15,18] predict organization into similar domains. Both subunits have an N-terminal signal peptide, a proregion, a catalytic astacin domain that shows significant sequence identity with astacin (a metallopeptidase from the crayfish *Astacus fluvialis* [19]), a MAM domain (MAM for Meprin, protein A5, protein tyrosine phosphatase μ) found in various extracellular molecules [20], an epidermal growth factor (EGF)-like domain close to the transmembrane region, and a short cytosolic segment (Figure 1A).

In spite of the presence of a putative C-terminal hydrophobic membrane-anchoring domain [15,18], the rat α subunit was secreted into the culture medium as α_2 dimers when expressed alone in heterologous COS-1 cells [21] or human kidney 293 cells [22], and no mature protein was found at the cell surface. We have recently shown that a furin-like convertase cleaved the rat meprin α subunit at the furin site, present only in the α subunit and located upstream from the EGF-like domain. Cleavage at this site resulted in the secretion of the α subunit [23]. In α/β co-transfection experiments, however, the α subunits were cleaved at different sites upstream from the furin site (S. Chevallier, unpublished work). The cleaved α subunits were then retained at the cell surface by disulphide linkage(s) to the plasma membrane-anchored β subunits [22,24].

Abbreviations used: DTT, dithiothreitol; EGF, epidermal growth factor; endo F, endo-α-*N*-acetylglucosaminidase F; endo H, endo-β-*N*-acetylglucosaminidase H; ER, endoplasmic reticulum; HCA, hydrophobic cluster analysis; RER, rough endoplasmic reticulum.

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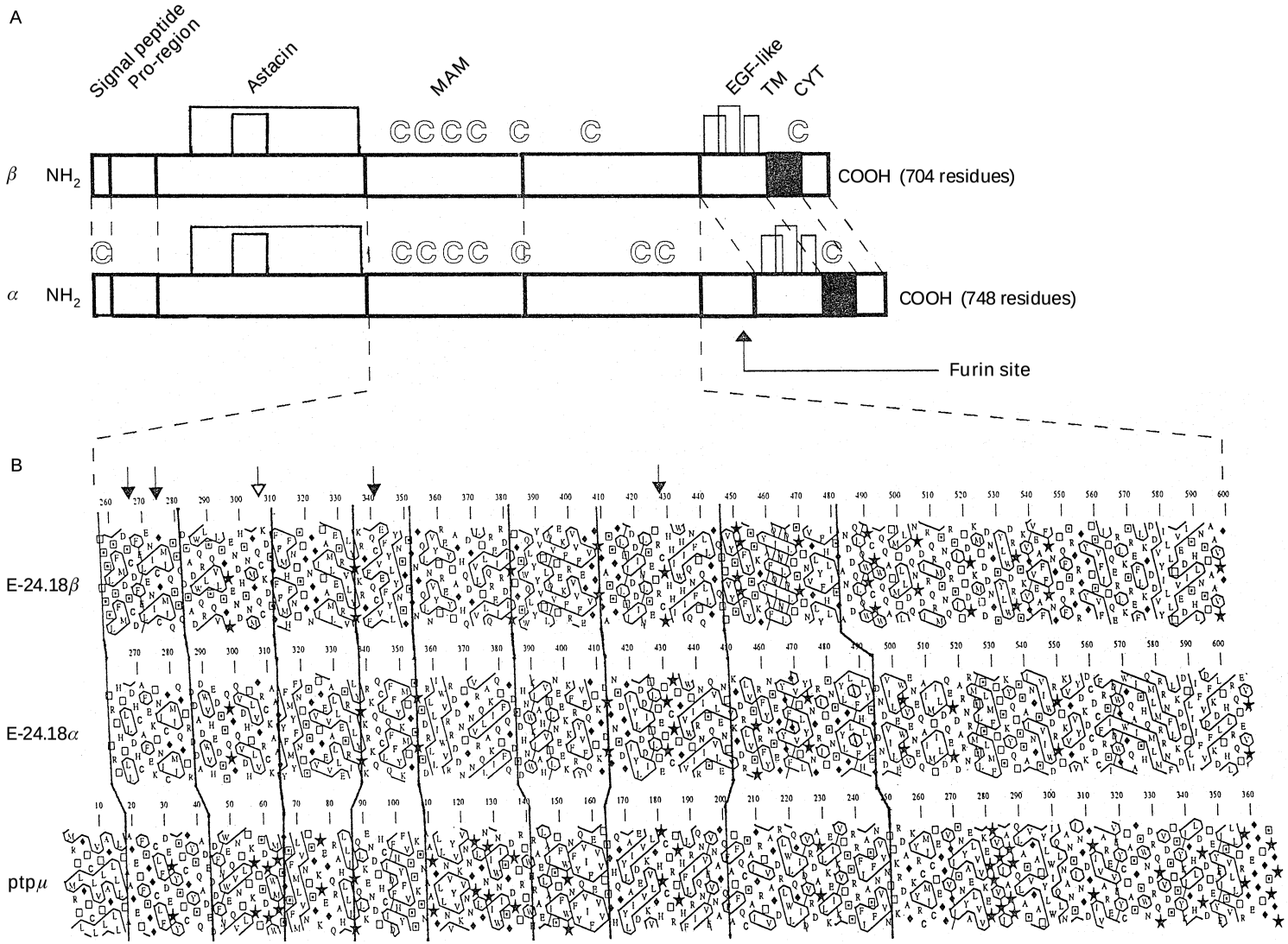

Figure 1 Schematic representation of rat meprin α and β subunits and partial HCA

(**A**) Domain organization of rat meprin α and β subunits predicted from their amino acid sequences. From the N-terminus to the C-terminus are represented the signal peptide, the proregion, the astacin-like catalytic domain, the MAM domain, the EGF-like domain, the transmembrane (TM) segment and the cytosolic (CYT) region. The potential intra-subunit disulphide bridges predicted from the known three-dimensional structures of EGF and astacin are indicated. The other cysteine residues are shown as C. (**B**) The sequences of the rat meprin α subunit (E-24.18α) from residues 261 to 597 and the β subunit (E-24.18β) from residues 257 to 586 were analysed by HCA. The resulting HCA plots are aligned with that of the mouse protein tyrosine phosphatase μ (ptpμ) sequence, residues 17–359. Amino acids are represented by their standard one-letter code with the exception of Pro (black star), Gly (black diamond), Thr (white square) and Ser (white dotted square). Vertical bars are used to facilitate the visualization of corresponding hydrophobic clusters. Black arrows indicate the four cysteine residues conserved between the meprin α and β subunits and the protein tyrosine phosphatase μ. The white arrow designates the cysteine residues at positions 309 and 306 in the α and β subunits respectively, for which no homologous cysteine residue can be found in the ptpμ.

Meprin and enteropeptidase EC 3.4.21.9 are the two known mammalian ectopeptidases showing a heteromeric structure [2,25]. Enteropeptidase EC 3.4.21.9 is present in the intestinal brush-border membranes and is composed of two distinct disulphide-linked subunits [26], only one of which is active [25,27]. In contrast, both α and β subunits of meprin show catalytic activity [24], making meprin unique among cell-surface proteases. The aim of the present study was to identify the cysteine residues implicated in the formation of α_2 and α/β dimers and to investigate the effects of the dimerization on intracellular transport and processing of the α subunit. The results presented in this paper demonstrate that Cys^{309} is the only residue of the α subunit implicated in the formation of both α_2 and α/β dimers. In α/β heterodimers this residue is covalently linked to Cys^{306} of the β subunit.

MATERIALS AND METHODS

Hydrophobic cluster analysis (HCA)

Protein sequences were analysed and compared by HCA, with HCA-PLOT software from Doriane (Le Chesnay, France) running on a Macintosh computer.

Site-directed mutagenesis and plasmid construction

The construction of the α and β subunit expression vectors (pSVP4-α and pSVP4-β respectively) has been described previously [21,24]. For conversion of Cys^{560} and Cys^{562} to Ser in the α subunit (αC560S and αC562S mutants respectively), a 1153 bp *Bam*HI–*Hin*dIII DNA fragment from pSVP4-α was subcloned into M13mp18 RF DNA. Conversion of Cys^{309} to Ser in the α subunit (αC309S mutant) was accomplished with an M13 subclone previously described (clone 6.2) [18]. For conversion of Cys^{306} to Ser in the β subunit (βC306S mutant), the β subunit (2491 bp) was excised from pSVP4-β with *Bgl*II and *Hpa*I and ligated to the *Bam*HI–*Hin*cII sites of M13mp18. Site-directed mutagenesis was performed on single-stranded recombinant M13 DNA by the method described by Taylor et al. [28], with a Sculptor kit purchased from Amersham Inc. Oligodeoxyribonucleotides for mutagenesis were synthesized by the phosphoramidite procedure with a Pharmacia Gene Assembler. Recombinant M13 phages carrying the mutations were screened directly by sequencing [29]. For the αC560S and αC562S mutants a 951 bp *Xmn*I–*Sca*I DNA fragment containing the mutation was substituted for the wild-type fragment in pSVP4-α. For the αC309S mutant, a 637 bp *Ssp*I-*Xmn*I DNA fragment containing the mutation was substituted for the non-mutated fragment in pSVP4-α. For the βC306S mutant a 1854 bp *Xba*I fragment containing the mutation replaced the wild-type fragment in pSVP4-β. The presence of the mutations was confirmed by double-stranded DNA sequencing [30] of the resulting plasmids.

Transfection of COS-1 cells

Transfection of COS-1 cells has been described in detail previously [31]. Dulbecco's modified Eagle's medium supplemented with fetal bovine serum (Gibco, Grand Island, NY, U.S.A.) was replaced 24 h after transfection with synthetic medium [24]. After 16 h the medium was concentrated as described previously [24]. Transfected cells were scraped into ice-cold PBS (137 mM NaCl, 2.7 mM KCl, 7.3 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4) and centrifuged; membrane proteins were solubilized with 2 % (w/v) *N*-octyl-β-*N*-glucopyranoside (octylglucoside) in Tris-buffered saline (50 mM Tris/HCl, 150 mM NaCl, pH 7.4).

Immunoblotting procedure

Proteins from cell extracts and culture media were separated by SDS/PAGE [32] and transferred to nitrocellulose membranes (0.45 μm pore size) as described previously [33]. Meprin subunits were then detected by using either the polyclonal antibody L5α, which specifically recognizes the α subunit [21], or the polyclonal antibody RRt-173, which recognizes both α and β subunits [16]. Biotinylated immunoglobulins against rabbit IgG were used to reveal antigen–antibody complexes (Vectastain ABC-Immunoperoxidase kit; Vector Laboratories, Burlingame, CA, U.S.A.). For densitometry the blots were developed with peroxidase-conjugated anti-rabbit IgG (DAKO, Glostrup, Denmark) and chemiluminescence reagent (Dupont–NEN, Boston, MA, U.S.A.).

Endoglycosidase digestions

Proteins recovered from octylglucoside solubilizates of COS-1 cells 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 in accordance with the manufacturer's instructions. Control samples were incubated under the same conditions but without enzyme. Proteins were analysed by immunoblotting with the polyclonal antibody L5α as described above.

Dithiothreitol (DTT) treatment of intact COS-1 cells

Transfected cells were scraped and harvested by centrifugation. Cell pellets were resuspended in Tris-buffered saline and DTT was added to a final concentration of 10 mM. After 30 min at 25 °C in an end-over-end shaker, 10 mM iodoacetamide was added to stop the reaction. Treated cells were centrifuged for 20 min (12000 ***g***, 4 °C) in an Eppendorf microcentrifuge (Brinkmann, Mississauga, Ontario, Canada). Supernatants and solubilized cell extracts were then analysed by SDS/PAGE and immunoblotting with the polyclonal antibody L5α as described above.

Enzyme assay

The azocasein-degrading activity of meprin and meprin subunits was measured by the production of trichloroacetic acid-soluble peptides after limited trypsin activation of the zymogens as described previously [24]. One unit of enzyme activity was defined as the amount of enzyme producing an increase of 0.001 in the absorbance at 340 nm. For each transfection experiment, the relative amounts of meprin in the different cell extracts were determined by immunoblotting and densitometric scanning of the film (LKB Ultroscan XL Enhanced Laser Densitometer). The specific activity was expressed as enzyme units per amount of meprin, where one arbitrary unit of meprin was defined as the amount of immunoreactive material present in COS-1 cells transfected with the wild-type α subunit alone. Finally, each specific activity was expressed as a ratio over the enzymic activity of the α subunit from singly transfected COS-1 cells.

RESULTS

Sequence analysis of the rat meprin α and β subunits

To identify the cysteine residues implicated in the formation of the α_2 and α/β dimers it was necessary to analyse more precisely the primary structure of both α and β subunits. In our study we have considered only the rat subunits but similar results could be obtained by analysing the sequences of mouse and human

meprins. The protein sequences were compared by HCA, which is a method suitable for detecting similarities in secondary and tertiary structures between proteins sharing a low sequence identity level (usually less than 10–20%). Schematically, individual amino acids in the sequence are classified as either hydrophobic or hydrophilic. The hydrophobic residues form clusters generally representative of the internal faces of regular secondary structures [34]. The sequence is represented as a classical α-helix, which is smoothed on a cylinder, cut parallel to its axis and duplicated to restore the neighbourhood of each residue [35,36]. The two-dimensional helical mode of representation used in HCA facilitates the identification of distinct domains and the comparison between structurally related proteins. Amino acids are represented by their one-letter code with the exception of glycine (black diamond), proline (black star), serine (white dotted square) and threonine (white square).

The meprin α and β subunits contain 19 and 17 cysteine residues respectively. In the α subunit, one of these cysteine residues is located within the signal peptide, which is cleaved during protein translocation. Thus this residue cannot be responsible for meprin dimerization. The catalytic domains of the α and β subunits contain four cysteine residues conserved in the astacin sequence. By comparison with astacin, for which the tridimensional structure has been determined by X-ray diffraction studies [37], these cysteine residues of the meprin subunits should form two internal disulphide bridges (Cys^{108}–Cys^{260} and Cys^{129}–Cys^{148} for the α subunit, and Cys^{104}–Cys^{256} and Cys^{125}–Cys^{145} for the β subunit). Moreover dimerization is not dependent on the presence of the EGF-like, transmembrane and cytosolic domains because deletion of all of these in the α subunit still resulted in the formation of α_2 and α/β dimers [24]. Thus the cysteine residues located at positions 676, 681, 687, 696, 698, 711 and 727 in the α subunit are not potential candidates for formation of interchain disulphide bonds.

The region comprising the MAM domain (about 170 residues), and an intervening segment (215 and 160 residues in the α and β subunits respectively) located between the catalytic and the EGF-like domains, are the less well characterized parts of the meprin α and β subunits. Until now all the proteins thought to belong to the MAM family (α and β subunits of rat, mouse and human meprin; human and mouse protein tyrosine phosphatase μ; protein A5; bovine enterokinase) have been identified on the basis of their low sequence similarities [6,20,38]. With classical methods of sequence alignments, these proteins show less than 10% identity (14 identical amino acids over 170), which is usually not considered significant. The HCA method was thus more suitable for the comparison of the meprin sequences in this region of the molecule. The sequences of the rat α (residues 261–608) and β subunits (residues 257–599) were analysed and compared with the corresponding sequence of the mouse protein tyrosine phosphatase μ (residues 17–367) by the HCA method (see above). This is shown in Figure 1B, in which vertical lines are drawn to reveal the correspondence between the hydrophobic clusters. The similitude of the cluster sizes and shapes between these proteins indicate that the structural similarities are much more evident than the sequence similarities. From this analysis it seems that cysteine residues 270, 278, 344 and 432 in the meprin α subunit are analogous to cysteine residues 27, 36, 96 and 182 in the protein tyrosine phosphatase μ. As the latter protein is a monomer, these cysteine residues in the sequence of the meprin α subunit are probably not involved in any interchain disulphide bonds. A similar analysis comparing the MAM domain of the meprin α subunit with those of protein A5 and enterokinase yielded similar conclusions (results not shown). Consequently only three cysteine residues in the α subunit (at positions 309, 560 and 562) do not correspond to any cysteine residues in the sequence of the protein tyrosine phosphatase μ. A similar conclusion was reached for the cysteine residues at positions 306 and 492 in the β subunit.

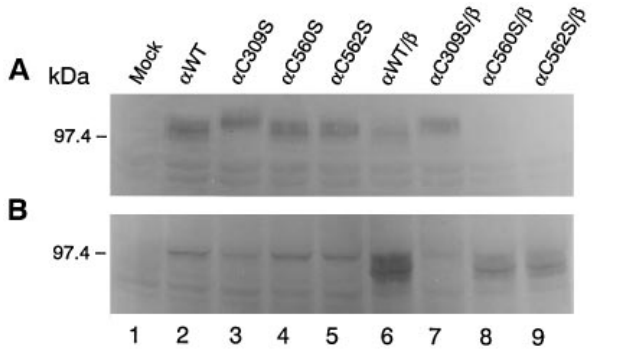

Figure 2 Immunoblotting analysis of mutants and wild-type α subunits

Proteins from media (**A**) and cell extracts (**B**) of transfected COS-1 cells were analysed by SDS/PAGE [7.5% (w/v) acrylamide gel] under reducing conditions as described in the Materials and methods section and detected by immunoblotting with the polyclonal antibody L5α, which is specific for the α subunit. Abbreviation: αWT, wild-type α subunit. Positions of size markers are indicated at the left.

Of these three, only one cysteine residue in the α subunit is found at an equivalent position in the β subunit (Figure 1B). Thus the cysteine residues located at positions 309 and 306 in the α and β subunits respectively are more likely to be implicated in the α/β covalent dimerization. However, for the α_2 dimers we cannot at this stage exclude the possibility that more than one cysteine residue is implicated. Consequently we decided to mutate independently the three cysteine residues at positions 309, 560 and 562 in the α subunit, and investigate the effects of these mutations on the formation of α_2 homodimers and α/β heterodimers.

Expression of wild-type or mutated meprin α subunits with or without the β subunit

Wild-type and mutated (αC309S, αC560S and αC562S) α subunits of rat meprin were expressed alone or with the wild-type β subunit in COS-1 cells. Cell extracts and media were analysed by SDS/PAGE under reducing conditions, and proteins were detected by immunoblotting with the polyclonal antibody L5α, which is specific for the meprin α subunit.

When expressed alone, most of the wild-type and mutated α subunits were secreted in the incubation medium. Wild-type α subunit and αC560S and αC562S mutants appeared as a broad band with apparent molecular masses of 90–100 kDa (Figure 2A, lanes 2, 4 and 5), whereas the αC309S mutant migrated with a slightly higher molecular mass (Figure 2A, lane 3). All these proteins were endo H-resistant (results not shown), suggesting that they had all received complex oligosaccharides. Digestion of the oligosaccharide side chains with endo F resulted in protein species with identical electrophoretic mobilities (results not shown). This suggested that differential oligosaccharide processing of the αC309S mutant most probably accounted for the small decrease in electrophoretic mobility. When the cell extracts were analysed, the majority of both wild-type and mutated (αC309S, αC560S and αC562S) α subunits were found to migrate with molecular masses of 98 kDa (Figure 2B, lanes 2–5). The 98 kDa species had previously been found to be an unprocessed

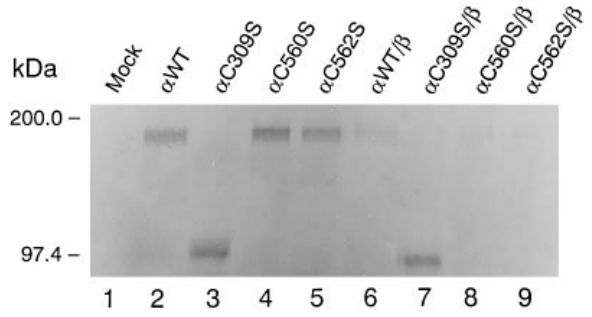

Figure 3 Oligomerization of mutated α subunit

Proteins from spent media of transfected COS-1 cells were analysed by SDS/PAGE [5% (w/v) acrylamide gel] under non-reducing conditions and detected by immunoblotting with the polyclonal antibody L5α. Abbreviation: αWT, wild-type α subunit. Positions of size markers are indicated at the left.

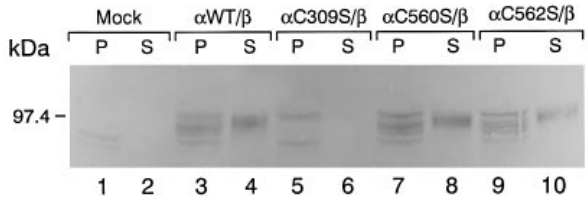

Figure 4 DTT treatment of intact cells expressing the mutated α and wild-type β subunits

Intact cells expressing the α (wild-type or mutated) and wild-type β subunits were treated with DTT as described in the Materials and methods section. After centrifugation, the proteins in the pellet (P) or supernatant (S) were separated by SDS/PAGE [7.5% (w/v) acrylamide gel] and detected by immunoblotting with the polyclonal antibody L5α. Abbreviation: αWT, wild-type α subunit. Positions of size markers are indicated at the left.

endo H-sensitive form of the α subunit present in the rough endoplasmic reticulum (RER) [21].

When wild-type α and β subunits were coexpressed in the same cells, small amounts of the α subunit were detected in the medium (Figure 2A, lane 6) whereas most of the immunoreactive material was found in cell extracts. As described previously [24], this heterogeneous immunoreactive material was found to consist of both the unprocessed 98 kDa intracellular precursor and additional processed forms of the α subunit present at the cell surface as α/β disulphide-linked heterodimers migrating as a 210 kDa band in non-reducing gels. In αC560S/β- and αC562S/β-transfected cells, most of the α-immunoreactive material was found to be associated with cell extracts (Figure 2A, lanes 8 and 9) with mobilities very similar to the wild-type α subunit of α/β transfected cells. Most of this material was also resistant to endo H (results not shown). In contrast, most of the αC309S mutant coexpressed with the β subunit was secreted in the medium (Figure 2A, lane 7). Only the 98 kDa form was detected in cell extracts (Figure 2B, lane 7). This protein was found to be endo H-sensitive (result not shown), supporting the idea that it corresponded to the unprocessed RER-associated form of the α subunit precursor mentioned above.

Dimerization of wild-type and mutated meprin α subunits

To study the effects of the mutations on meprin dimerization, wild-type or mutated α subunits of rat meprin were expressed in COS-1 cells. Secreted proteins were separated by SDS/PAGE under non-reducing conditions and analysed by Western blotting with the polyclonal antibody L5α. A broad band of 180 kDa corresponding to secreted α_2 dimers was detected in the spent culture medium of COS-1 cells expressing the wild-type α subunit (Figure 3, lane 2). Similar results were obtained with the αC560S and αC562S mutants (Figure 3, lanes 4 and 5). With αC309S-transfected cells, however, no band corresponding to α_2 dimers was detected (Figure 3, lane 3). Rather, the mutated α subunits were secreted as monomers that appeared as a broad band of approx. 105 kDa (Figure 3, lane 3). In double-transfection experiments, very small amounts of the α subunit were detected in the culture medium of αWT/β-expressing COS-1 cells (Figure 3, lane 6) because most of the α subunit was retained at the cell surface as disulphide-linked heterodimers with the membrane-anchored β subunit [24] (see above). Similar results were obtained in αC560S/β- and αC562S/β-expressing cells (Figure 3, lanes 8 and 9). In contrast, when the αC309S mutant was coexpressed with the β subunit, the α subunit was detected in the culture medium with a mobility very close to that of the subunit found in simple αC309S-transfection experiments (compare lanes 3 and 7 in Figure 3).

Cellular localization of mutated meprin α subunits

We have previously shown that in co-transfection experiments most of the wild-type α subunits are present at the cell surface as part of a disulphide-linked heterodimer with the β subunit. The α subunits can, however, be solubilized and released into the supernatant by treating intact cells with 10 mM DTT (Figure 4, lane 4) [24]. The same observation was made with αC560S and αC562S mutants coexpressed with the wild-type β subunit (Figure 4, lanes 8 and 10). In contrast, when the αC309S mutant was coexpressed with the β subunit, no immunoreactive material was released into the supernatant by treatment of intact cells with DTT (Figure 4, lane 6). This indicates that the αC309S that was found in the cell pellet (Figure 4, lane 5) is not present at the cell surface. This material most probably represents intracellular forms of the enzyme in transit to the plasma membrane. These intracellular forms of the α subunit were also observed when both wild-type α and β subunits were expressed by co-transfection.

Expression of mutated meprin β subunit with the wild-type α subunit

As shown in Figure 1, Cys^{306} of the β subunit seemed to be similar to Cys^{309} of the α subunit. If cysteine residues α309 and β306 are linked by a disulphide bond in the structure of the heterodimer, mutations of Cys^{306} in the β subunit should also abolish the formation of α/β heterodimers. To test this hypothesis we constructed the βC306S mutant and coexpressed this mutated β subunit with the wild-type α subunit in COS-1 cells.

When expressed alone in COS-1 cells, the wild-type β subunit migrated predominantly as 110 kDa monomers on non-reducing SDS gels although 235 kDa homodimers were also present (Figure 5A, lane 2) [24]. In contrast, all of the βC306S mutant migrated as monomers under these conditions (Figure 5A, lane 4). Cells co-transfected with wild-type α and β subunits expressed 210 kDa α/β heterodimers (Figure 5A, lane 3) whereas those transfected with α and βC306S expressed mainly monomers (Figure 5A, lane 5). The multiple bands appearing at approx. 200 kDa in extracts from α/βC306S-transfected cells (Figure 5A, lane 5) were tentatively identified as α/α dimers because they

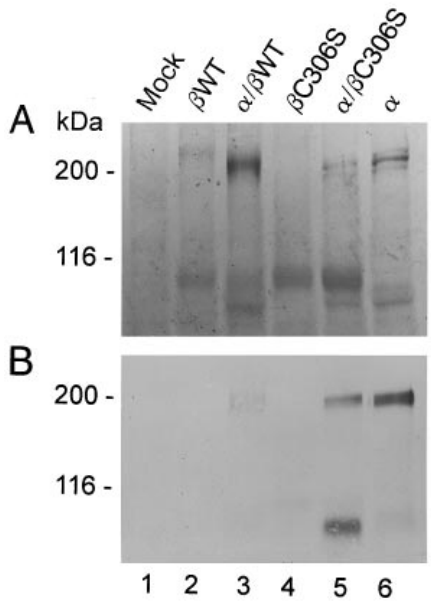

Figure 5 Immunoblot of wild-type and mutated β subunits coexpressed with wild-type α subunit

Proteins from cell extracts (**A**) and media (**B**) of transfected COS-1 cells were analysed as for Figure 3, except that the polyclonal antibody RRt173 was used to detect both α and β subunits. When a blot identical to that shown in (**B**) was incubated with the α-specific antibody L5α, similar results were obtained (results not shown), confirming that only the α subunit is secreted into the medium. Abbreviation: βWT, wild-type β subunit.

comigrated with the dimers from cells transfected with the α subunit alone (Figure 5A, lane 6) and could be detected with the α-subunit-specific antibody L5α (results not shown).

If the α subunit is unable to form a covalent bond with the mutated β subunit, we should expect the α subunit to remain soluble and to be secreted into the culture medium just as when the protein is expressed alone in transfected COS-1 cells. As shown in Figure 3 above, the wild-type β subunit prevented secretion of the α subunit when the two subunits were coexpressed in COS-1 cells (compare lanes 3 and 6 in Figure 5B). In contrast, the βC306S mutant did not prevent secretion of the α subunit (Figure 5B, lane 5). Surprisingly, however, in the presence of the mutated β subunit about half of the α subunit was secreted as monomers (Figure 5B, lane 5) rather than as homodimers (Figure 5B, lane 6). Thus although no stable disulphide bonds were formed between the α and β subunits, the mere presence of a β subunit inhibited α/α dimerization.

Enzymic activity of αC309S and αC306S mutants

To determine whether covalent dimerization is required for the meprin subunits to be functional, we compared the azocasein-degrading activities of the cysteine mutants with those of the wild-type enzyme. To compensate for variations in the expression levels from one transfection to the other, the specific activities were expressed as units per amount of immunoreactive meprin. Table 1 shows that azocasein-degrading activity was below detection levels for the mutant monomeric form of the α subunit (αC309S). The activity of the βC306S mutant was also significantly decreased. In doubly transfected cells the activity of the cysteine mutants was less than that of the α/β dimers.

Table 1 Enzymic activity of meprin cysteine mutants

COS-1 cells were transfected with the following meprin subunits and assayed for azocasein-degrading activity after solubilization in octylglucoside and activation by trypsin. The release of trichloroacetic acid-soluble peptides was measured with mock-transfected cells as the blank. The specific enzyme activities relative to the wild-type α subunit were determined for each experiment. The results are means $\pm$ S.D. for four separate experiments. Abbreviation: n.d., not detectable.

Subunit	Relative enzyme activity (arbitrary units)
α	1.0 ± 0.1
αC309S	n.d.
β	1.7 ± 0.7
βC306S	0.5 ± 0.2
$\alpha + \beta$	1.0 ± 0.4
αC309S + βC306S	0.5 ± 0.2

DISCUSSION

Meprin is the only known mammalian ectopeptidase that comprises two distinct, catalytically active subunits [24]. The membrane-bound enzyme is composed of disulphide-linked α/α, α/β, or β/β dimers that associate non-covalently to form homo- or hetero-tetramers [17,24]. Soluble α_2 homodimers have also been observed in the culture medium of transfected COS-1 cells [21]. In the present study we used site-directed mutagenesis to identify the cysteine residues implicated in the formation of covalent α_2 and α/β dimers. The results indicate that Cys^{309} of the α subunit, located within the MAM domain, is the unique residue responsible for the covalent α_2 dimerization, and that a single disulphide bridge between this residue and Cys^{306} of the β subunit results in the formation of α/β dimers. These conclusions are supported by several observations summarized below.

When expressed alone in COS-1 cells, the αC560S and αC562S mutants formed dimers and were secreted into the culture medium just like the wild-type α subunit. In contrast, the αC309S mutant was found in the incubation medium only as monomers. In cells co-transfected with the αC309S mutant and the wild-type β subunit, the mutant α subunit was also found in the medium in its monomeric form. The wild-type α subunit and the αC560S and αC562S mutants, on the other hand, were all present at the surface of α/β-transfected cells and could be released from intact cells with the reducing agent DTT. Thus Cys^{309} of the α subunit, but not Cys^{560} or Cys^{562}, is essential for both α/α and α/β covalent dimerization. By mutating Cys^{306} of the β subunit (which is similar to Cys^{309} of the α subunit), we have shown that this residue is responsible for the formation of covalent β_2 homodimers. When coexpressed with the wild-type α subunit, the membrane-bound mutant β subunit could not prevent secretion of the α subunit.

In the present study, we have not proved directly that Cys^{309} of the α subunit or Cys^{306} of the β subunit actually forms a bond between subunits in the native meprin. The introduction of a single point mutation could significantly alter the tertiary structure of the subunit such that intermolecular disulphide bridges that normally involve other cysteine residues are prevented from forming in the mutant protein. However, our results show that mutations of the cysteine residues at positions 309, 560 or 562 do not affect the expression level of the α subunit, its exit from the endoplasmic reticulum (ER), acquisition of complex oligo-

saccharides in the Golgi apparatus and secretion into the culture medium. Moreover, mutation of βCys306 does not seem to disrupt the non-covalent interactions between the α and β subunits because the βC306S mutant inhibited α_2 dimerization, presumably by associating directly with the α subunit (discussed below). These observations are consistent with the mutant proteins' being correctly folded. The only detectable difference in expression was a decrease in the mobility of the secreted form of the αC309S mutant; this decrease disappeared after deglycosylation with endo F. Perhaps the α subunit monomer, unlike α/α dimers, receives additional sugar residues during its transit through the Golgi apparatus.

We cannot exclude the possibility that other disulphide bonds form between subunits, in addition to that involving αCys309 and βCys306, but these are not sufficient by themselves because mutation of the crucial αCys309 or βCys306 resulted in monomeric forms of the subunits. It is indeed possible that covalent dimerization is a multistep process where the initial bond between αCys309 and βCys306 is required before other bonds form between subunits. After the other disulphide bridges are in place, the αCys309–βCys306 bond might be reduced so that other cysteine residue(s) link the two subunits of the dimer together in the final product. A similar mechanism can be proposed for α/α dimerization. Although we cannot ignore these possibilities, we can conclude that Cys^{309} and Cys^{306} in the α and β subunits respectively are the essential residues implicated in covalent dimerization.

It is interesting to note that the inability to form disulphide bonds does not seem to prevent the C-terminal processing of the α subunit. Two hypotheses can be proposed: cleavage at the C-terminus might occur before the dimerization process, or the α subunits may form non-covalent dimers that are readily cleaved by the furin-like enzyme. These non-covalent dimers of mutated subunits would be stable in the intracellular environment but would dissociate after their secretion into the incubation medium.

In a recent model of the biosynthesis of mouse meprin, Marchand et al. [39] suggested that the formation of covalent dimers took place in the ER and was a prerequisite for the transport to the Golgi apparatus. Taken together, our results instead demonstrate that covalent dimerization of rat meprin is required for neither the exit from the ER nor transport through the Golgi apparatus where complex oligosaccharides are acquired. Moreover our experiments indicate that, although no stable intermolecular disulphide bonds were formed between the wild-type α subunit and the βC306S mutant, the formation of α_2 homodimers was significantly inhibited by the presence of the mutated β subunit. Thus it appears that α–β interactions can compete with α–α interactions when these subunits are coexpressed in COS-1 cells. These non-covalent associations may be favourable in the intracellular environment but not at the cell surface, where the unanchored α subunit is released into the medium as monomers. Furthermore those α subunits that self-associate intracellularly form intermolecular disulphide bonds and are secreted from the cell as homodimers.

Covalent dimerization does seem to play a functional role because the enzymic activities of the αC309S and βC306S mutants were lower than the activities of the wild-type disulphide-bond-forming subunits. Most of the α subunits from transfected COS-1 cells migrate as dimers on non-reducing gels and this, but not the α monomer, is the active form. Even though the β subunit migrates mostly as monomers on non-reducing gels, cells transfected with the wild-type β subunit displayed higher activity than cells transfected with the βC306S mutant, an increase that is explained by the presence of β/β dimers in cells expressing the wild-type β subunit. The specific enzyme activity of the β subunit alone was also significantly greater than that of α/α or α/β dimers, suggesting subtle differences in the enzymic properties of the various forms of meprin.

In conclusion, meprin is mainly expressed as membrane-associated α/β heterodimers that are linked by disulphide bonds. As mentioned above, this type of oligomeric organization is unique among cell-surface proteases. Different hypotheses can be proposed for the functional significance of such an organization: individually, the α and β subunits might harbour enzymic activities distinct from those of the heteromeric protein and the full catalytic activity and/or the substrate specificity of meprin could only be demonstrated by the enzyme in its oligomeric form. Alternatively, α/β dimerization might be a mechanism for maintaining the α subunit at the cell surface, where it must be localized to fulfil a particular function. The presence of soluble α_2 dimers in rat urine [40] and membrane-associated β_2 dimers in the kidney brush border would be especially important if these forms also had distinct enzyme kinetics and/or physiological functions. The oligomeric structure of the extracellular matrix-degrading enzyme purified by Kaushal et al. [12] is a tetramer composed of two disulphide-linked α subunit dimers. It is not known which forms are expressed and play a role during embryonic development.

To address these different points, the enzymic activities and specificities of the monomeric forms of the α and β subunits have to be investigated and compared with those of the different dimeric forms. The present work has identified the cysteine residues in the α and β subunits implicated in the formation of covalent dimers of rat meprin and demonstrated that these residues are required for full enzymic activity, with azocasein as the substrate. Whether covalent dimerization is important for the degradation of other substrates or the physiological function of the α and β subunits remains to be determined.

We thank Dr. A. J. Kenny for the gift of Rrt173 antibody and Christiane Nault for her expert technical assistance in constructing some of the meprin mutants. This work was supported by a grant from the Medical Research Council of Canada to P.C. and G.B.; J.A. was the recipient of a postdoctoral training award from Le Fonds de la Recherche en Santé du Québec (FRSQ); G.B. was the recipient of a Chercheur-Boursier Award from FRSQ.

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Received 26 October 1995/20 March 1996; accepted 3 April 1996