Cysteine Mutations in the MAM Domain Result in Monomeric Meprin and Alter Stability and Activity of the Proteinase*

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Meprins are oligomeric, glycosylated cell surface or secreted metalloendopeptidases that are composed of multidomain disulfide-linked subunits. To investigate whether subunit oligomerization is critical for intracellular transport or for the enzymatic and/or physical properties of the proteinase, specific cysteine residues were mutated, and the mutants were expressed in 293 cells. Mutation of mouse meprin α Cys-320 to Ala in the MAM domain (an extracellular domain found in meprin, A-5 protein, and receptor protein-tyrosine phosphatase μ) resulted in expression of a monomeric form of meprin, as determined by SDS-polyacrylamide gel electrophoresis and nondenaturing gel electrophoresis. The monomeric subunits were considerably more vulnerable to proteolytic degradation and heat inactivation in vitro compared with the oligomeric form of the enzyme. Proteolytic activity of the monomeric meprin using a bradykinin analog or aminobenzoyl-Ala-Ala-Phe-p-nitroanilide as substrate was similar to that of disulfide-linked oligomeric meprin; however, activity against azocasein was markedly decreased. Mutation of another cysteine residue in the MAM domain (C289A), predicted to be involved in intrasubunit disulfide bridging, resulted in disulfide-linked oligomers and monomers. These results indicated that the mutant was capable of forming intersubunit disulfide bonds but less efficiently than wild-type meprin subunits. Mutant C289A also retained activity toward peptides but not the protein substrate and was more vulnerable to proteolytic degradation and heat inactivation compared with the wild-type enzyme. Both Cys mutants were expressed and secreted into the medium at levels comparable with the wild type and had slightly altered glycosylation. This work indicates that 1) Cys-320 of mouse meprin α is most likely responsible for the covalent interactions of the subunits; 2) covalent dimerization of subunits is not essential for efficient biosynthesis, trafficking, or posttranslational processing of the secreted protease; and 3) mutations in the MAM domain affect noncovalent interactions of the subunits and the stability and activity of the protease domain, indicating that domain-domain interactions are critical for structure and function of the enzyme.

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1 The abbreviations used are: EGF, epidermal growth factor; BK+, bradykinin analog 2-aminobenzoyl-Ala-Ala-Phe-p-nitroanilide; PAGE, polyacrylamide gel electrophoresis; X domain, unknown domain; and Meprin/A-5 protein/receptor protein tyrosine phosphatase μ; I domain, inserted domain.
translational levels differentially.

The related crayfish protease astacin (EC 3.4.24.21), which in its mature form is comprised of only the protease domain, is a nonmoneric enzyme (17). In contrast, all mature secreted and membrane-associated forms of the meprin α subunit contain the protease, MAM, and X domains. The functions of the latter two domains are unknown, and no matching sequences for the X domain have been identified in the data banks. MAM domains, however, are found in otherwise unrelated proteins, such as enteropeptidase, tyrosine phosphatase μ and κ, and A5 protein and have been suggested to serve as “adhesion” domains (16, 18–20). There is some evidence that indicates that MAM domains can mediate specific noncovalent interactions between the tyrosine phosphatase μ and κ receptors (21). Comparisons of MAM domains of the different proteins indicate that they contain four conserved cysteine residues. Meprin MAM domains contain a fifth cysteine residue not present in the MAM subunit of mice, rats, and humans. Therefore, the Cys residues that are the most promising candidates for formation of disulfide bridges between mature subunits of mice, rats, and humans.

Experimental Procedures

**Plasmid Construction and Mutagenesis**—The pcDNA I/Amp (Invitrogen) plasmid expressing full-length wild-type mouse meprin α subunit cDNA was described previously (15). The COOH-terminal truncation mutant was generated by the polymerase chain reaction using a mutagenic antiserum primer that changed the codon for Cys-571 to a stop codon. Meprin α cysteine mutants were constructed according to the method of Deng and Nickoloff (22) using the Transformer site-directed mutagenesis kit (Clontech). Two separate mutagenic primers were designed to target either the Cys-289 or the Cys-320 codon. The selection primer was designed to change a XhoI site in the multiple cloning site of pcDNAI/Amp to a SalI site. The same selection primer was used together with either one of the two mutagenic primers in the mutagenesis reactions. The presence of an Ala codon at position 289 or 320 was determined using a Hitachi F2000 fluorescence spectrometer.

**RESULTS**

**Rationale for Mutagenesis Studies**—There are 19 cysteine residues in the meprin α subunit (Fig. 1, top). Of those, four cysteines are in the protease domain, and they are highly conserved in all members of the astacin family of metalloendopeptidases (27). In astacin, they are known to be involved in intradomain disulfide bridges, and this is most probably true and is important for the proteolytic activity of all eukaryotic members of the family (17, 27). There are five Cys residues in the MAM domain; four of them are conserved in all other MAM domains, and those cysteines are most likely in intradomain bridges because they are part of extracellular proteins that do not have covalent interactions with other proteins (16). The fifth Cys in the meprin MAM domain, Cys-320 in α, is unique to meprins and is conserved among the six meprin subunits cloned to date (α and β subunits of mice, rats, and humans).

There are two cysteines in the X domain of α subunits, Cys-571 and Cys-573; these two are not conserved in meprin β subunits. There are six Cys in the EGF-like domain; by analogy with other EGF-like units, all are intradomain bridged (28). There is one Cys in the signal sequence that is cleaved off during biosynthesis and one in the COOH-terminal transmembrane domain of meprin α that is removed during maturation (3, 14). Therefore, the Cys residues that are the most promising candidates for formation of disulfide bridges between mature subunits are Cys-320 of the MAM domain and Cys-571 and Cys-573 of the X domain.

**Cys-571 and Cys-573 Are Not Required for Disulfide Linkage of a Subunits**—Previous studies had shown that transcripts truncated just after the X domain were secreted as disulfide-linked dimers (15). The truncated products of the X domain that were candidates for intersubunit disulfide bridges are present near the COOH terminus of the X domain. Truncated subunits that were terminated immediately NH₂-terminal to...
Cys-571 and Cys-573 were expressed in 293 cells (Fig. 1). The secreted truncated meprin α protein, like the wild type, was predominantly a disulfide-linked oligomer as assessed by mobility after SDS-PAGE. Therefore, Cys-571 and Cys-573 are not essential for covalent interactions between subunits. The truncated mutant was expressed at high levels and, when activated, had a specific activity for the substrate azocasein that was comparable with wild type (data not shown). The level of expression of wild-type and truncated mutants varied from one experiment to another; the higher level of mutant compared with wild-type protein. The data lend further support to the conclusion that Cys-320 is likely responsible for intersubunit S–S bridging.

Mutagenesis of Cys-320 to Alanine Results in Monomeric Meprin a—The mobility of both cysteine mutants when subjected to SDS-PAGE appeared to be somewhat decreased compared with wild-type protein. To determine whether this mobility change was due to glycosylation differences, the wild-type and mutant proteins were deglycosylated with endoglycosidases F and H and subjected to SDS-PAGE as described previously (15). Treatment of the proteins with endoglycosidase F, which removes high mannose and most complex N-linked oligosaccharides, decreased the molecular masses of all the proteins to approximately 67 kDa and eliminated the mobility differences between the proteins (data not shown). All three proteins were resistant to endoglycosidase H, indicating they were complex-glycosylated. These data indicate that both cysteine mutations altered the complex glycosylation patterns.

Cys Mutations in the MAM Domain Affect the Noncovalent Oligomerization of Meprins—Previous work has established that the covalently linked dimers of meprin α associate noncovalently with other dimers to form tetramers and higher oligomeric complexes (14). In addition, the MAM domain has been implicated as an adhesion domain (16, 21), and in spite of the fact that the cysteine mutants formed monomeric units in SDS gels, it is possible that they exist as oligomers under nondenaturing conditions. To determine the monomeric/oligomeric state of native cysteine mutant proteins, they were subjected to electrophoresis on nondenaturing gradient gels (Fig. 3). Under the conditions used, native proteins could be separated mainly on the basis of size (26). These data show that the C320A mutant protein is a monomer under nondenaturing conditions, with an apparent molecular mass of about 130 kDa. The observed subunit molecular mass in the nondenaturing gel was larger than that observed in SDS-PAGE (95 kDa); however, the value is consistent with previous results for monomeric meprin subunits in this gel system (14). The wild-type α protein exists predominantly in a higher oligomeric state, and the C289A mutant protein is a mixture of monomers and oligomers (dimers) under these conditions. Meprin A, purified from

dimers as assessed by SDS-PAGE in the absence of β-mercaptoethanol (Fig. 2). One interpretation of these data is that the C289A mutation allowed covalent S–S bridging between subunits but that covalent dimerization was a less efficient process with the mutant compared with wild-type protein. The data lend further support to the conclusion that Cys-320 is likely responsible for intersubunit S–S bridging.

Cys Mutations in the MAM Domain Affect Glycosylation of Meprin α—Expression of meprin α cysteine mutants in 293 cells. Wild-type (wt) or mutant meprin α subunits in which specific Cys residues were altered to Ala (C289A and C320A mutants) were transiently expressed in 293 cells. The tissue culture media containing the secreted expressed subunits were subjected to SDS-PAGE (7.5% gels) in the presence or absence of β-mercaptoethanol (β-ME); immunoblots were performed using anti-meprin α antibodies.
Table I

| Substrate | wt meprin | C289A meprin | C320A meprin | Kidney meprin A |
|-----------|-----------|--------------|--------------|----------------|
| Specific activity |          |              |              |                |
| BK+       | 6.06      | 10.9         | 8.32         | 6.78           |
| ABz-AAF-pNA | 9.7       | 7.7          | 7.1          | 11.1           |
| Azocasein | 10 × 10^5 | ND           | ND           | 12 × 10^5      |
| kcat/Km | 3.7 × 10^3 | 10.1 × 10^5 | 7.0 × 10^5 | 6.1 × 10^5 |

The cysteine mutants and wild-type meprin had comparable specific activities against the bradykinin analog and ABz-AAF-pNA (Table I). Analysis of the catalytic efficiencies (kcat/Km values) using the BK+ substrate also indicated that the mutant proteins were as active as wild-type proteins. Activity of the mutant subunits against azocasein, however, was markedly decreased. No activity of the mutants could be detected with azocasein as substrate at 30 or 37 °C; at these temperatures at least 1,000 units/mg would have been measurable. The peptide-degrading activity of wild-type and mutant meprins was stable at 30 °C over 30 min, the same conditions under which azocaseinase activity was measured. The results shown on Table I were typical for several different transfection preparations.

The recombinant meprins and purified kidney meprin A were also tested for activity against gelatin on zymographs (29). Purified kidney meprin and the recombinant wild type degraded gelatin, whereas no gelatinase activity was observed for the cysteine mutants (data not shown).

Because the cysteine mutant proteins were considerably more vulnerable to proteolytic degradation than the wild-type protein, the vulnerability of the proteins to heat inactivation was further tested (Fig. 5). The cysteine mutant proteins were inactivated, as determined by activity against BK+, much faster than wild-type protein at temperatures of 40 °C and above. Half the activity of the cysteine mutant proteins was lost in 2 min at 40 °C, whereas the half-life for the wild-type protein

were found (5–10 ng of trypsin/ml), indicating that the mutants were totally degraded. Further investigations of the susceptibility of the Subunit to Proteolysis and Heat Inactivation—To determine whether the mutants correctly fold into catalytically active conformation, proteolytic activity was measured for the wild-type and mutant meprins. Meprin subunits are secreted as inactive proproteases in cultured cells and have to be treated with trypsin-like proteinases to remove the prosequence and activate the protein (1,15). Trypsin activation decreases the molecular mass by approximately 5 kDa and results in a mobility shift on SDS-PAGE (Fig. 4). Under conditions that were usually used to activate wild-type meprin α in the culture medium (40 ng of trypsin/μl), the cysteine mutants were totally degraded. Further investigations of the susceptibility of the proteins to trypsin indicated that the mutants were more susceptible to proteolytic degradation than the wild type (Fig. 4). Conditions were found (5–10 ng of trypsin/μl of sample) where the mutants were activated but not degraded. Alternatively, the protease Arg-C (40 ng/μl) could be used to activate wild-type and mutant enzymes without degrading the bulk of the protein (data not shown).

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The recombinant meprins and purified kidney meprin A...
The folding of monomers reflects different environments and interactions with other proteins. The work clearly shows that MAM domain cysteine mutations on the ability of meprin to hydrolyze azocasein implicate complex domain or subunit interactions for this activity. It is possible that there are interactions between subunit active sites for protein hydrolysis or that there is a different conformation of the active site in monomers and oligomers. There has been no indication of cooperative interactions between subunits in kinetic studies of meprin A (8). However, studies with inhibitors have yielded unexpected results that may indicate complex interactions at the active sites. For example, the inhibitor actinonin is a simple competitive inhibitor of astacin but shows complex inhibition patterns with meprin A (competitive/noncompetitive), and products of bradykinin hydrolysis that would be predicted to be simple competitive inhibitors show mixed inhibition patterns for meprin A.

The differences in the glycosylation of the mutants and wild-type meprin subunits could contribute to differences in resistance to proteases and oligomerization. There is at least one N-linked glycosylation site in the MAM domain near Cys-320; the Cys mutations could affect the interaction of that site with cellular glycosidases, which could in turn expose normally protected peptide bonds to proteases. Oligomerization to high molecular weight complexes may also confer increased proteolytic resistance by protecting areas of the protein that are vulnerable to proteases. Native forms of meprin are quite resistant to proteases; for example, functionally intact meprin can be solubilized from membranes by treatment with high concentrations of papain or trypsin/toluene. Many brush border enzymes exist as oligomers, and a standard treatment for solubilization of the bulk of these enzymes is to incubate membranes with proteases. Intracellular enzymes may also be stabilized by oligomerization, as in the case of cathepsin E. The latter aspartic proteinase is a homodimer in which the subunits are linked by a disulfide bond. Mutation of a specific cysteine residue resulted in the formation of some dimers but also resulted in dramatic effects on oligomerization, activity, and stability of the subunit indicating that the covalent intersubunit disulfide bridges stabilize units to allow noncovalent interactions of the subunits or domain swapping. Whatever the mechanism, it is clear that the MAM domain acts as an adhesion domain and stabilizes subunit interactions, protein structure, and the function of the enzyme. The role of the disulfide interactions between subunits may be to stabilize subunit interactions so that higher oligomeric forms are favored.

MAM domains have been identified recently as distinct protein modules in several proteins, and one study has specifically addressed the role of this domain in protein structure and function. Zondag et al. (21) demonstrated that the MAM domains of receptor protein-tyrosine phosphatase μ and κ are essential for mediating specific homophilic cell-cell interactions in transfected nonadherent insect cells. The present study provides an example where the MAM domain mediates specific homooligomeric interactions of a secreted protein. In vivo, MAM domain interactions result in the concentration of meprin α subunits at the cell surface in association with integral membrane β proteins.

The effect of MAM domain cysteine mutations on the ability of meprin to hydrolyze azocasein implicates complex domain or subunit interactions for this activity. It is possible that there are interactions between subunit active sites for protein hydrolysis or that there is a different conformation of the active site in monomers and oligomers. There has been no indication of cooperative interactions between subunits in kinetic studies of meprin A (8). However, studies with inhibitors have yielded unexpected results that may indicate complex interactions at the active sites. For example, the inhibitor actinonin is a simple competitive inhibitor of astacin but shows complex inhibition patterns with meprin A (competitive/noncompetitive), and products of bradykinin hydrolysis that would be predicted to be simple competitive inhibitors show mixed inhibition patterns for meprin A.

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enzymes may be to allow survival of active enzymes in a protease-rich environment. While meprin structures are unique for known proteases, they may serve as excellent models for other mosaic, multimeric proteins.

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