The carboxyl-terminal tail of kinase splitting membranal proteinase/meprin \( \beta \) is involved in its intracellular trafficking

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The kinase splitting membranal proteinase (KSMP), was recently shown to be identical with the \( \beta \)-subunit of meprin. Meprin is a metalloendoproteinase located in brush border membranes and composed of the two types of subunits, \( \alpha \) and \( \beta \). Despite their high sequence homology and similar domain organization, meprin subunits are differentially processed during maturation; meprin \( \alpha \) is retained in the endoplasmic reticulum (ER), and undergoes a proteolytic removal of the transmembrane and cytoplasmic domains, prior to its export from this organelle. In contrast, meprin \( \beta \) retains these domains even after reaching its final destination in the plasma membrane. Using truncated mutants of rat meprin \( \beta \) expressed in Cos-7 and human embryonic kidney (HEK) 293 cells, we show here that the cytoplasmic tail is indispensable for its exit from the ER. A meprin \( \beta \) mutant lacking the last 25 amino acids is shown to be transport-incompetent, although it does not contain any of the known ER retention signals. Systematic analysis of the rate of the ER to Golgi transport using a series of mutants with Ala or Pro substitutions in the tail, suggests that while no specific amino acid residue by itself is imperative for normal intracellular trafficking of meprin \( \beta \), the insertion of a bend at a distinct position in the tail (specifically by a Y685P mutation) suffices to retain this protein in the ER. We propose that the very length of the cytoplasmic tail, as well as its secondary structure are essential for the ER to Golgi transport of meprin \( \beta \), possibly by allowing an interaction with a cargo receptor.

The presence of a kinase splitting membranal proteinase (KSMP)\(^1\) in brush border membranes was demonstrated as early as 1979 (1, 2). The proteinase was shown to clip the catalytic subunit of PKA (C-subunit) with the formation of a distinct cleavage product devoid of kinase activity (2). Recently we demonstrated that the C-subunit degrading activity of KSMP can be quantitatively reproduced by the \( \beta \)-subunit of meprin (3) and reported a unique specificity of the enzyme toward substrates containing clusters of acidic (Glu and Asp) amino acid residues, decorated with hydrophobic amino acid residues (4). KSMP/meprin is a \( \text{Zn}^{2+}/\text{Mg}^{2+} \) metalloendoproteinase located in brush border membranes of the small intestine (rat, mouse, guinea pig, rabbit, and man) and of the kidney (rat, mouse, guinea pig, and man) (5–10). Meprins belong to the astacin family of endopeptidases (6, 8) and are composed of two types of subunits: \( \alpha \) and \( \beta \), that exist as homo- and heterotetramers (7, 9). It was previously proposed that meprins might be involved in the degradation of some biologically active peptides such as bradykinin, angiotensin, and substance P (6), emphasizing that meprin subunits differ in their specificity. For example, it was shown that bradykinin is a good substrate for mouse meprin A (which is assumed to be either a hetero-oligomer of \( \alpha \)- and \( \beta \)-subunits or homo-oligomer of \( \alpha \)-subunits), but not for meprin B (a homo-oligomer of \( \beta \)-subunits) (11). Furthermore, we have recently demonstrated that while the rat meprin \( \beta \)-subunit cleaves the C-subunit of PKA, the meprin \( \alpha \)-subunit did not cleave this kinase under the same experimental conditions (4). The physiological targets and the exact role of meprins are still not established. Nevertheless, specificity studies carried out recently in our laboratory showed that the peptide hormone gastrin (which is physiologically accessible to this ecto-proteinase), has a significantly lower \( K_m \) than any other previously tested substrate of meprin \( \beta \). Since this peptide hormone is inactivated by such cleavage, we proposed that gastrin may well be an in vivo substrate of meprin \( \beta \) (4).

Despite the high sequence homology and similar domain structure, the different meprin subunits undergo different post-translational proteolytic processing. The cDNA structure of both \( \alpha \)- and \( \beta \)-subunits of meprin predicts the presence of carboxyl-terminal, cytoplasmic, transmembrane, and epidermal growth factor-like domains, which are all still present in the mature \( \beta \)-subunit, but removed from the \( \alpha \)-subunit in the course of its maturation (10, 12–14). In addition, the \( \alpha \)-subunit of meprin also differs from the \( \beta \)-subunit by possessing a domain (denoted the I domain) which is both necessary and sufficient for the proteolytic processing of the immature \( \alpha \)-subunit (15).

It was recently reported that the \( \alpha \)-subunit of the human analog of meprin is retained in the endoplasmic reticulum until the proteolytic removal of the transmembrane and cytoplasmic domains is completed, and that the cytoplasmic tail of this subunit of human meprin is responsible for its retention in the endoplasmic reticulum (16). Since only the \( \beta \)-subunit of meprin possesses the cytoplasmic tail in its mature form, we attempted to establish the functional assignment of this tail, using constructs of meprin \( \beta \) with various mutations in this region (expressed in mammalian cells), and monitoring their properties.
and their localization. Here we show that the segment Tyr<sup>679</sup> to Asn<sup>700</sup> of the rat meprin β is indispensable for its transport of from the endoplasmic reticulum to the Golgi complex. A meprin β mutant, lacking the last 25 amino acids, is shown to be transport-incompetent, although it does not contain any of the known ER retention signals. It seems, therefore, that not only the two meprin subunits have different mechanisms of maturation and targeting, but that their carboxy-terminal tail is involved in this process in a different manner. In the α-subunit, the tail is involved in its retention in the endoplasmic reticulum (16) to allow it to undergo the proteolytic cleavage(s) necessary for the export of this subunit to the cell exterior. The results presented here show that, in the case of the β-subunit, the carboxy-terminal tail is essential for the export of the enzyme from the ER and its subsequent transport to the Golgi apparatus.

MATERIALS AND METHODS

Construction of Meprin β Mutants—Mutants of meprin β truncated at their carboxyl terminus were constructed by introducing stop codons at the positions 680, 690, 695, and 700 in the sequence of rat meprin β by PCR using PCR primers (Boehringer Mannheim, Germany). For all reactions, the sense oligonucleotide primer 5'-CTGAGTCTGACGATTGCAACTTTGAAAGCCAC-3' carrying a KpnI restriction site were designed as follows: 5'-ATTGGATCCCGGTGTCATGAGCCGCTGATA-3' (stop 680), 5'-GCTGAGATCCTGTCTAACGATCTGATGCAATCTGCTGTC-3' (stop 690), 5'-GGTTTTCTGGATCCATTTATGACCAATTTATATAGCCGAT-3' (stop 695), 5'-AAGCAGATCTGGTTATATTGAGGTCCATGTTGAC-3' (stop 700). PCR amplified fragments were digested by the indicated restriction enzymes, and subcloned into a pcDNA3 vector (Stratagene, La Jolla, CA) served as a template. Antisense oligonucleotides with a BamHI restriction site were designed as follows: 5'-ATTGGATCCCGGTGTCATGAGCCGCTGATA-3' (stop 680), 5'-GCTGAGATCCTGTCTAACGATCTGATGCAATCTGCTGTC-3' (stop 690), 5'-GGTTTTCTGGATCCATTTATGACCAATTTATATAGCCGAT-3' (stop 695), 5'-AAGCAGATCTGGTTATATTGAGGTCCATGTTGAC-3' (stop 700). PCR amplified fragments were digested by the indicated restriction enzymes, and subcloned into a pcDNA3 vector (Stratagene, La Jolla, CA) served as a template. Antisense oligonucleotides with a BamHI restriction site were designed as follows: 5'-ATTGGATCCCGGTGTCATGAGCCGCTGATA-3' (stop 680), 5'-GCTGAGATCCTGTCTAACGATCTGATGCAATCTGCTGTC-3' (stop 690), 5'-GGTTTTCTGGATCCATTTATGACCAATTTATATAGCCGAT-3' (stop 695), 5'-AAGCAGATCTGGTTATATTGAGGTCCATGTTGAC-3' (stop 700). PCR amplified fragments were digested by the indicated restriction enzymes, and subcloned into a pcDNA3 vector (Stratagene, La Jolla, CA) served as a template. Antisense oligonucleotides with a BamHI restriction site were designed as follows: 5'-ATTGGATCCCGGTGTCATGAGCCGCTGATA-3' (stop 680), 5'-GCTGAGATCCTGTCTAACGATCTGATGCAATCTGCTGTC-3' (stop 690), 5'-GGTTTTCTGGATCCATTTATGACCAATTTATATAGCCGAT-3' (stop 695), 5'-AAGCAGATCTGGTTATATTGAGGTCCATGTTGAC-3' (stop 700). PCR amplified fragments were digested by the indicated restriction enzymes, and subcloned into a pcDNA3 vector (Stratagene, La Jolla, CA) served as a template. Antisense oligonucleotides with a BamHI restriction site were designed as follows: 5'-ATTGGATCCCGGTGTCATGAGCCGCTGATA-3' (stop 680), 5'-GCTGAGATCCTGTCTAACGATCTGATGCAATCTGCTGTC-3' (stop 690), 5'-GGTTTTCTGGATCCATTTATGACCAATTTATATAGCCGAT-3' (stop 695), 5'-AAGCAGATCTGGTTATATTGAGGTCCATGTTGAC-3' (stop 700). PCR amplified fragments were digested by the indicated restriction enzymes, and subcloned into a pcDNA3 vector (Stratagene, La Jolla, CA) served as a template. Antisense oligonucleotides with a BamHI restriction site were designed as follows: 5'-ATTGGATCCCGGTGTCATGAGCCGCTGATA-3' (stop 680), 5'-GCTGAGATCCTGTCTAACGATCTGATGCAATCTGCTGTC-3' (stop 690), 5'-GGTTTTCTGGATCCATTTATGACCAATTTATATAGCCGAT-3' (stop 695), 5'-AAGCAGATCTGGTTATATTGAGGTCCATGTTGAC-3' (stop 700). PCR amplified fragments were digested by the indicated restriction enzymes, and subcloned into a pcDNA3 vector (Stratagene, La Jolla, CA) served as a template. Antisense oligonucleotides with a BamHI restriction site were designed as follows: 5'-ATTGGATCCCGGTGTCATGAGCCGCTGATA-3' (stop 680), 5'-GCTGAGATCCTGTCTAACGATCTGATGCAATCTGCTGTC-3' (stop 690), 5'-GGTTTTCTGGATCCATTTATGACCAATTTATATAGCCGAT-3' (stop 695), 5'-AAGCAGATCTGGTTATATTGAGGTCCATGTTGAC-3' (stop 700). PCR amplified fragments were digested by the indicated restriction enzymes, and subcloned into a pcDNA3 vector (Stratagene, La Jolla, CA) served as a template. Antisense oligonucleotides with a BamHI restriction site were designed as follows: 5'-ATTGGATCCCGGTGTCATGAGCCGCTGATA-3' (stop 680), 5'-GCTGAGATCCTGTCTAACGATCTGATGCAATCTGCTGTC-3' (stop 690), 5'-GGTTTTCTGGATCCATTTATGACCAATTTATATAGCCGAT-3' (stop 695), 5'-AAGCAGATCTGGTTATATTGAGGTCCATGTTGAC-3' (stop 700). PCR amplified fragments were digested by the indicated restriction enzymes, and subcloned into a pcDNA3 vector (Stratagene, La Jolla, CA) served as a template. Antisense oligonucleotides with a BamHI restriction site were designed as follows: 5'-ATTGGATCCCGGTGTCATGAGCCGCTGATA-3' (stop 680), 5'-GCTGAGATCCTGTCTAACGATCTGATGCAATCTGCTGTC-3' (stop 690), 5'-GGTTTTCTGGATCCATTTATGACCAATTTATATAGCCGAT-3' (stop 695), 5'-AAGCAGATCTGGTTATATTGAGGTCCATGTTGAC-3' (stop 700).

Cell Cultures and Transfections—The human cell line HEK 293 and monkey Cos-7 cells were grown on 35 mm plastic tissue culture dishes (Nunc, Napperville, CT) in DMEM with 10% fetal calf serum and standard antibiotics. For transient transfection experiments, subconfluent cells were incubated for 5 h at 37 °C with the precipitate prepared for 2 min with the fixing solution containing 0.1° Triton X-100. The cells were then incubated as described for anti-meprin β immune serum (diluted 1:500) in PBS, and goat anti-rabbit TRITC conjugated antibodies (Sigma). Immunofluorescence was monitored using a fluorescence microscope (Nikon, Japan). Photographs were taken with a LJS-700 video camera (APPLItec, Israel).

Immunoelectron Microscopy—HEK 293/β (3) and 293/Δ2525 cells were grown to confluence on 15-cm tissue culture dishes in DMEM, supplemented with 10% fetal calf serum, a standard mixture of antibiotics, and 0.2 mg/ml G418. Cells were fixed on the plates with a PBS buffer (containing 1% glutharaldehyde and 3% paraformaldehyde) for 1 h at room temperature, and the ultrathin frozen sections were prepared as described by Himmelhoch (21). The anti-meprin β immune serum or normal rabbit serum (diluted 1:25), and goat anti-rabbit IgG (10-nm gold-conjugated, Zymed Laboratories Inc. Laboratories, San-Francisco, CA), were used for the detection of the immunocomplexes. Electron micrographs were taken at magnifications of 15,000–34,000 with a Philips 410 electron microscope operated at 80 kV. SDS-PAGE and Western Blotting—Protein samples were analyzed by 10% SDS-PAGE under reducing conditions, as described in the literature (20). The proteins were either stained by Coomassie Blue R-250 (Serva, Germany), or subjected to Western blotting according to Harlow and Lane (19). Typically, a dilution of 1:15,000 of anti-meprin β immune serum was used. For visualization of the cross-reactive material, horseradish peroxidase-conjugated goat anti-rabbit antibodies (Transduction Laboratories, Lexington, KY), and an ECL detection system (Amerham, UK) were used.

RESULTS

Construction and Expression of the Meprin β Mutants Truncated in the Cytoplasmic Domain—In an attempt to assess the possible functional assignment of the cytoplasmic tail of KSMP/meprin β we prepared meprin β mutants systematically truncated in their carboxy-terminal part (see Scheme 1). A comparison of the carboxy-terminal sequences of the human (PFPHβ) and of rat meprin β (9, 22) revealed three distinct segments within the tail having a different level of sequence identity, a highly conserved cluster of basic amino acids following the transmembrane domain, another conserved region in the last six-amino acid fragment of the tail, and an in-between segment with no homology. On this basis, stop codons were introduced by PCR into positions between these regions, and the mutant enzyme molecules were then subcloned into a pcDNA3 vector for expression in mammalian cells.

To test the general integrity of the cloned proteins, we translated them in vitro using the rabbit reticulocyte lysate system (Fig. 1A). By supplementing the reaction mixture with the purified canine microsomal membranes (CMM), and subsequent purification of the membranal fraction by centrifugation, we monitored the co-translational translocation of the proteins
The Tail of Meprin \( \beta \) Is Essential for Trafficking

**Scheme 1.** Structures of meprin \( \beta \) (human and rat) and of the mutants of rat meprin \( \beta \) truncated in the cytoplasmic tail which were prepared for the work described here. The domain structure of meprins is designated as follows: S, signal peptide; P, prosequence segment, catalytic domain; TM, transmembrane domain, CYT, cytoplasmic tail. The alignment of the carboxyl-terminal segments (the "tails") of the human and the rat meprin \( \beta \) was performed using the Bestfit program (GGG, Madison, WI). Double dots indicate identical, and single dots, homologous amino acid residues. The structure of the deletion (truncated mutants \( \beta \Delta 5, \beta \Delta 10, \beta \Delta 15, \) and \( \beta \Delta 25 \) is given.

![Diagram](image)

**Fig. 1.** Glycosylation of meprin \( \beta \) and its truncated mutants *in vitro* and *in vivo*. A, cell-free expression of the full-length meprin \( \beta \) and its truncated mutants. The proteins were translated *in vitro* in the presence or absence of CMM, using the rabbit reticulocyte lysate system described under "Materials and Methods." The membranal fraction (where present) was separated from the reaction mixture by centrifugation for 30 min at 22,000 \( \times \) g. The samples prepared from translation mixture without CMM, and the isolated CMM (intact or treated with PNGase F) were analyzed by SDS-PAGE (10% gel) (see "Materials and Methods"). The precipitated proteins were then treated by endo H and PNGase F in order to analyze their processing. Since Endo H is able to remove only the high mannose oligosaccharide side chains added to the proteins upon maturation in the endoplasmic reticulum, the appearance of the Endo H-resistant forms indicates the processing of the oligosaccharides by Golgi-associated enzymes. As shown in Fig. 1B, whereas most of the amount of the full-length meprin \( \beta \) and \( \beta \Delta 5 \) mutant protein exhibited an Endo H-resistant glycosylation (this is evident from the appearance of slow migrating forms of the protein sensitive to the treatment with PNGase F), essentially no Endo H-resistant form of the \( \beta \Delta 25, \beta \Delta 15, \) and \( \beta \Delta 10 \) mutant proteins was accumulated under the same experimental conditions. The lack of complex glycosylation of the \( \beta \Delta 25, \beta \Delta 15, \) and \( \beta \Delta 10 \) proteins suggests that they are transport-incompetent, *i.e.* that they retained in the endoplasmic reticulum.

This result was further confirmed by immunocytochemical visualization of the distribution of the truncated and the non-truncated meprin \( \beta \) within the cell. For indirect immunofluorescence studies we used transiently transfected Cos-7 cells (see "Materials and Methods"), since these cells were found to be more suitable than the HEK 293 fibroblasts for microscopy studies. The full-length meprin \( \beta \) is detected on the surface of the nonpermeabilized cells (Fig. 2a), and the pattern of staining was essentially not changed upon permeabilization of the cell membrane before incubation with antibodies (Fig. 2b). The distribution of the \( \beta \Delta 5 \) mutant was similar to that of the wild type, but with the highlighted Golgi-like compact structure. In agreement with the Endo H assay results, we observed that the \( \beta \Delta 25, \beta \Delta 15, \) and \( \beta \Delta 10 \) mutant proteins were accumulated in the perinuclear zone of the cell exhibiting a characteristic localization in the endoplasmic reticulum (Fig. 2). Thus, the carboxyl-terminal truncation of more than 10 amino acids resulted in accumulation of meprin \( \beta \) in the ER.

**Stable Expression of the Meprin \( \beta \) Mutant Lacking the 25 Carboxyl-terminal Amino Acids**—To gain an insight into the possible reason for the retention of the meprin \( \beta \Delta 25 \) mutant in

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The carboxyl-terminal truncations did not impede the initial maturation of the meprin \( \beta \) mutants.

The constructs thus obtained were utilized to stably transfected HEK 293 fibroblasts, monitoring the expression of the cloned proteins with polyclonal antibodies raised against purified meprin \( \beta \) (4). In an attempt to assess the effect of carboxyl-terminal truncations on the enzyme activity, we tested the proteolytic activities of nonactivated and of trypsin-activated wild type meprin \( \beta \) and of its mutants on two previously characterized KSMP/meprin \( \beta \) substrates, the catalytic subunit of PKA (3) and azocasein (18). The level of expression of each construct was determined by enzyme-linked immunoassay with polyclonal antibodies raised against meprin \( \beta \), using as a reference the meprin \( \beta \) purified from the stably transfected cell line (described previously in Chesnutkin et al. (4)). As shown in Table I, all the mutants were found to have a specific activity toward azocasein and toward the C-subunit of PKA, which was similar to that of the wild type meprin \( \beta \), clearly showing that the carboxyl-terminal truncations caused no major misfolding in the catalytic domain of the enzyme.

**Removal of 25, 15, or 10 Carboxyl-terminal Amino Acids**

Acell-free expression of the full-length meprin \( \beta \) and its truncated mutants. The proteins were translated *in vitro* in the presence or absence of CMM, using the rabbit reticulocyte lysate system described under "Materials and Methods." The membranal fraction (where present) was separated from the reaction mixture by centrifugation for 30 min at 22,000 \( \times \) g. The samples prepared from translation mixture without CMM, and the isolated CMM (intact or treated with PNGase F) were analyzed by SDS-PAGE (10% gel) (see "Materials and Methods" for details). Note the deglycosylation-sensitive increase in the molecular mass of the translation product when CMM is included in the reaction mixture. B, glycosylation of the truncated meprin \( \beta \) mutants expressed in mammalian cells. Transiently transfected HEK 293 fibroblasts were labeled with \( ^{35} \text{S} \) methionine pulse-chase labeling, followed by immunoprecipitation with anti-meprin \( \beta \) antibodies (for details, see "Materials and Methods"). The precipitated proteins were then treated by Endo H and PNGase F in order to analyze their processing. Since Endo H is able to remove only the high mannose oligosaccharide side chains added to the proteins upon maturation in the endoplasmic reticulum, the appearance of the Endo H-resistant forms indicates the processing of the oligosaccharides by Golgi-associated enzymes. As shown in Fig. 1B, whereas most of the amount of the full-length meprin \( \beta \) and \( \beta \Delta 5 \) mutant protein exhibited an Endo H-resistant glycosylation (this is evident from the appearance of slow migrating forms of the protein sensitive to the treatment with PNGase F), essentially no Endo H-resistant form of the \( \beta \Delta 25, \beta \Delta 15, \) and \( \beta \Delta 10 \) mutant proteins was accumulated under the same experimental conditions. The lack of complex glycosylation of the \( \beta \Delta 25, \beta \Delta 15, \) and \( \beta \Delta 10 \) proteins suggests that they are transport-incompetent, *i.e.* that they retained in the endoplasmic reticulum.

This result was further confirmed by immunocytochemical visualization of the distribution of the truncated and the non-truncated meprin \( \beta \) within the cell. For indirect immunofluorescence studies we used transiently transfected Cos-7 cells (see "Materials and Methods"), since these cells were found to be more suitable than the HEK 293 fibroblasts for microscopy studies. The full-length meprin \( \beta \) is detected on the surface of the nonpermeabilized cells (Fig. 2a), and the pattern of staining was essentially not changed upon permeabilization of the cell membrane before incubation with antibodies (Fig. 2b). The distribution of the \( \beta \Delta 5 \) mutant was similar to that of the wild type, but with the highlighted Golgi-like compact structure. In agreement with the Endo H assay results, we observed that the \( \beta \Delta 25, \beta \Delta 15, \) and \( \beta \Delta 10 \) mutant proteins were accumulated in the perinuclear zone of the cell exhibiting a characteristic localization in the endoplasmic reticulum (Fig. 2). Thus, the carboxyl-terminal truncation of more than 10 amino acids resulted in accumulation of meprin \( \beta \) in the ER.

**Stable Expression of the Meprin \( \beta \) Mutant Lacking the 25 Carboxyl-terminal Amino Acids**—To gain an insight into the possible reason for the retention of the meprin \( \beta \Delta 25 \) mutant in
the ER and to minimize the possible effect of overexpression, we prepared the HEK 293 fibroblasts cell line stably expressing this mutant (293/bD25 cells). The meprin-bD25 protein was expressed at approximately the same level as the wild type meprin-b in 293/b cells (Fig. 3A) and was found to be associated with the total membranal fraction of these cells. It was not present in the culture medium or the cytoplasm, thus exhibiting essentially the same distribution as the wild type enzyme (data not shown). The apparent molecular mass of the bD25 mutant expressed in 293 cells was calculated to be 78 kDa by SDS-PAGE (Fig. 3A). Such a 37-kDa decrease in molecular mass (from 115 kDa for the full-length meprin-b) cannot be attributed merely to the removal of 25 amino acids. The susceptibility of the bD25 mutant to the Endo H treatment proved the absence of the complex glycosylation (Fig. 3A), which is in agreement with the results on the transiently expressed bD25 described above. The possibility that the molecular weight of the protein is reduced due to an additional deletion from the amino terminus was ruled out by NH2-terminal amino acid sequence of the meprin-b and of the bD25 mutant purified from the cell lines described above. In both cases, the analysis revealed an identical amino acid sequence which started from Leu21, the amino acid following the predicted signal peptide cleavage site (23).

**Deletion of the Cytoplasmic Tail Does Not Affect the Life Span and Folding of Meprin b**—It is now known that the ER can be regarded as a “quality control station” for newly synthesized proteins, rapidly disposing of those with incorrect folding (24).

### Table I

| Enzyme     | Azocasein<sup>a</sup> | C-subunit of PKA<sup>b</sup> |
|------------|-----------------------|------------------------------|
|            | units/mg              | units/mg                     |
|            | Nonactivated | Activated | Nonactivated | Activated |
| Meprin b   | 46 ± 25            | 224 ± 3  | ND           | 717 ± 68  |
| Meprin bΔ5 | 40 ± 18            | 239 ± 14 | ND           | 952 ± 134 |
| Meprin bΔ10| 60 ± 33            | 237 ± 19 | ND           | 975 ± 70  |
| Meprin bΔ15| 26 ± 30            | 246 ± 1  | ND           | 781 ± 89  |
| Meprin bΔ25| 31 ± 23            | 226 ± 13 | ND           | 949 ± 115 |

<sup>a</sup> Activity was measured in total 1% octyl-β-D-glucopyranoside extracts of transfected HEK 293 cells according to Wolz and Bond (18). Proteolytic activity of the extract of mock-transfected cells was used as a background. Specific activity was calculated per mg of expressed meprin b protein (quantitated by enzyme-linked immunosorbent assay using meprin b purified from stably transfected HEK 293 cells as a reference). Values are mean of the two independent measurements.

<sup>b</sup> C-degrading activity was measured as described previously (2, 3). Briefly, 0.5 μg of purified bovine catalytic subunit of PKA was incubated with 0.2 μg of the total 1% octyl-β-D-glucopyranoside extracts of transfected HEK 293 cells for different times. The samples then were subjected to SDS-PAGE and visualized with Coomassie Blue staining. Activity was determined by densitometric quantitation of the C<sup>9</sup> to C (clipped form) conversion according to equation: activity (%) = C<sup>9</sup>/C<sup>9</sup> + C<sup>1</sup>. One unit of the C-degrading activity was defined as amount of the enzyme degrading 1 μg of the catalytic subunit of PKA/min at 22 °C. C-degrading activity of the extracts without trypsin activation was below the level of detection.

<sup>c</sup> ND, not detectable.
Considering the possibility that the βΔ25 mutant may be improperly folded and therefore destined for degradation, we performed a long [35S]methionine pulse-chase labeling experiment of the HEK 293/β and 293/βΔ25 cells and monitored the content of both the full-length meprin β and of the βΔ25 mutant by immunoprecipitation with anti-meprin β antibodies. Since we did not observe any significant decrease in the amount of both proteins in the course of a 12-h chase (Fig. 3D), we concluded that the mutant protein does not have a significantly shorter life span. It should also be noted that, even after the 12-h chase, the molecular mass of meprin Δ25 did not increase, whereas 1 h of chase was sufficient to achieve a complete glycosylation of meprin β, as evident from the increase of the apparent molecular mass from 97 to 115 kDa.

Since it is known that for oligomeric proteins the process of subunit assembly is tightly associated with the folding of its subunits, and their interaction with the chaperones, we attempted to compare the kinetics of dimerization of the full-length meprin β and the βΔ25 mutant. We monitored the formation of the dimer after the pulse-labeling of the 293/β and 293/βΔ25 cells with [35S]methionine followed by the chase for different times and immunoprecipitation by anti-meprin β antibodies (for details, see “Materials and Methods”). The immunoprecipitated proteins were analyzed by SDS-PAGE (under nonreducing conditions) followed by autoradiography. As seen in Fig. 3C, no significant difference in the kinetics of accumulation of the dimer form of the protein was observed, suggesting that the truncation of the tail did not affect the overall folding of the mutant protein. Interestingly, the dimer form of both wild type and truncated meprin β migrates on the gel as two bands. A possible explanation for this observation might be that meprin undergoes core glycosylation at the same time as it forms the disulfide-linked dimers (as previously shown for human meprin by Sterchi et al. (25)), and that the two bands seen in Fig. 3C may represent differently glycosylated forms of the meprins.

**FIG. 3.** Characterization of the meprin βΔ25 mutant. A, stable expression of meprin β and the βΔ25 truncated mutant in HEK 293 cells. The cells were treated with 1% octyl-β-D-glucopyranoside, and the extracted proteins (2 μg/lane) were incubated overnight either in the absence (lines “-”), or in the presence of Endo H (lines “H”) or PNGase F (lines “F”), prior to separation by SDS-PAGE (7.5% gel). After separation, proteins were transferred onto nitrocellulose paper and incubated with anti-meprin β immune serum, then separated by SDS-PAGE (10% gel) and visualized by autoradiography as described under “Materials and Methods.” B, pulse-chase labeling of the meprin β and the βΔ25 truncated mutant stably expressed in HEK 293 cells. Confluent 293/β and 293/βΔ25 cells were grown overnight on a methionine-depleted medium, then incubated with [35S]methionine for 10 min, and chased with complete medium for the indicated time before lysis. Proteins were immunoprecipitated with 1 μl of anti-meprin β immune serum, then separated by SDS-PAGE (10% gel) and visualized by autoradiography as described under “Materials and Methods.” C, kinetics of the dimerization of the meprin and βΔ25 mutant stably expressed in HEK 293 cells. Cells were grown as in previous experiment, labeled with [35S]methionine for 5 min, and lysed in the immunoprecipitation buffer (containing 0.1 M iodoacetamide) after a chase by complete medium for the indicated time. Proteins were then immunoprecipitated as described under “Materials and Methods” and analyzed by autoradiography after SDS-PAGE (7.5% gel) under the nonreducing conditions.

| Time, hr | 0 | 1 | 2 | 3 | 4 | 6 | 12 | 20 |
|---------|---|---|---|---|---|---|----|----|
| meprin β | H | F | H | F | H | F | H | F |
| meprin βΔ25 | 0 | 1 | 2 | 3 | 4 | 6 | 12 | 20 |

**FIG. 4.** Immunoelectron microscopy of 293/β (panels 1A and 1B) and 293/βΔ25 (panels 2A and 2B) HEK 293 cells. The cells were incubated with anti-meprin β immune serum diluted 1:25 in PBS (panels 1A and 2A), or normal rabbit serum (panels 1B and 2B), before staining with immunogold-conjugated goat anti-rabbit IgG. The abbreviations are: PM, plasma membrane; V, vacuole; N, nucleus. ER, endoplasmic reticulum. Bar, 0.5 μm.
From a comparison of the maturation of the meprin β protein already in this early stage of maturation. Short time metabolic labeling of the expressed proteins, immunoprecipitation, Endo H resistance assay, and quantitation of the Endo H-resistant and -sensitive forms were performed as described under “Materials and Methods.” Presented results are the mean of the three independent experiments, error bars show S.D.

Evidence Suggesting That No Specific Amino Acid Residue in the Tail of Meprin β Is Imperative for the Successful ER to Golgi Transport—From a comparison of the maturation of the meprin βΔ5 and βΔ10 mutants, it appeared that the segment 695AMNL699 in the cytoplasmic tail, which is chopped off in βΔ10 but not in βΔ5 (see Scheme 1 and Fig. 5A) may accommodate at least an important constituent of the signal sequence necessary for the exit of this protein from the ER. In an attempt to identify the specific amino acids which might constitute such a sorting signal, we introduced a series of mutations in this region by replacing the wild type amino acid residues by alanine (Fig. 5B), and then tested the kinetics of maturation of the mutant proteins transiently expressed in HEK 293 cells. In this set of experiments, maturation was monitored by the ratio between Endo H-resistant and -sensitive forms of the mutants (taking the wild type meprin β as a reference in each series of experiments) with time (0–60 min). The bars in Fig. 5 represent the slopes calculated from a linear curve fitting of the results and expressed as percent of the value obtained in the same experiment with the wild type meprin β. As evident from Fig. 5B, alanine substitutions in the 695AMNL699 region caused no dramatic effect on the ability of the expressed mutant to translocate from the ER to the Golgi (see mutants M696A/N697A and L698A/E699A), although the mutant M696A/N697A exhibited a slightly decreased rate of acquisition of an Endo H resistance.

In view of this finding we considered the possibility that a putative sorting signal may be located upstream of Ala694, and that the βΔ10 truncation may render meprin β transport-incompetent by placing this signal too close to the COOH terminus. Therefore we also tested other mutants in the segment between Tyr679 and Ala694. As seen in Fig. 5B we found a –25% decrease in the translocation rate in the R686A/K687A/K688A mutant. This effect was even more pronounced when all the amino acid residues creating the cluster of basics in the tail (as in the RRK,RKK to Ala mutant) were substituted with alanine. Replacement by alanine of the Tyr685 residue in the middle of the cluster of basics (a cluster conserved also in the human meprin β; Scheme 1), also resulted in no impediment to translocation from the ER to the Golgi. It should be noted that in the case of the R682A/R683A/K688A mutant, the protein was actually able to undergo glycosylation even faster than the wild type meprin β as judged by the Endo H resistance. The reason for this apparent acceleration is not clear to us at this stage, but from the mechanistic point of view it is quite intriguing. It should be noted, however, that most of the mutants in which charged amino acids were replaced by the nonpolar alanine residue (K692A/T693A, L698A/E699A, and R682A/R683A/K684A) exhibited a somewhat increased rate in the ER to Golgi translocation. In conclusion it can be said that this series of
experiments indicate that the substitution of the any of the tested amino acid residues in the cytosolic tail of meprin β does not dominantly affect the transport of this protein from the ER to the Golgi apparatus.

A very intriguing observation we made in the course of these experiments was with the ΔAla689–Met696 mutant (Fig. 5A). As a result of this mutation, the rate of the ER to Golgi translocation of the protein was decreased significantly (40% of the wild type), although the deleted amino acid residues by themselves were found to be nonessential for transport by alamine scanning. Therefore, we considered the possibility that the retention of the βΔ15 and βΔ10 mutants might be due to a recognition of the 685KK688 segment as an ER-retrieval signal as reported for several proteins that are known to reside in the endoplasmic reticulum (26, 27). To test this possibility, we constructed two mutants in which the segment accommodating the last 10 amino acid residues (which is truncated in βΔ10), or the segment Ala689–Met696, were deleted alongside with the substitution of the 685KK688 by alanine residues. By analysis of the kinetics of Endo H resistance, we found that, indeed, in those mutants, the same deletion and truncation that caused an impaired transport, resulted now in no ER retention (Fig. 6).

In both cases, the ability to undergo complex glycosylation was reconstituted, showing that, although the diysine motif is localized in an unfavorable position in relation to the carboxy-terminal tail (KKX)_β and (KKX)_β in meprin βΔ10 and ΔAla689–Met696 mutants, respectively), it is able to cause retrieval of these mutants from the ER, therefore preventing them from further transport to the plasma membrane.

Possible Contribution of the Secondary Structure of the Tail and Its Flexibility to the Successful ER to Golgi Transport—
Since the alanine substitutions of the amino acids in the tail which were considered likely to participate in protein-protein interaction (charged, polar or hydrophobic residues), did not influence significantly the ER to Golgi rate of translocation, we attempted to find out whether a mutation which is known to affect the local secondary structure would affect this translocation. Indeed, as seen in Fig. 5C a specific single amino acid mutation (Y685P, but not Y685A) in the middle of the cluster of basic amino acids resulted in a dramatic decrease in the rate of exit from the ER, reducing the rate of the translocation to essentially that of the βΔ25 mutant. The structural importance of this exact position in the sequence was indicated (i) by the finding that mutation to proline of its two adjacent amino acids (K684P and R686P) also caused a significant delay (about 50% of the wild type) in the ER to Golgi transfer, but did not essentially abolish the transport completely as found in case of the Y685P mutant, and (ii) by the fact that the introduction of this mutation at the position of two other alanines in the tail (A691P and A695P) did not result in a significant decrease in the rate of the translocation. It should be noted that the clear-cut effect of the Y685P mutation on the transfer could not be attributed to an interference with the dimerization of meprin β since the rate of dimerization of this mutant protein was found to be essentially identical to that of the wild type meprin β (data not shown).

On the basis of the results presented above we conclude that the introduction of the kink forming proline residue at position 685 or at its immediate vicinity alters the secondary structure and maybe also the flexibility of the tail, and that such alterations interfere with the ER-to-Golgi transfer. This conclusion is in line with the analysis of the alanine and proline substitutions in this region, which show that while alanine substitution of the one (Y685A), three (R682A/R683A/K684A and R686A/K687A/K688A) and even six (RRK/RKK to Ala) amino acids can be tolerated, single-site mutations such as K684P, R686P, and especially Y685P affect the transport dramatically.

In line with this proposal we would like to point out that the prediction of the secondary structure of the cytoplasmic tail by the method of Chou and Fasman (28) (Pepplot software, GCG, Madison, WI) suggests a high probability for the existence of an α-helix in the tail of meprin β that is reduced significantly upon introduction of a helix-breaking proline residue in the sequence of the tail.

**DISCUSSION**

Until recently it was commonly accepted that the transport of proteins to the plasma membrane through the ER to Golgi vesicular pathway occurs “by default,” or by “bulk flow.” However, it now seems that there are diverse routes of transport, and that proteins may be specifically sorted before they are targeted to the appropriate membranal compartment or organelle (for a recent review see Kuehn and Schekman (29)). In addition, it was recently shown that membranal, as well as secreted, proteins are first concentrated in the ER before being transferred to the Golgi apparatus (30, 31). This finding raises the possibility that there may be specific receptors for cargo proteins that selectively anchor to these molecules upon interaction with appropriate transport signals. For example, the Emp24 protein, a known component of the COPII-coated vesicles, was shown to function as such a receptor in yeast cells (32). A family of structurally related proteins (the p24 family), has already been identified, indicating that the occurrence of such specific receptors may well be more widespread than previously thought (33). Many of the transport studies were focused on the protein traffic through the early secretory pathway, namely from the ER to Golgi apparatus and inside the Golgi apparatus. It is now believed that this transport is mediated by the coatamer complexes COPI (participating both in the anterograde and retrograde transport of vesicles between the ER and Golgi apparatus) (34), and COPII (primarily involved into the anterograde vesicular flow) (35). Several anter-
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...leading to pathological traffic jams. It may indicate, for example, that the interaction of a putative sorting or transport protein with the tail of meprin β is particularly sensitive to the conformation of the juxtanemembranal region accommodating the cluster of basic amino acids. Another possibility that we cannot neglect at this stage is that the putative sorting signal may be composed of a unique combination of distant amino acids in the sequence. An extensive and complex, pairwise scanning of mutations in the tail may be required to unveil this signal.

The results reported here indicate that in the case of meprin β, the carboxy-terminal tail plays a role which is actually opposite to the role of the corresponding region in meprin α, specifically we propose that in meprin β the tail is necessary for the export of the newly synthesized enzyme molecule from the ER to Golgi apparatus, possibly through an interaction with one or several cargo receptor proteins. The question of why these two structurally similar subunits have such different mechanisms for their intracellular transport and maturation is not clear at this stage, but it seems to us that our findings shed light on the trafficking of meprin α and meprin β in a way that may have a general mechanistic significance.

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