Role of the COOH-terminal Domains of Meprin A in Folding, Secretion, and Activity of the Metalloendopeptidase*

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Secreted forms of the α subunit of recombinant mouse meprin A include an NH₂-terminal prosequence, a catalytic domain, and three COOH-terminal domains designated as MAM (meprin, Δ-5 protein, receptor protein-tyrosine phosphatase), MATH (meprin and TRAF homology), and AM (after MATH). In this study, the importance of these COOH-terminal domains for biosynthesis of secreted, activable forms of the protease was investigated. Transcripts of the meprin subunit truncated after the protease (α(1–275)), MAM (α(1–452)), and MATH (α(1–528)) domains or with individual domains deleted (ΔMAM, ΔMATH, and ΔAM), were transfected into human embryonic kidney 293 cells. The wild-type subunit, ΔMATH, ΔAM, α(1–452), and α(1–528) were secreted into the media, although the ΔAM mutant was secreted at very low levels. The ΔMATH and α(1–452) mutants were not activable by limited proteolysis. The α(1–528) mutant was as active as wild-type meprin α against a bradykinin substrate, but had no activity against azocasein, and is, as all other mutants, more vulnerable to extensive degradation by proteases than the wild-type protein. Pulse-chase experiments revealed that the ΔMAM and α(1–275) mutants were rapidly degraded within cells. Treatment with lactacystin, a specific inhibitor of the proteasome, significantly decreased the proteasome degradation, indicating that the mutants lacking the MAM domain are degraded by the proteasome as misfolded proteins. These results indicate that the MAM domain is necessary for correct folding and transport through the secretory pathway, the MATH domain is required for folding of an activable zymogen, and the AM domain is important for activity against proteins and efficient secretion of the protein. The work demonstrates the interdependence of the domains for correct folding of an activable, stable, mature enzyme.

Meprins belong to the “astacin family” of metalloendopeptidases and the “metzinin superfamily” (1, 2). The prototype of the family is the crayfish astacin (EC 3.4.24.21), a 20-kDa monomer isolated from the hepatopancreas. The meprins, meprin A, EC 3.4.24.18; and meprin B, EC 3.4.24.63) are multidomain, oligomeric proteases that are secreted or highly expressed in mammalian brush-border membranes of the intestine or kidney (1, 3–7). The meprins are complex and structurally unique proteases: homo- or heterotetrameric glycoproteins composed of evolutionarily related α and/or β subunits that contain astacin-like catalytic domains and disulfide-bridged dimers (6–10). They are capable of degrading proteins such as collagen and gelatin, hormones such as parathyroid hormone, luteinizing hormone-releasing hormone, and melanoctye-stimulating hormone, and small peptides such as bradykinin, angiotensins, and gastrin (11, 12). The meprins may, therefore, be involved in activation or inactivation of important extracellular proteins and peptides, and are highly regulated at transcriptional and posttranscriptional levels themselves. They are tissue-specific proteases that are implicated in developmental processes, as well as in normal and pathological processes in adult tissues (1, 13).

Studies of recombinant forms of meprins expressed in mammalian cells have yielded important information about critical features of the enzyme (8–10, 14, 15). For example, it has been demonstrated that removal of a 56-amino acid domain in the α subunit (the I domain; see Fig. 1) results in retention of the subunit in the endoplasmic reticulum (ER), 1 and that mutation of Cys₁²₀₀ in the MAM domain to Ala results in a monoclonal secreted, activable form of the protease that is unstable to heat and proteolytic degradation. Attempts to express enzymatically active or activable forms of the recombinant protease domain of meprin or the protease domain with signal and/or prosequences (in the absence of COOH-terminal domains) have been unsuccessful, in that these proteins are either degraded in the cells or accumulate in inclusion bodies depending on the cell type.2 These results implied that the noncatalytic, COOH-terminal domains of meprins are important for the secretion and correct folding of the enzyme, although this has not been specifically investigated previously.

The deduced amino acid sequences of meprin α and β subunits from mouse, rat, and human cDNA have been determined (Refs. 3, 4, 6, and 16; see Fig. 1). The subunits encode an amino-terminal signal sequence (S), a prosequence (Pro), a protease (astacin-like) domain, a MAM (meprin, Δ-5 protein, receptor protein-tyrosine phosphatase), MATH (meprin and TRAF homology) domain, an AM (after MATH) domain, an MATH domain, an epidermal growth factor-like domain, a putative transmembrane-spanning (T) domain, and a cytoplasmic (C) domain (15). The domain structure of the meprin α and β subunits are similar except that meprin α encodes a 56-amino acid sequence (the I or inserted domain) located between the AM and the epidermal growth factor-like domains that is absent in the β subunit (9). The I domain enables COOH-terminal

1 The abbreviations used are: ER, endoplasmic reticulum; MAM, meprin, Δ-5 protein receptor protein-tyrosine phosphatase, MATH, meprin and TRAF homology; TRAF, tumor necrosis factor receptor-associated factor; AM, after MATH; Endo-H, endoglycosidase H; Endo-F, endoglycosidase F/N-glycosidase F; PCR, polymerase chain reaction; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; BK⁺, bradykinin analog, 2-aminobenzoyl-Arg-Pro-Pro-Gly-OH, where Dnp is dinitrophenyl.
2 W. Jiang and J. S. Bond, unpublished observations.
**Protein**

Proteolyticprocessingofthemeprina subunit at residue Arg^615^ (near the end of the AM domain) in the ER, so that this subunit is secreted if not associated with a β subunit at the cell surface (9, 15). The MAM and MATH domains occur in several other proteins, and have been implicated as “interaction” or “adh- esion” domains (17–20). The MAM domain, which is present in cell surface proteins, consists of about 170 amino acids, contains four or five cysteine residues, and has been shown to play a role in homodimerization of protein-tyrosine phosphatase μ (17, 18). The MATH domain is found in cytosolic proteins such as TRAF (tumor necrosis factor receptor-associated factor) and has been shown to be essential for homodimerization and heterologous interactions with receptor proteins (19, 20). Functions of the AM domain are unknown, and no homologous sequences have been identified for this domain in proteins other than meprin.

In the present study, we investigated the role of the MAM, MATH, and AM domains in secretion, folding, and activity of the meprin α subunit by mutational (see Fig. 1) and transfection analyses.

### EXPERIMENTAL PROCEDURES

**Reagents and Materials**—[^S]Methionine/cysteine was purchased from ICN Pharmaceuticals, Inc. Lactacystin and Pansorbin were from Calbiochem. The pcDNA I/Amp plasmid was from Invitrogen. Dulbecco’s medium, methionine-free Dulbecco’s modified Eagle’s medium, and Opti-MEM were from Life Technologies, Inc. The tissue culture media were purchased in response to native meprin antigen (anti-meprin α antibody); the second antibody was produced in response to native meprin antigen (anti-α antibody). Meprin subunits were detected using the enhanced chemiluminescence method (Pierce).

**Plasmid Construction and Mutagenesis**—The pcDNA I/Amp plasmid was from Invitrogen. Dulbecco’s medium, methionine-free Dulbecco’s modified Eagle’s medium, and Opti-MEM were from Life Technologies, Inc. The tissue culture media were collected after 48 h of transfection and were subjected to centrifugation at 16,000 × g for 20 min. The supernatant fractions were concentrated by ultrafiltration using 30-kDa microcon-30 concentrators (Amicon, Inc.). The cells were washed twice with phosphate-buffered saline (PBS), removed from the plates with a rubber scraper, and subjected to centrifugation at 300 × g for 5 min. The sediments were suspended in PBS containing 0.1% Triton X-100, sonicated for 1 min at 4 °C, and subjected to centrifugation at 100,000 × g for 1 h; the supernatant fraction is referred to as the cell lysate.

**Pulse-Chase Experiments**—The transfected cells were preincubated for 1 h at 37 °C in Dulbecco’s modified Eagle’s medium lacking methionine supplemented with 10% dialyzed fetal bovine serum. The cells were pulse-labeled for 10- to 30-min (with [^S]methionine/cysteine (100 μCi/ml/dish), and then chased in serum-free Opti-MEM (1.5 ml/plate). At indicated times, the cells were collected after 16 h. The immunocomplexes were precipitated with 40 μl of Protein A-Sepharose beads (50% gel suspension) for 1 h at 4 °C with gentle agitation. The beads were washed three times with PBS, lysed in PBS containing 1% Triton X-100, 0.5% sodium deoxycholate, 0.02% sodium azide, and proteinase inhibitors (antipain, chymostatin, leupeptin, pepstatin, phenylmethylsulfonyl fluoride), and subsequently sonicated for 1 min. The media were subjected to centrifugation at 6,500 × g for 10 min.

**Immunoprecipitation of Labeled Meprins**—The cells and media were mixed with 40 μl of Pansorbin for 1 h at 4 °C to prevent nonspecific binding to IgG-protein A complexes, and then centrifuged at 5,000 × g for 20 min. The supernatant fractions were incubated with 15 μl of anti-meprin α IgG at 37 °C for 10 min, and then stored at 4 °C for 1 h. The immunocomplexes were mixed with 40 μl of Protein A-Sepharose beads (50% gel suspension) for 3 h at 4 °C with gentle agitation. The beads were washed three times with 0.1% SDS, 0.1% Triton X-100, 200 mM EDTA, 10 mM Tris-HCl (pH 7.5), washed another three times with buffer containing 1% Triton X-100, 0.1% sodium deoxycholate, 0.02% sodium azide, and proteinase inhibitors (antipain, chymostatin, leupeptin, pepstatin, phenylmethylsulfonyl fluoride), and subsequently sonicated for 1 min. The media were subjected to centrifugation at 16,000 × g for 10 min.

**SDS-PAGE and Immunoblotting**—The supernatant fractions were subjected to electrophoresis using 7.5% SDS-polyacrylamide gels (22). Immunoblotting was performed as described previously (9). The proteins were probed with one of two different antibodies, which had been produced by injection of meprin antigen into rabbits. In one instance, the purified mouse meprin had been deglycosylated prior to injection (referred to as anti-deglycosylated antibody); the second antibody was produced in response to native meprin antigen (anti-α antibody). Meprin subunits were detected using the enhanced chemiluminescence method (Pierce).

**Endoglycosidase Digestion**—The immunoprecipitated or secreted proteins were denatured by boiling for 5 min at 100 °C with 5% Tris-HCl (pH 8.0) containing 0.2% SDS. The samples were adjusted to a final concentration of 50 μg/ml sodium acetate buffer (pH 6.0), containing 0.75% Triton X-100 and 100 μg/ml protease inhibitors such as antipain, chymostatin, leupeptin, pepstatin, and phenylmethylsulfonyl fluoride. The mixtures were adjusted to 10 ml of Endo-H or 1.0 unit of Endo-D, and incubated at 37 °C for 18 h. Reactions were stopped by boiling the samples in SDS-PAGE sample buffer.

**Trypsin and Arg-C Treatment**—Trypsin was added to the secreted proteins at the final concentration of 10 ng/ml in 20 mM Tris-HCl, pH 7.5. After incubation at 25 °C for 30 min, soybean trypsin inhibitor...
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RESULTS

Wild-type Meprin α and Mutants α(1–528), α(1–452), and ΔMATH Were Secreted into the Media—When the wild-type mouse meprin α subunit cDNA was transfected into human embryonic kidney 293 cells, the protein was secreted into the medium with a molecular mass of approximately 95 kDa (Fig. 2A, lane 2). A small amount of the expressed meprin α subunit was found associated with cell lysates (Fig. 2A, lane 1); this component had a molecular mass of approximately 90 kDa. Truncated mutants α(1–528), α(1–452), and the ΔMATH deletion mutant were also detected as immunoreactive, secreted proteins with molecular masses of approximately 90, 75, and 92 kDa, respectively. By contrast, mutants α(1–275), ΔMAM, and ΔAM were undetectable by immunoblot analysis of the cell lysates and media (Fig. 2A, lanes 7–10, 13, and 14). Repeated analyses revealed that the expression levels of all the mutants in the media were lower than the wild-type, but deletion of the MAM domain (ΔMAM), truncation just before the MAM domain (Δ(1–275)), and deletion of the AM domain (ΔAM) yielded the lowest levels of secreted protein (Fig. 2B).

When the secreted proteins were subjected to SDS-PAGE in the absence of β-mercaptoethanol, all migrated as dimers (data not shown). Thus, the intersubunit S-S bridging was maintained in secreted mutants as in the wild-type.

The Secreted Proteins Contain Complex-type Oligosaccharide Chains—To determine whether the secreted mutants contained complex oligosaccharides as the wild-type (9), they were treated with Endo-H and Endo-F (Fig. 3). The α(1–528), ΔMATH, and α(1–452) proteins were all resistant to Endo-H, and sensitive to Endo-F, indicating complex glycosylation had occurred. Endo-F treatment decreased the molecular mass of the wild-type from about 95 to 75 kDa (Fig. 3, lanes 1 and 3); the α(1–528) mutant from approximately 90 to 70 kDa (Fig. 3, lanes 4 and 6); the ΔMATH mutant from about 92 to 72 kDa (Fig. 3, lanes 7 and 9); and the α(1–452) mutant was shifted from approximately 75 to 65 kDa (Fig. 3, lanes 10 and 12). The deglycosylated proteins all migrated slower on gels than their predicted molecular sizes (by about 10 kDa), but as predicted relative to each other. Endo-F treatment decreased the molecular masses of the wild-type, α(1–528), and ΔMATH mutants approximately 20 kDa, as assessed by mobility after SDS-PAGE. The α(1–452) mutant lost approximately 10 kDa upon treatment with Endo-F. The decreased mass lost from the latter mutant relative to others may be due to the absence of the MATH and AM domains, both of which contain potential glycosylation sites.

The MATH Domain Is Required for Biosynthesis of an Activable Form of Meprin—Meprin α subunits are secreted as inactive proenzymes and must be treated with trypsin-like proteinases for the conversion of the zymogen to the active form.
mature enzyme (11–15). To test whether secreted mutants \(\alpha(1–528)\), \(\alpha(1–452)\), and \(\Delta\text{MATH}\) were correctly folded proteins and active enzymes, the mutants were treated with trypsin and Arg-C, under conditions used for limited proteolysis of the wild-type enzyme, and catalytic activities were measured against a bradykinin analog (BK\(^+\)) and azocasein. After incubation with 40 ng/\(\mu\)l endoprotease Arg-C at 37 °C for 30 min, the wild-type and the secreted mutants \(\alpha(1–528)\), \(\alpha(1–452)\) and \(\Delta\text{MATH}\) migrated faster on polyacrylamide gels than non-treated proteins, indicating limited proteolysis (Fig. 4A). Prior to treatment with Arg-C neither wild-type nor mutants had activity. After the limited proteolysis, the wild-type and \(\alpha(1–528)\) mutant proteins had comparable activities against the bradykinin substrate. However, the mutants lacking the MATH domain (\(\Delta\text{MATH}\) and the \(\alpha(1–452)\) mutant) had no detectable activity (Table I). Activity of all mutants against azocasein was markedly decreased. The \(\alpha(1–528)\) mutant had a 10-fold decreased activity against the protein substrate compared with the wild-type, and no activity of the mutants lacking MATH domain could be detected with azocasein. These results indicate that the mutants were synthesized as proforms, that the MATH domain is necessary for synthesis of an enzymatically activable protease (against peptide and protein substrates), and that the AM domain is necessary for generation of activity against protein substrates but not against small peptides such as bradykinin.

All Mutants Are More Susceptible to Degradation by Trypsin than the Wild-type—Notable differences in the vulnerability to extensive degradation by trypsin were also observed between the wild-type and secreted mutant proteins. The wild-type and \(\alpha(1–528)\) mutant were resistant to general proteolysis when treated with 10 ng/\(\mu\)l trypsin, while limited proteolysis at this trypsin concentration shifted the proforms to the enzymatically active forms (Fig. 4B, lanes 1–4). However, under the same conditions, the \(\Delta\text{MATH}\) and \(\alpha(1–452)\) mutants were extensively degraded (Fig. 4B, lanes 5–8). Differences in the susceptibility between the wild-type and the \(\alpha(1–528)\) mutant were found when they were treated with a higher concentration of trypsin (Fig. 4C). The \(\alpha(1–528)\) mutant was extensively degraded by 20–40 ng/\(\mu\)l of trypsin (Fig. 4C, lanes 7 and 8), whereas the wild-type was resistant to proteolysis (Fig. 4C, lanes 5 and 4). These results indicate that the mutants lacking the MATH domain were not correctly folded to generate active enzymes, while the mutant lacking the AM domain (\(\alpha(1–528)\)) had a correctly folded protease domain for generation of peptidase activity, but lacked proper structure for activity against protein substrates and resistance to degradation.

Pulse-Chase Experiments Reveal That the \(\Delta\text{AM}\) Mutant Was Secreted at Low Levels, and That \(\Delta\text{MAM}\) and \(\alpha(1–275)\) Were Rapidly Degraded within Cells—To determine whether mutants undetectable by immunoblot analysis (see Fig. 2) were synthesized and rapidly degraded, pulse-chase experiments were performed (Fig. 5). The wild-type protein associated with cells was initially observed as a protein of approximately 100 kDa, and then converted to a 95-kDa form; by 2 h after the

![Figure 3. Deglycosylation of the secreted wild-type and mutant meprins.](http://www.jbc.org) The secreted meprin subunits in media were incubated at 37 °C for 18 h with or without Endo-H (H) and Endo-F (F). The mixtures were subjected to SDS-PAGE, and analyzed by immunoblotting using anti-meprin α. Wt, wild-type.

![Figure 4. Limited and extensive proteolysis of the secreted wild-type and mutant meprins.](http://www.jbc.org) A, the secreted wild-type (Wt) and mutants were incubated at 37 °C for 30 min with or without 40 ng/\(\mu\)l protease Arg-C. The Arg-C was then inactivated with 0.3 mM 3,4-dichloroisocoumarin. B, the secreted wild-type and mutants were incubated at 25 °C for 30 min with or without 10 ng/\(\mu\)l trypsin. The samples were then inactivated with a 2-fold excess of soybean trypsin inhibitor. C, the secreted wild-type and mutants were incubated at 25 °C for 30 min with 10, 20, and 40 ng/\(\mu\)l trypsin. The incubated samples were then inactivated with a 2-fold excess of soybean trypsin inhibitor. The mixtures were subjected to SDS-PAGE followed by immunoblotting.

### Table I

| Samples     | BK+ (\(\mu\)mol/min/mg) | Azocasein (units/mg) |
|-------------|-------------------------|----------------------|
| Wild-type   | 2.10 ± 0.21             | 8485 ± 265           |
| \(\alpha(1–528)\) | 1.89 ± 0.20             | 324 ± 176            |
| \(\alpha(1–452)\) | ND                      | ND                   |
| \(\Delta\text{MATH}\) | ND                      | ND                   |

Specific activity of secreted wild-type and mutant meprins

Secreted wild-type and mutants were activated with endoproteinase Arg-C as described under “Experimental Procedures.” Activity was determined using 24 \(\mu\)M fluorogenic bradykinin analog (BK\(^+\)) or 11 \(\mu\)g/\(\mu\)l azocasein as substrates. The amount of meprin protein was estimated from immunoblotting analyses using a purified mouse meprin A preparation as a standard as described previously (10). Activities are expressed as the mean ± S.E. ND, not detectable.
radiolabeled pulse, the protein was detectable in the medium (Fig. 5A). All the mutants that were undetectable by immunoblot analysis were detected in cells after the radiolabel pulsetime was increased from 10 to 30 min. The radiolabeled ΔAM mutant was also detected in the medium; the cell-associated and secreted proteins were approximately 88 kDa (Fig. 5C). This indicates that the protein can fold and be transported without the AM domain but that the protein is inefficiently secreted without this domain.

The α(1–275) mutant was observed as two polypeptide forms of approximately 45 and 42 kDa after the 30-min radiolabeling period (Fig. 5B). After 2 h, only a small amount of the protein was retained within the cell, and no protein was detected in the medium (Fig. 5B, lane 2). Similarly, the ΔMAM mutant was initially synthesized with a molecular mass of about 85 kDa, and then rapidly disappeared within the 2-h chase period (Fig. 5D). Over a 10-h period of incubation with unlabeled amino acids, no radiolabeled protein was secreted in the medium (Fig. 5D, lanes 5–8). These results indicate that the mutants α(1–275) and ΔMAM were degraded within the cell.

To determine whether the cell-associated mutant proteins were retained in a pre-Golgi compartment, deglycosylation experiments were performed. After a 2-h pulse with radiolabeled amino acids, the ΔMAM and the α(1–275) were deglycosylated with Endo-H and Endo-F. The proteins of these mutants were sensitive to Endo-H and Endo-F, indicating that they were high mannose-type forms (Fig. 6A). Moreover, when cells were treated with brefeldin A, a compound that prevents the transport in the secretory pathway from the ER to the Golgi complex (23), the degradation of the mutants of the ΔMAM and the α(1–275) mutants was not inhibited (data not shown). These results indicate that degradation of the mutants ΔMAM and α(1–275) occurs in a pre-Golgi compartment. When the ΔAM mutant was treated with Endo-H and Endo-F, the cell-associated protein was sensitive to both endoglycosidases. The 88-kDa protein was converted into two forms of approximately 70

![Fig. 5. Pulse-chase experiments of the wild-type and meprin mutants.](http://www.jbc.org/)

The transfected cells were pulse-labeled with [35S]methionine/cysteine for 10 min (wild-type) or for 30 min (α(1–275), ΔAM, and ΔMAM) and chased with non-radiolabeled medium for 2, 5, or 10 h. The cell lysate and the media were immunoprecipitated with anti-meprin α. The immune complexes were analyzed by SDS-PAGE, followed by fluorography. A, wild-type; B, α(1–275); C, ΔAM; D, ΔMAM.

![Fig. 6. Deglycosylation of cellular forms of radiolabeled mutant meprins.](http://www.jbc.org/)

The transfected cells were pulse-labeled with [35S]methionine/cysteine for 2 h (A) or 30 min (B). The cell lysates or media were immunoprecipitated with anti-meprin α. The immune complexes were incubated at 37°C for 18 h with or without Endo-H (H) and Endo-F (F). The mixtures were analyzed by SDS-PAGE followed by fluorography.

and 68 kDa (Fig. 6B). However, the secreted ΔAM protein was resistant to Endo-H, indicating that it had acquired complex-type oligosaccharide chains.

The Mutants Lacking the MAM Domain Are Degraded by the Proteasome as Misfolded Proteins—Recent studies have re-
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Fig. 7. Effect of lactacystin on the degradation of the mutants lacking the MAM domain. The transfected cells were preincubated for 4 h in the absence or presence of 10 μM lactacystin. The cells were pulse-labeled with [35S]methionine/cysteine for 30 min and chased for 2 or 5 h in the continued absence or presence of lactacystin. Cell lysates and the media were immunoprecipitated with anti-meprin α. The immune complexes were analyzed by SDS-PAGE, followed by fluorography. A, wild-type (Wt); B, ΔMAM; C, α(1–275) mutant.

revealed that mutants of several membrane-bound and soluble proteins that enter the secretory pathway are degraded by the proteasome located in the cytosol (24, 25). To examine whether the proteasome is involved in the degradation of the ΔMAM and α(1–275) mutants, cells were incubated with lactacystin, a specific inhibitor of the proteasome (26). In the presence of lactacystin, the rates of degradation of the ΔMAM and α(1–275) mutants were significantly decreased (Fig. 7, B and C). Treatment with lactacystin resulted in retention of the ΔMAM mutant within the cells for at least 5 h and the α(1–275) mutant for 2 h (Fig. 7, B and C). Under the same conditions, treatment of cells with lactacystin had no notable effect on the biosynthesis or secretion of the wild-type protein (Fig. 7A). These results indicate that the mutants lacking the MAM domain are degraded at least in part by the proteasome.

DISCUSSION

The work herein clearly demonstrates that the MAM, MATH, and AM domains of meprin α are essential for efficient transport of the protein to the cell surface and/or correct folding to generate an enzymatically active protease. A summary of the effects of truncations of the subunit and deletions of the domains on expression, activity, and protease susceptibility is shown in Table II.

The only mutant in this series that was secreted (at levels detectable by immunoblotting) and enzymatically activable was α(1–528), the truncated mutant missing COOH-terminal domains after the MATH domain. The fact that this mutant displayed full activity against bradykinin, but very little activity against azocasein, is reminiscent of truncated mutants of MMP-13 in which the COOH-terminal hemopexin domain is removed (27). The latter mutant can be activated as the wild-type for activity against peptide substrates, but has no activity against collagen. For the MMPs, the hemopexin domain is critical for interaction with the collagen substrate, and in an analogous fashion, it is possible that the AM domain serves this type of function in meprin for protein substrates. In previous studies, monomeric forms of meprin, produced by mutations of cysteine residues in the MAM domain that prevent intersubunit disulfide bridges, were found to display this characteristic of activity against peptides but not proteins, which led to the suggestion that the oligomeric structure of the enzyme was important for interaction of the enzymes with protein substrates (10). The data herein provide further evidence that meprin activity against proteins is determined by domain-domain interactions that are critical for structure and full function of the enzyme.

Previous studies have shown that the meprin α(1–570) mutant, truncated near the end of the AM domain, was secreted at levels comparable to the wild-type protein (10). The α(1–528) mutant in the present study was secreted at a lower level, approximately 20% of wild-type. Thus, residues 528–570 of the AM domain appear to be important for the efficient secretion of the subunit. This segment of the AM domain contains at least one glycosylated residue (Asn546 and/or Asn554), determined from carbohydrate analyses of mouse meprin α (3), and may be important for interaction with chaperones or other factors in the ER involved in transport from the ER to the Golgi apparatus. The loss of the oligosaccharide chain in mutants missing the AM domain may also increase the susceptibility of the protein to proteolytic degradation.

The ΔAM mutant was secreted at an even lower level than α(1–528): ΔAM was only detectable with radiolabeling techniques. The difference between the ΔAM mutant and α(1–528), is that the former is synthesized with all domains COOH-terminal to AM, and because of this contains a transmembrane-spanning segment. Previous studies have established that the COOH terminus of the secreted mouse kidney meprin α subunit is at Arg<sup>615</sup> in the AM domain, and that the I domain is essential for the COOH-terminal proteolytic processing of the wild-type protein to occur in the ER (9, 15). Deletion of the AM domain may also impede the efficient COOH-terminal proteolysis of the subunit, and thereby retard its movement through the secretory pathway. Taken together, all the data indicate that the AM domain plays a role in secretion due to its importance in COOH-terminal proteolytic processing and glycosylation of the subunit.

Mutants lacking the MATH domain are folded sufficiently for secretion (at approximately 40–70% of the wild-type level), but not correctly to generate an active protease from the proform. In addition, both the ΔMATH and MATH truncation mutant α(1–452) were very vulnerable to extensive degradation by trypsin, which is another indication of incorrect folding. Meprins are dependent on external proteases for removal of the prosequence; they do not have a cysteine switch mechanism for
activation (28), as the matrixinxs, and cannot be auto- or self-activated (14). The MATH domain likely plays a role in domain-domain interactions that allow limited proteolysis, for removal of the prosequence, and also prevent extensive proteolysis of the subunit. It has been suggested that prosequence removal in meprins allows the formation of hydrogen bonds involving the two NH$_{2}$-terminal residues that are critical for enzyme structure (14). The MATH domain may be important for correct dimerization or oligomerization of the enzyme, as it plays this role in other proteins. There is approximately 30% identity in the MATH domain between TRAFs and meprins (19). Deletion of the MATH domain of CRAF (CD40 receptor-associated factor 1), which is a relative of TRAF, revealed that the MATH domain in the cytosolic proteins is essential for homodimerization and heterologous interactions with receptor proteins in acute phase responses, lymphocyte activation, and nerve cell growth (20). It is notable that the MATH domain is conserved between TRAFs and meprins, since TRAFs are intracellular binding proteins in the cytosol whereas meprins are extracellular metalloproteinas. However, there are no cysteine residues in the MATH domain, therefore folding does not depend on the reducing/oxidizing environment.

The MAM domain in meprin α appears to be particularly important for correct folding of the protein during biosynthesis, as without this domain the protein is degraded via a proteasomal route. These data imply that meprins are members of a growing group of membrane and secreted proteins in which chaperones bind to misfolded proteins during biosynthesis, and facilitate retrograde transport to the cytosol for degradation by the proteasome (24, 25). Examples of this group include unassembled subunits of T cell receptor (29), a mutant of the cystic fibrosis transmembrane conductance regulator (30), 3-hydroxy-3-ethylglutaryl-CoA reductase (31), apolipoprotein B100 (32), and α$_{1}$-antitrypsin Z (33). Wiertz et al. (34) demonstrated that misfolded proteins are exported across the ER membrane to the cytosol for degradation through a proteinaceous channel, the Sec61p translocon. In meprin mutants containing the MAM domain, chaperones may interact with folding intermediates and facilitate formation of correct conformations that lead to COOH-terminal proteolytic processing and transit through the ER. When the MAM domain is not present, chaperones may bind to misfolded protein and enable retrograde transport of the protein from the ER to the cytoplasm.

Studies with a recombinant form of human meprin α, α(1–325), which was truncated after the first 62 amino acids residues of the MAM domain, so that approximately one third of the MAM domain was present in the protein, indicated that this mutant was secreted efficiently from Madin-Darby canine kidney cells (35). Thus, it is possible that only the first third of the MAM domain is critical for secretion. The first third of the MAM domain contains three cysteine residues, and studies with mouse and rat meprin α transcripts have indicated that one of those residues (human Cys$^{308}$, equivalent to Cys$^{320}$ in the mouse and Cys$^{309}$ in the rat) is responsible for intersubunit S-S bridging. The folding of the MAM domain during biosynthesis and covalent dimerization of meprin molecules could impact on the interaction of the protein with chaperones and other molecules in the ER that determine retrograde transport versus Golgi transport. Hahn et al. (35) also reported that calnexin, a membrane-bound and lectin-type chaperone (36), binds to a human meprin α truncated mutant containing a transmembrane domain. This chaperone interaction may be important for correct folding and exit of the glycoprotein from the ER.

The MAM domain in proteins other than meprin does not necessarily function to prevent retrograde transport to the cytosol. For example, when the MAM domain was deleted from receptor protein-tyroine phosphatase μ, the protein was well expressed at the plasma membrane (18). The expressed protein lacking the MAM domain, however, lost the ability of specific hemophilic cell-cell interactions in the transfected cells. Thus, while the MAM domain in receptor protein-tyroine phosphatase μ plays a role in protein-protein interactions at the cell surface, the phosphatase proteins lacking MAM are not detected as misfolded proteins during biosynthesis. By contrast, meprin mutants lacking MAM are detected as misfolded proteins by the “quality control” systems of the ER. This implies a dependence of the protease domain on the MAM domain to fold correctly.

These results indicate a similarity of the astacin family enzymes to the subtilisin family of proteases (37). Mammalian proprotein convertases are multidomain subtilisin-like endopeptidases with noncatalytic domains COOH-terminal to the catalytic domain. Furin, for example, is composed of signal and prosequences NH$_{2}$-terminal to the serine-type protease domain, which is absent in bacterial subtilisins, is essential for catalytic activity (38). Mutation of the homo B domain results in loss of catalytic activity and miss-sorting of the convertase in the secretory pathway. Astacin family and subtilisin family members have evolved so that there is an important interdependence of domains for correct folding. The complex interactions between domains and putative chaperones add another dimension to the regulation of these proteinases.

**TABLE II**

Summary of effects of truncation and deletion mutants of mouse meprin α on expression, activity, and susceptibility to extensive degradation.

| Wt or mutants | Expression of recombinant proteins | Analyses | For secreted proteins |
|---------------|-----------------------------------|----------|-----------------------|
|               | Final fate | Type of glycosylation | Enzyme activity$^{a}$ | Susceptibility to extensive degradation by trypsin |
| Wt(1–760)     | Secreted to medium | Complex |  + + R$^{b}$ | 10 ng/ml |
| α(1–528)      | Secreted to medium | Complex |  + Low R | 40 ng/ml |
| α(1–452)      | Secreted to medium | Complex |  No No S |  |
| α(1–275)      | ER degradation | High mannose |  No No S |  |
| ΔMATH         | Secreted to medium | Complex |  No No S |  |
| ΔMAM          | ER degradation | High mannose |  No No S |  |

$^{a}$ Enzyme activity measured after limited proteolysis with Arg-C.

$^{b}$ R, resistant; S, susceptible.

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