MEPRIN A OLIGOMERIZATION:
CONTRIBUTIONS OF N-LINKED GLYCOSYLATION AND
THE MAM DOMAIN

A Thesis in
Integrative Biosciences

by
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ABSTRACT

The oligomerization of proteins can serve to activate or inhibit functional properties of the proteins, create new functions of the oligomeric complex based on subunit composition, concentrate enzymatic activity, and localize the proteins to a particular region of the cell or extracellular space. Meprins are zinc metalloproteases of the astacin family and metzincin superfamily that are obligate oligomers, targeted to the cell surface of brush border epithelial cells in the kidney and intestine, as well as to the surface of certain leukocytes and cancer cell lines. Meprins are composed of two related subunits, α and β. Depending on the subunit composition, the meprin oligomers range in size from a membrane-bound, disulfide-bonded dimer (homooligomeric meprin B), to tetramers of heterooligomeric meprin A, to large, secreted α oligomers of ten or more subunits (homooligomeric meprin A). The work herein investigated the contributions of N-linked glycosylation and the MAM (meprin, A5 protein, protein tyrosine phosphatase μ) domain to the formation and activity of the meprin A homooligomer.

Meprins are highly glycosylated, with carbohydrate accounting for approximately 20% of the subunit molecular mass. Using chemical deglycosylation and electrospray mass spectrometry (ESI/MS), it was shown that at least seven of the ten potential N-linked glycosylation sites of mouse meprin A are glycosylated. The glycans are mainly composed of N-acetylglucosamine (GlcNAc), mannose, galactose, and a small amount of fucose. Three glycosylation sites, N234 and N270 in the protease domain and N452 in the TRAF (tumor necrosis factor receptor associated factor) domain, were found to be removed by PNGaseF under native conditions. Removing these glycans did not
markedly affect the protein conformation or oligomeric state, but decreased the stability of the oligomer as assessed by urea and heat denaturation.

A series of single and double glycosylation site mutants were used to determine the contribution of glycosylation to the formation of the meprin oligomer. No one individual glycosylation site affected formation of the oligomer, although one glycosylation site (N41) influenced the amount of secreted protein in the cell culture system. Interestingly, two protease domain glycosylation sites (N152 and N270) were implicated in oligomerization. The N152Q/N270Q and N152Q/N234Q/N270Q mutants were greatly impaired in their ability to form not only the high molecular mass oligomers, but also the disulfide-bonded dimers. These mutations, unlike the other single and double mutants examined, did not retain proteolytic activity, demonstrating that glycosylation of the protease domain is involved in correct tertiary and quaternary structure formation.

The noncatalytic domains of meprin, MAM and TRAF, have previously been shown to contribute to oligomerization of meprin A. Here, a series of single point mutants at charged residues in the putative MAM noncovalent interface (K352G, K361Y, R369Q, R376E, D377Y, D378T, R384G, K388L) were created. All mutants were shown to affect formation of the noncovalent interface, with the exception of K361Y. These mutants did not affect disulfide bond formation or proteolytic activity. However, as several variants at position R384 demonstrate, the effect of these residues is not based solely on the presence or absence of charge, but may reflect a strict sequence requirement in the MAM domain of the α subunit.
As these studies demonstrate, charged residues of the MAM domain as well as posttranslational modifications influence the oligomeric structure of meprin A. However, as these and previous studies have shown, it is unlikely that one particular feature of the α subunit can be introduced into the β subunit to promote oligomerization of meprin B to higher order oligomers. This highlights the highly complex folding process that must occur for each meprin subunit.
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<tr>
<td>BCA</td>
<td>bicinchoninic acid</td>
</tr>
<tr>
<td>BK+</td>
<td>an internally quenched fluorescent bradykinin analog</td>
</tr>
<tr>
<td>BME</td>
<td>β-mercaptoethanol</td>
</tr>
<tr>
<td>BMPH</td>
<td>N-(β-maleimidopropionic acid) hydrazide trifluoroacetic acid</td>
</tr>
<tr>
<td>C</td>
<td>cytoplasmic domain</td>
</tr>
<tr>
<td>CD</td>
<td>circular dichroism</td>
</tr>
<tr>
<td>CNX</td>
<td>calnexin</td>
</tr>
<tr>
<td>CRT</td>
<td>calreticulin</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle’s Medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethylsulfoxide</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>EDEM</td>
<td>ER degradation-enhancing α-mannosidase-like protein</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGF</td>
<td>epidermal growth factor-like domain</td>
</tr>
<tr>
<td>EndoH</td>
<td>endoglycosidase H</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>ERAD</td>
<td>ER-associated degradation</td>
</tr>
<tr>
<td>ESI/MS</td>
<td>electrospray ionization mass spectrometry</td>
</tr>
<tr>
<td>FAB/MS</td>
<td>fast atom bombardment mass spectrometry</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>GalNAc</td>
<td>N-acetylgalactosamine</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>GlcNAc</td>
<td>N-acetylglucosamine</td>
</tr>
<tr>
<td>GPI</td>
<td>glycosyl phosphatidylinositol anchor</td>
</tr>
<tr>
<td>HEK-293</td>
<td>human embryonic kidney-293 cells</td>
</tr>
<tr>
<td>HNK-1</td>
<td>human natural killer-1</td>
</tr>
<tr>
<td>HPAE</td>
<td>high pH anion exchange</td>
</tr>
<tr>
<td>HPLC</td>
<td>high performance liquid chromatography</td>
</tr>
<tr>
<td>MALDI/TOF</td>
<td>matrix assisted laser desorption ionization/time-of-flight</td>
</tr>
<tr>
<td>MAM</td>
<td>meprin, A5 protein, protein tyrosine phosphatase μ domain</td>
</tr>
<tr>
<td>MBP</td>
<td>mannose binding protein</td>
</tr>
<tr>
<td>MDCK</td>
<td>Madin-Darby canine kidney cells</td>
</tr>
<tr>
<td>MS</td>
<td>mass spectrometry</td>
</tr>
<tr>
<td>OCK+</td>
<td>an internally quenched fluorescent analog of orcokinin</td>
</tr>
<tr>
<td>OST</td>
<td>oligosaccharide transferase</td>
</tr>
<tr>
<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PDI</td>
<td>protein disulfide isomerase</td>
</tr>
<tr>
<td>PNGaseF</td>
<td>peptide N-glycosidase F</td>
</tr>
<tr>
<td>Pro</td>
<td>prosequence</td>
</tr>
<tr>
<td>PTPμ</td>
<td>protein tyrosine phosphatase μ</td>
</tr>
<tr>
<td>RPTP</td>
<td>receptor-like protein tyrosine phosphatase</td>
</tr>
<tr>
<td>S</td>
<td>signal sequence</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>TBS-T</td>
<td>Tris-buffered saline with 0.1% Tween-20</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
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<td>----------------------------------------</td>
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<tr>
<td>TCEP</td>
<td>Tris-(2-carboxyethyl)phosphine hydrochloride</td>
</tr>
<tr>
<td>TFA</td>
<td>trifluoroacetic acid</td>
</tr>
<tr>
<td>TFMS</td>
<td>trifluoromethane sulfonic acid</td>
</tr>
<tr>
<td>TM</td>
<td>transmembrane domain</td>
</tr>
<tr>
<td>TRAF</td>
<td>tumor necrosis factor receptor-associated factor domain</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
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Finally, I am truly grateful for this opportunity to learn and to achieve this degree, in addition to the many wonderful blessings that God pours out upon me every day in the people and experiences of my life.
1.1 Meprin Metalloproteases

1.1.1 Classification and Expression

Meprins are cell-surface and secreted metalloproteases that contain a single active site zinc to facilitate peptide bond hydrolysis and are members of the metzincin superfamily of proteases. The metzincin superfamily includes the matrixin (e.g. matrix metalloproteases), reprolysin (e.g. adamlysin), serralysin, autolysin, small neutral protease, and astacin (e.g. meprins) families (1). Like most metalloproteases, metzincins are characterized by the zinc binding motif HEXXH that contains two of the zinc ligands. The third zinc ligand is a His for all metzincins, while meprins and other astacins have a conserved Tyr residue and a water molecule as the fourth and fifth ligands. This arrangement creates an unusual trigonal bipyramidal geometry (1,2). Metzincins are also characterized by a conserved Met residue that creates a “Met turn” in the structure, allowing the third His ligand to coordinate the zinc (3). The astacin family encompasses proteases with distinct functions in development and maturation from a wide range of organisms. The first members identified included astacin, a digestive enzyme in the European crayfish, the extracellular matrix reshaping protease bone morphogenetic protein-1 (BMP-1), a developmental protein in *Xenopus laevis*, and meprins (4). In addition to
expression of astacin family members in humans, rodents, and other mammals, astacins have also been identified in fish, sea urchin, quail and chicken, hydra, squid, Drosophila melanogaster, Caenorhabditis elegans, and even a prokaryotic flavobacterium (5).

Even though other astacins are multidomain proteins, meprins are unique in that they exist as oligomers. Astacin, consisting of a single catalytic domain, is monomeric. Meprin oligomers are composed of two related subunits, \( \alpha \) and \( \beta \), that are encoded on separate genes and are expressed in humans, mice, and rats. The \( \alpha \) subunit is encoded on chromosome 17 in mouse (chromosome 6 in humans), near the major histocompatibility complex, while the meprin \( \beta \) subunit is encoded on chromosome 18 in both mice and humans (6-8). Meprins are most abundantly expressed in the specialized brush border membranes of epithelial cells in the kidney juxtamedullary proximal tubules and throughout the intestine and colon (7,9-12). Under normal, healthy conditions, meprins are targeted exclusively to the apical side of these polarized epithelial cells (13,14). However, in certain diseased states the proteins can be found on the basolateral side of the cell, exposing the basement membrane to the action of this protease (15,16).

Recently, meprins were shown to be expressed on leukocytes and in certain cancer cells, thus expanding their role to inflammatory processes and cancer metastasis (5,12,15,17). Both meprin \( \alpha \) and \( \beta \) subunits are expressed in mouse and rat embryonic kidney and intestine, although the expression pattern of each subunit changes during distinct phases of pre- and post-natal development (18,19). Meprin expression has also been detected in rat embryonic neuroepithelial cells and certain tissues of the craniofacial region (20).
While the most abundant expression occurs in the kidney and intestine of adult rodents and humans, meprins have also been detected in adult mouse salivary glands (9).

1.1.2 Meprin Protein Structure and Substrate Specificity

The meprin α and β subunits have similar cDNA deduced domain structures (Figure 1). There is an N-terminal signal sequence that directs the protein into the endoplasmic reticulum (ER) and is subsequently cleaved during biosynthesis. A prosequence maintains the protease in a latent state until it is proteolytically removed. In tissue culture, meprins are synthesized in the latent form; the prosequence can be removed by limited proteolysis with trypsin. In kidney and intestinal tissues, meprins exist in both latent and active forms; these active forms are due to action of proteases in the cell surface environment (21-25). A catalytic domain containing the zinc active site is followed by MAM (meprin, A5 protein, protein tyrosine phosphatase μ) and TRAF (tumor necrosis factor receptor associated factor) domains. The latter two domains are not themselves catalytic, but they direct protein folding and oligomerization of meprin subunits into active enzymes (26,27).

The main difference between the α and β subunits is the presence of a 56 a.a. inserted (I) domain in the α subunit that directs proteolytic removal of the EGF (epidermal growth factor-like), TM (transmembrane), and C (cytosolic) domains (Figure 1). This creates a secreted meprin α subunit, while the β subunit remains membrane-bound as a type I integral membrane protein (28). However, a small portion of human meprin β is constitutively shed from the cell surface by cleavage in a 13 a.a. “stalk”
**Figure 1. Domain organization and processing of the meprin α and β subunits.** The meprin α subunit is processed to a secreted form by cleavage in the inserted (I) domain, eliminating the EGF (epidermal growth factor-like), TM (transmembrane), and C (cytoplasmic) domains, while the meprin β subunit retains these domains and is a type I integral membrane protein. S, signal sequence; Pro, prosequence; Protease, catalytic domain; MAM, meprin, A5 protein, protein tyrosine phosphatase μ; TRAF, tumor necrosis factor receptor associated factor; I, inserted; EGF, epidermal growth factor-like; TM, transmembrane; C, cytoplasmic.
removed during biosynthesis

β
S Pro Protease MAM TRAF EGF TM C

α
S Pro Protease MAM TRAF I EGF TM C

proteolytic processing in the ER

Inserted domain

mature α
Pro Protease MAM TRAF
region (29). In addition to processing at the amino acid level, meprins are highly glycosylated and contain multiple intra- and intersubunit disulfide bonds.

Unlike many other proteases, meprins are capable of cleaving both peptide and protein substrates. The distinct specificities of the α and β subunits allow meprins to cleave a wide range of substrates, including basement membrane proteins (gelatin, collagen, laminin, nidogen), cell surface proteins (an epithelial sodium channel), cytokines (interleukin-1β, osteopontin, MCP-1), and other bioactive peptides (bradykinin, gastrin, glucagons) (5,30,31). The meprin α subunit has a preference for small or hydrophobic residues in the P1 and P1’ subsites and proline in the P2’ subsite, while the meprin β subunit prefers to cleave substrates with acidic residues in the P1 and P1’ subsites (31). These differences in substrate specificity have been mapped to key residues within the active of the subunits (32). Meprin β is most active at acidic pH (pH 5-6), while meprin α has a neutral pH optimum (33).

1.1.3 Meprins and Disease

By combining the distinct specificities of the meprin subunits, these proteases have the potential to play a role in several disease processes, especially ones that are characterized by aberrant proteolysis. Meprin α has been implicated in cancer metastasis, where it is redistributed to the basolateral surface of cultured intestinal carcinomas and increases tumor invasiveness in vitro (5,15). This redistribution of meprin from the apical side of the cell brings it in contact with many basement membrane proteins that have been shown to be good meprin substrates. Expression of meprin α and
β has been detected in certain leukocytes of the intestinal lamina propria, and α expression decreases during intestinal inflammation (12,17). In addition, leukocytes in the meprin β knockout mouse have a decreased ability to move through a synthetic extracellular matrix (17). It is possible that meprins also cleave critical cytokines that regulate inflammation. Meprin β has been shown to cleave the proinflammatory cytokine interleukin-1β in vitro, which in turn activates the cytokine (30). Meprin α is capable of removing a short peptide from the N-terminus of monocyte chemoattractant protein-1 (MCP-1). Removal of this peptide decreases the binding of MCP-1 to one of its major receptors (5). Both of these processes (invasiveness and cytokine cleavage) can greatly influence the immune response. Recent experiments with the meprin β knockout mice show that there is a difference in the response of β null and wild-type mice in the dextran sodium sulfate-induced model of inflammatory bowel disease (C. Valeski and G. Bradley, personal communication). In the kidney, meprin β has been identified as a candidate gene in diabetic nephropathy (34,35). Meprins are also redistributed to the basolateral surface of kidney cells during acute renal failure, and it has been observed that certain strains of inbred mice with low meprin α expression have decreased renal damage (16,36). Furthermore, meprins also had a cytotoxic effect on renal cells in vitro (37). Elevated levels of meprin α have been detected in the urine of woman with acute urinary tract infections, suggesting a role for meprins in this disease process as well (38).
1.1.4 Meprin Oligomerization

Meprin α and β subunits are approximately 40-50% identical at the amino acid level, yet they have distinct oligomeric properties. Meprins form oligomers that vary in size depending on subunit composition (Figure 2). The basic unit for all forms is a disulfide-bonded dimer. Meprin B, composed of only β subunits, is a membrane-bound, disulfide-linked dimer that does not form any larger species even at high protein concentrations (39,40). The three intersubunit disulfide bonds of the meprin B dimer impart stability to the oligomer (39).

Heterooligomeric meprin A is composed of both α and β subunits and forms tetramers both in vivo and in vitro, either α₂β₂ or α₃β (28,40,41). Since mature meprin α does not retain a transmembrane domain and is normally secreted, the association of meprin α with meprin β in the heterooligomer localizes meprin α (and thus its proteolytic activity) to the cell surface (13,28,42,43).

The meprin A homooligomer is composed solely of α subunits. Unlike meprin B, this homooligomer can associate noncovalently to form large crescent, ring, spiral, and barrel structures of 10-100 subunits (Figure 2) (40,44). This oligomerization serves to concentrate meprin activity in the extracellular milieu, where it can be activated at a site downstream of the proximal tubules or intestine (45). Meprin A can be detected as a large oligomer in the urine of rats, mice, and humans (38,40,45,46). In the absence of meprin β expression, as in the β knockout mouse, meprin α is exclusively secreted into the lumen of the kidney (47). Exclusive secretion of meprin A also occurs in the human colon since meprin β subunit expression is lacking (12). The distribution of meprin
Figure 2. Oligomeric structure of the meprin isoforms. The meprin subunits form homo- and heterooligomers through disulfide bonds (S=S) as well as noncovalent interactions to create distinct isoforms that are membrane-bound and secreted. A tetramer of homooligomeric meprin A is enlarged to illustrate the covalent and noncovalent interfaces; this isoform of meprin forms an octamer or larger. *Light blue, α* subunits; *magenta, β* subunits.
Secreted homooligomeric meprin A

brush border membrane

heterooligomeric meprin A

homooligomeric meprin B

covalent interface
noncovalent interface

S—S
S—S
S—S
isoforms that are at the cell surface or secreted into the lumen depends on the relative expression of each subunit. In mouse kidney, there is a two-fold higher rate of $\alpha$ subunit synthesis that leads to the presence of both heterooligomers and homooligomers of meprin A (48). Interestingly, the homooligomer of meprin B, a $\beta_2$ dimer, is not found in kidneys when meprin $\alpha$ is expressed, suggesting that the $\beta$ subunit preferentially associates with the $\alpha$ subunit when both subunits are synthesized (28). The size of the meprin A homooligomer is sensitive to the salt concentration, which may affect its function in the kidney (40).

Several factors have been identified that affect the oligomerization of meprin subunits. Intersubunit disulfide bonds form in the MAM domain, creating the disulfide-bonded dimer. Mapping studies of rat meprin B indicate that there are two intersubunit disulfide bonds in the MAM domain, a finding that is also supported by site-directed mutagenesis (39,49). Cysteine mutants of homooligomeric mouse meprin A also implicate two MAM domain cysteines in intersubunit disulfide bonds (50). However, meprin B contains an additional intersubunit disulfide bond in the TRAF domain, at C492, that is absent in meprin A. This difference may be one factor that prevents meprin B from forming larger oligomers by altering the overall protein fold. A short sequence of 9 a.a. is required in the meprin $\alpha$ TRAF domain to promote oligomerization and create a stable, active enzyme (27). Even the protease domain promotes oligomerization; mutation of one of the zinc ligands (H167) in the active site of mouse meprin A resulted in a protein that was secreted as a monomer lacking any proteolytic activity (51).
As these studies have shown, several factors influence the ability of meprin subunits to oligomerize. Since the meprin A homooligomer is the largest known protease, it is of interest to further define characteristics of the meprin α subunit that drive oligomerization. In particular, N-linked glycosylation has the potential to influence protein structure in several manners. Although it is known that the presence of the meprin α MAM domain is critical for biosynthesis of the protein, specific features of this domain that influence oligomerization have not been defined. Thus, it was the aim of these studies to determine the role of N-linked glycosylation and the MAM domain in the quaternary structure of meprin A.

1.2 Glycans and Their Role in Protein Structure and Function

1.2.1 Types of glycosylation

It is predicted that more than half of eukaryotic proteins are glycoproteins (52). Although there are at least 41 documented modifications of proteins by carbohydrates in eukaryotes, archaeabacteria, and bacteria (53), the most common of these is the attachment of an oligosaccharide at either Asn or Ser/Thr residues. The former modification is known as N-linked glycosylation, since the oligosaccharide is attached via an amide bond to the side chain of asparagines residues in a consensus sequence of N/X/(S/T), where X is any amino acid except proline (Figure 3a). The main characteristic of N-linked glycosylation is the presence of an oligosaccharide core consisting of three mannose residues and two GlcNAc residues, one of which is attached directly to the
Figure 3. Types of glycosylation. The two types of glycosylation are named for the atom of the amino acid side chain through which the glycans are connected. 

a) N-linked glycans are attached to an Asn residue in the consensus sequence of N/X/(S/T), where X is any amino acid except proline. N-linked glycans are characterized by an innermost N-acetylglucosamine residue (GlcNAc).

b) O-linked glycans are attached to Ser or Thr residues through the side chain hydroxyl group, and are characterized by an innermost N-acetylgalactosamine (GalNAc).

Figure adapted from www.ionsource.com/Card/carbo/nolink.htm
Asn residue (Figure 4, residues shown in red). The N-linked oligosaccharides are synthesized in a high mannose form (Glc₃Man₉GlcNAc₂), transferred en bloc to a nascent polypeptide in the ER, and subsequently modified by glycosidases and glycosyltransferases in the Golgi apparatus to form the three types of N-linked carbohydrates. The three types of glycans are defined by the composition of their outer antennae (Figure 4). Complex glycans have no mannose (Man) residues except in the glycan core. They have the most structural variation, and can have one to five antennae modified by galactose (Gal), N-acetylmuramic acid (NeuNAc, or sialic acid), GalNAc, and fucose (Fuc), in addition to GlcNAc (Figure 4a). The antennae of high mannose glycans contain only mannose, as the name implies (Figure 4b), while hybrid glycans have features of both other types (Figure 4c). Each of the monosaccharides depicted in Figure 4 are attached in specific linkages and on particular hydroxyl groups of each sugar. These linkages positions are not shown for the sake of simplicity. However, these linkages dictate the overall presentation of the glycan, which is particularly important for recognition of the glycans on the protein’s surface by carbohydrate binding molecules.

O-linked glycosylation is defined as modification of Ser/Thr residues with an oligosaccharide via the hydroxyl group of the side chain (Figure 3b). The structures of these oligosaccharides are less well defined although several different core groups are observed. The innermost residue of O-linked oligosaccharides is a GalNAc attached to the Ser/Thr side chain, instead of GlcNAc. O-linked glycans are found in mucins, but proteins can carry both N-linked and O-linked glycans, as in the case of the model glycoprotein, fetuin. O-linked glycosylation is a Golgi event that occurs after
**Figure 4. Types of N-linked glycosylation.** The N-linked glycans are further categorized into three groups based on the monosaccharide composition of the antennae. Examples of *a*) complex, *b*) high mannose, and *c*) hybrid glycans are shown. Residues in red indicate the core oligosaccharide. *GlcNAc*, N-acetylglucosamine; *Man*, mannose; *Gal*, galactose; *NeuNAc*, N-acetylneuraminic acid (sialic acid); *Asn*, asparagine.
proteins have folded. In one of several variations on O-linked glycosylation, nuclear and cytoplasmic proteins can be modified by the addition of a single GlcNAc residue on Ser and Thr. This type of glycosylation has been proposed to be involved in regulation of protein function, similar to phosphorylation. However, this modification does not occur in the Golgi compartment (53).

With the exception of the N-linked glycan core structure, glycosylation is not template driven, as is transcription and translation of genes to form proteins. Thus, the extent of glycosylation, as well as the composition of the glycans themselves, is largely dependent on the cell type and cellular conditions such as concentration of sugar donors, expression of glycosylation machinery enzymes, structure of the protein being modified, and the amount of time spent in the Golgi compartment (54,55). Hence, glycosylation is often heterogeneous and this heterogeneity is defined at two levels (56). First, there can be variation in the usage of a particular glycosylation site in a protein (macroheterogeneity). Second, when a glycan is present, there can be differences in the composition of the branched antennae (microheterogeneity). Thus, a given glycosylation site in three different molecules of a protein can each have a different oligosaccharide structure.

This heterogeneity at both the protein and glycan levels can be used to fine tune protein function, as well as to change recognition of the protein by other molecules. However, it also complicates the analysis of glycans and their role in protein structure and function. Defects in the formation of this glycan fine structure can lead to diseases such as leukocyte adhesion deficiency type II (LAD II), which results from a deficiency in the fucosylation of glycans (57). In LAD II patients, the sialyl-Lewis X epitope is
altered, and a group of molecules called selectins are not able to recruit neutrophils to the sites of infection. This leads to severe infections with high circulating neutrophil counts, mental retardation, and skeletal abnormalities (57,58).

1.2.2 Roles of glycosylation in protein biosynthesis and function

The biological role of glycosylation is diverse (59). One of its most important functions is in the ER quality control system, where protein folding is monitored and assisted by resident ER chaperones. It can also influence the physico-chemical properties of a protein, such as stability and solubility. At the cell surface, it can mediate interactions between proteins, and even entire cells, by serving as determinants of recognition and binding.

1.2.2.1 Glycosylation in protein biosynthesis and ER quality control

N-linked glycans are added to nascent proteins cotranslationally as the proteins emerge from the ribosomes and pass into the ER lumen through the Sec61 translocon (60). As mentioned above, the addition of glycans to Asn residues occurs with the transfer of a preformed core oligosaccharide \((\text{Glc}_3\text{Man}_9\text{GlcNAc}_2)\), Figure 5) from a dolichol carrier via the action of the multisubunit oligosaccharide transferase (OST) (61). Problems with this initial transfer lead to underglycosylation of proteins and Congenital Disorders of Glycosylation Type I (62). As soon as a potential glycosylation site is 12-14
Figure 5. A core oligosaccharide is transferred *en bloc* to nascent proteins. This oligosaccharide has three glucoses (circles), nine mannoses (triangles), and two GlcNAc (squares) residues. The numbered glucose residues are sensitive to the ER glucosidases, while the mannose residues are sensitive to the ER and Golgi mannosidases that recognize the $\alpha$-1,2 linkage through which these mannoses are attached.
UDP-glc:glycoprotein glucosyltransferase

α-1,2-mannosidases

Asn
residues from the translocon, it can be glycosylated (63). This allows the protein to enter
the ER quality control cycle by binding to the pair of ER resident chaperones, membrane-
bound calnexin (CNX) and its soluble analog, calreticulin (CRT). These lectin-based
chaperones bind nascent glycoproteins and monitor their folding, recruit other ER
proteins such as certain members of the protein disulfide isomerase (PDI) family, and
help to target terminally misfolded glycoproteins for ER-associated degradation (ERAD)
(61).

Interactions between nascent glycoproteins and CNX or CRT require a
monoglucosylated core oligosaccharide. The combined actions of glucosidases I and II
remove the first two glucoses, leaving a single glucose on the core glycan
(Glc$_1$Man$_9$GlcNAc$_2$; Figure 5). The monoglucosylated core glycan is then recognized by
CNX and CRT (64). Removal of the final glucose residue by glucosidase II releases the
glycoprotein from CNX and CRT, but if the protein is still not correctly folded it can be
re-glucosylated by UDP-Glc:glycoprotein glucosyltransferase that recognizes the unfolded
protein conformation (Figure 5) (65). Glycoproteins can continue to be de-glucosylated
and re-glucosylated, allowing them to enter and exit the chaperone cycle multiple times
(66). In this way, glycosylation serves to retain glycoproteins in the ER until they
complete proper folding.

Despite multiple rounds of CNX/CRT binding, some proteins form terminal
misfolds and need to be degraded. This is mediated by trimming of four mannose
residues (Figure 5, mannoses numbered 1-4) from the glycan by the slow action of ER
mannosidases. It was originally thought that the signal for degradation was the removal
of the mannose from the middle arm of the glycan (Figure 5, mannose #2) by ER
mannosidase I, thus creating a $\text{Man}_9\text{GlcNAc}_2$ structure. This $\text{Man}_9\text{GlcNAc}_2$ structure is a poor substrate for both UDP-Glc:glycoprotein glucosyltransferase and glucosidase II and would lead to “molecular capture” by CNX/CRT since the protein cannot be released from CNX and CRT without the action of glucosidase and it cannot reenter another folding cycle without UDP-Glc:glycoprotein glucosyltransferase (64). In another version of this molecular capture model, endomannosidase I releases Glc-Man (Figure 5, glucose #3 and mannose #1) that also creates the $\text{Man}_9\text{GlcNAc}_2$ signal (64). However, a more recent model proposed by Lederkremer and Glickman (67) suggests that it is the extensive trimming of the glycan to $\text{Man}_6\text{GlcNAc}_2$ that commits the substrate to ERAD, because the $\text{Man}_9\text{GlcNAc}_2$ and $\text{Man}_7\text{GlcNAc}_2$ glycans, though poorer substrates than $\text{Man}_9\text{GlcNAc}_2$, can still be deglucosylated and reglucosylated. The removal of two other mannoses to create $\text{Man}_6\text{GlcNAc}_2$ removes the protein from the folding cycle permanently because it removes the mannose that is the glucose acceptor (Figure 5, mannose #1). Thus, the removal of the first few mannoses slows down the process of degradation and allows the protein ample time to fold by entering successive rounds of the CNX/CRT cycle, but once the critical mannose is removed, the protein can be recognized by EDEM (ER degradation-enhancing $\alpha$-mannosidase-like protein) and targeted for retrograde transport through the Sec61 channel for degradation by the proteasome (68,69). If a protein is folded correctly, further removal of the remaining mannose residues by other Golgi mannosidases and the action of Golgi glycosyltransferases leads to the three different types of N-linked glycans (70).

ER chaperones affect the folding of proteins mainly by slowing down the folding process. Although the rate of folding is decreased, this increases folding efficiency
because it prevents competing, nonproductive folds or aggregates from occurring, as well as nonnative disulfide bonds from forming (65). Actual folding catalysts then accelerate the slow steps, such as disulfide bond formation (71). Since CNX and CRT can bind to a protein as soon a consensus sequence is glycosylated and deglucosylated, they can, in a sense, sequester more N-terminal regions of the protein while the C-terminal regions are being synthesized. For example, tyrosinase, an enzyme involved in melanogenesis, can fold more quickly in the absence of CNX interactions, but this higher rate of folding causes the protein to misfold and certain glycans are implicated in this interaction (72,73). Not all glycoproteins require CNX and CRT to achieve a native, or near native, conformation. Treatment of cells in culture with glucosidase inhibitors such as castanospermine or expression of proteins in glucosidase-deficient cells has protein-dependent effects. For instance, meprins can fold in the absence of CNX and still retain activity, but tyrosinase-related proteins are misfolded and less active (14,72-75). In the case where proteins can fold in the absence of CNX and CRT, other ER resident chaperones such as BiP can be utilized (75,76). In fact, CNX and CRT have been shown to be part of a large network of ER proteins, including BiP and GRP94 (77). Despite this, CRT knockout mice are lethal due to impaired cardiac development, and CNX knockout mice die early after birth due to motor disorders (78,79).

The N-linked glycans of a glycoprotein also direct chaperone selection. The work of several groups has showed that CNX and CRT act on different parts of the influenza hemagglutinin protein and the Sendai virus fusion protein, and are recruited to these regions by the key positioning of several glycans (80-82). Positioning of the glycans
within a protein sequence also determines whether the protein will utilize CNX/CRT or BiP for early folding events (76).

1.2.2.2 Structure and stability of glycoproteins

Studies of model glycopeptides have shown that glycosylation can alter the conformational preferences of the peptides without inducing any permanent secondary structure, while glycosylation does not significantly alter the overall protein fold of a glycoprotein (83). Instead, glycosylation most often stabilizes an already existing protein fold, by decreasing protein conformational dynamics and imparting backbone rigidity. A study of five partially deglycosylated glycoproteins showed no effects on the circular dichroism (CD) spectra of the proteins, but they were all less thermostable (84). Individual glycoforms can also have variable stability (85,86).

With the exception of the innermost residues, the glycans attached to proteins are fairly flexible and do not interact with the protein backbone (83). The basis for the increased stability of glycoproteins is believed to be an overall increase in entropy gained from the flexibility of the glycans, which compensates for the loss in entropy as the protein adopts a more rigid conformation (87). This effect may also be manifested in the rates of processes that require a conformational change; the more stable a conformation, the less likely it is to change (59). Since glycans can be rather large and extend out over the surface of a protein, they can also affect the half-life of the glycoprotein by shielding the backbone of the protein from proteolytic attack (86,88). In the case of serum IgG, glycans attached to Asn297 in the Fc region make extensive contacts between the
individual chains of the IgG molecule and influence the dynamics of the hinge region (Figure 6) (89). The positioning of the glycans between the subunits prevents them from coming too close together, which abolishes function. Differences in the composition of the IgG glycan are the underlying cause of rheumatoid arthritis. These changes expose a binding surface for the recognition of IgG by mannose-binding protein (MBP) and activation of the complement system (90,91).

The influence of glycosylation on the process of folding and oligomerization has been reported for the influenza hemagglutinin protein. When CNX/CRT interactions are disrupted with glucosidase inhibitors, the protein aggregates and is degraded (66). The glycan attached to Asn81 was also implicated in oligomerization by site-directed mutagenesis studies (80). In this case, the A81Q mutant could still bind to CNX and CRT, but it did not form intersubunit disulfide bonds or trimerize.

Glycosylation can also modulate protein activity. In glutamate carboxypeptidase II, glycans far from the catalytic domain affect activity (92). A subfamily of the transient receptor potential channels, which are receptor-activated cation channels, are modulated from a constitutively active to gated forms by the presence or absence of glycans (93). Several voltage gated sodium channels are also modulated by glycosylation (94).

1.2.2.3 Glycans at the cell surface and in the extracellular space

Glycosylation may also direct targeting of glycoproteins to particular regions of the cell, affect the clearance rates of proteins from the bloodstream, and mediate recognition and binding processes between proteins (56,95). The glycans of membrane-
Figure 6. The crystal structure of the IgG Fc region.  

a) The IgG immunoglobin is composed of four polypeptides, two heavy chains (blue and red) and two light chains (light blue and pink), with the protein backbone shown in a ribbon diagram.  

b) The C_H2 domains in the Fc region of the molecule are glycosylated at Asn297, and the glycans (shown in a surface model) occupy the space between the C_H2 domains from each chain. The IgG model was created using ViewerLite with the PDB coordinates for 1IGT.
bound proteins create a particular pattern on the cell surface that is distinct for each cell type. Cancer cell lines have different glycosylation patterns resulting from differences in the expression levels of Golgi glycosyltransferases, resulting in a change in the composition of the complex oligosaccharides presented on the cell surface (96,97). This change in glycosylation pattern is not merely a result of malignant transformation, but has functional consequences as well. Depending on the particular changes, the altered glycans can either promote invasion or prevent progression of the malignant cells (96,97). These changes often alter the interactions of malignant cells with neighboring cells, as well as cells of the immune system. Glycosylation of cell surface receptors and adhesion molecules also mediates the formation of the immunological synapse between T cells and antigen presenting cells by preventing aggregation of cell surface molecules, stabilizing the proteins at the cell surface, and orienting the proteins for optimal binding at the cell-cell junction (98).

1.2.3 Glycosylation of meprins

The role of glycosylation in protein structure and function varies from protein to protein; one or more of the roles mentioned above may come into play for any particular protein. Homooligomeric mouse meprin A has been shown to interact with both CNX and CRT, which accelerates the folding of the protein without affecting the rate of disulfide bond formation (74,99). A truncation mutant of homooligomeric mouse meprin A that retains only the protease and MAM domains, lacking all of the potential glycosylation sites in this region, was degraded intracellularly, indicating that some
glycosylation is needed for correct folding and secretion (14). The terminal misfolding and degradation of this mutant may also be influenced by the lack of the TRAF domain, since this domain has been shown to be required for proper folding of the protein to a native, active state (27). Tunicamycin, an inhibitor of N-linked glycosylation, also leads to degradation of meprin A (14).

A series of single N-linked glycosylation mutants of homooligomeric mouse meprin A affected activity and stability of the protein to different extents, but no one particular glycosylation site was responsible for targeting meprin to the apical membrane of polarized epithelial cells (14). After traversing through the Golgi compartment, both meprin A and meprin B have mainly complex carbohydrates, as demonstrated by their resistance to EndoH (a deglycosidase that removes high mannose and some hybrid glycans) as well as sensitivity to various lectins. Kidney isolated heterooligomeric meprin A and homooligomeric meprin B differ in their glycan composition, as determined by their sensitivity to lectins (100). Digestion of kidney meprin A with cyanogen bromide and lectin blotting indicated that N330 of the α subunit is glycosylated, as well as one or more of the potential sites in the TRAF domain (101). However, a full map of glycosylation sites has not been determined for any meprin isoform. Human, but not mouse, meprin B has been shown to carry O-linked glycosylation in a “stalk” region in the TRAF domain, and these glycans have been shown to protect meprin B from being shed from the membrane (102).

The location and composition of meprin’s glycans may also play an important role in recognition and regulation of activity at the cell surface. It was recently shown that both meprin α and β activity is decreased by binding of mannose binding protein
(MBP), a lectin involved in complement activation during innate immune responses (103). Furthermore, a nonsulfated form of the human natural killer antigen-1 (HNK-1) carbohydrate epitope has been found on mouse kidney meprins (104). This epitope is found on neural cell adhesion molecules that mediate cell-cell interactions (105).

Glycosylation has been shown to be at or near the noncovalent interface of the active meprin A homooligomer, where it has the potential to influence the quaternary structure of this meprin isoform (106). Glycosylation may be a factor that distinguishes the oligomeric states of meprin A and meprin B. From inspection of a sequence alignment of the meprin $\alpha$ and $\beta$ subunits, there are differences in the number and location of potential N-linked glycosylation sites between the subunits from mouse, rat, and human (Figure 7). Certain potential glycosylation sites are completely conserved in meprin $\alpha$ subunits and absent in meprin $\beta$ subunits, and vice versa. In particular, the site that is homologous to N152 in mouse meprin $\alpha$ is missing in meprin $\beta$ subunits. Likewise, the sites homologous to N422 and N547 in rat meprin $\beta$ are conserved in $\beta$ subunits but not $\alpha$ subunits. Interestingly, the lack of a consensus sequence at the first two sites is only due to a lack of Asn in the first position of the sequence (NIS for $\alpha$ subunits at N152 vs ELS for $\beta$ subunits; NLS for N422 in $\beta$ subunits vs TLT for $\alpha$ subunits). Presence or absence of a particular glycan may alter the folding pattern of meprins by stabilizing critical regions of the structure or altering interactions with the ER protein folding machinery.
Figure 7. Sequence alignment of meprin α and meprin β from mouse, rat, and human. The potential N-linked glycosylation sites are indicated in bold red. Cysteine residues are underlined blue. Glycosylation site are also boxed to highlight the sites that are conserved between species and subunits. Numbering above glycosylation sites is based on the mouse meprin α sequence. *mmep*, mouse; *rmep*, rat; *hmep*, human. *a*, α subunit; *b*, β subunit.
1.3 The MAM domain

MAM domains were defined based on homology of this region between meprins, the *Xenopus* A5 protein (also known as neuropilin), and a member of the receptor-like protein tyrosine phosphatase (RPTP) family, PTPμ (107). The two signature sequences for MAM domains are tChtFahxxtt and ttGhhxhD-hxh (where h is a hydrophobic residue, t is a turn-like or polar residue, +/- are charged residues, and capitalized, bolded letters are strictly conserved amino acids). Overall, there is only about 20-30% homology between proteins containing MAM domains, and the domain is found in proteins with very diverse functions. The intestinal activator of trypsinogen, enteropeptidase, contains one MAM domain (108). Pig and mouse zonadhesins also contain one and three MAM domains, respectively, and are involved in binding of sperm to the zona pellucida of eggs (109,110). Other proteins contain multiple MAM domains, such as apical endosomal glycoprotein from rat (6 MAMs) and the thyroid hormone induced protein B from *Xenopus laevis* (4 MAMs), as determined by a pattern and profile search using PROSITE (http://www.expasy.org/cgi-bin/nicedoc.pl?PD0C00604). The recently cloned MDGA 1 and 2 proteins (MAM domain GPI-anchored protein 1 and 2) are two related MAM domain-containing proteins expressed in neuronal cells, and they both have the potential to be involved in neural development (111). MAM domains have been identified in a hydra metalloprotease, an astacin-like squid metalloprotease, an integrin binding protein called POEM (preosteoblast epidermal growth factor-like repeat protein with MAM domain), and another GPI-anchored protein (GPIM) expressed in normal and cancerous tissues (112-115).
In PTPμ, the MAM domain has been shown to mediate the dimerization of PTPμ proteins on the same cell surface (cis binding), but it does not mediate the homophilic binding of PTPμ to PTPμ proteins on another cellular surface (trans binding) (116-118). The MAM domains of neuropilins 1 and 2 are also involved in homo- and heterodimerization of the proteins at the neuronal cell surface, which mediates interactions with semaphorins in axon growth cone collapse (119,120). The MAM domain in meprins is noncatalytic, but its presence is needed for synthesis of the protein. Truncating the protein after the protease domain or deleting the MAM domain leads to intracellular degradation of the protein (26). A truncation of human meprin α at 322 (approximately the first third of the domain) was secreted, implicating the very first portion of MAM domain in promoting the protease domain fold (99). In addition, the noncovalent dimer of mouse meprin A, produced by selective reduction of the intersubunit disulfide bonds, can be crosslinked via Cys355 in the MAM domain, implicating this region in the noncovalent interface of the protein (44).

Structural details of MAM domains have been obtained mainly from studies of PTPs and meprins. There are four conserved cysteines common to MAM domains of meprins and PTPμ. Based on work with the PTPμ MAM domain, the four conserved cysteines form intrasubunit disulfide bonds (117). However, meprins are unique in that they have a fifth cysteine in the MAM domain, and this cysteine has been shown to be involved in intersubunit disulfide bonding (50). In addition, mapping of the meprin B disulfide bonds by mass spectrometry showed that there are actually two intersubunit disulfide bonds in the MAM domain, as well as one free cysteine and one intrasubunit disulfide bond (39). This arrangement is probably similar for meprin A based on site-
directed mutagenesis studies, although the disulfides of this isoform have not been mapped directly (50). In this way, the MAM domain has been demonstrated to promote oligomerization of meprin B as well as meprin A.

The lack of a crystal structure from any MAM domain containing protein hinders attempts to propose a homology model of the meprin α or β MAM domains. Thus, an in-depth understanding of how this domain folds and how this fold promotes oligomerization is also lacking. Site-directed mutagenesis allows for an initial analysis of the contribution of the MAM domain to the oligomeric structure of meprin A. The size of the meprin A homooligomer is salt dependent, and formation of the oligomer may be driven by the presence of a large number of charged residues in the meprin α MAM domain (40). Mutants at single charged residues in the MAM domain were thus tested for their effect on the oligomerization of meprin A in order to define features or regions of this domain that contribute to oligomerization.
Chapter 2
MATERIALS AND METHODS

2.1 Expression and purification of meprins

Human embryonic kidney 293 cells (HEK-293; ATCC 1573 CRL) stably expressing the wild-type, full length meprin mouse α cDNA (121) or the wild-type mouse α protein truncated at R615 and His-tagged at the C-terminus (27) were maintained in Dulbecco’s modified Eagle’s Medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS) and antibiotics/antimycotics (100 units/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B, Gibco) at 37°C with 5% CO₂. The wild-type rat meprin α construct was truncated at R603 and His-tagged at the C-terminus (33). Wild-type rat meprin β, truncated at K648 in the EGF domain, was also His-tagged (33). Both rat meprins were stably transfected into HEK-293 cells and maintained as described above. When confluent, cells were incubated in DMEM without FBS for three to four days before collection of the media for purification. The culture medium from cells expressing the full length cDNA of mouse meprin α was subjected to anion exchange and size exclusion chromatography, as previously described (51). For His-tagged constructs, the medium was purified by nickel chelating column, as previously described (33). Purified proteins were stored at –80°C in 20 mM Tris, 150 mM NaCl, pH 7.5 until needed.
2.2 Site-directed mutagenesis

The single N-linked glycosylation site mutants and the N330Q/N426Q double mutant of mouse meprin A were made previously (14). The MAM truncation mutant (1-445/α) was also made previously (26). All other mutants were generated using the mouse meprin α full length cDNA or the His-tagged, secreted rat meprin β cDNA as template with Stratagene’s QuikChange site-directed mutagenesis kit, according to manufacturer’s instructions. All plasmids were sequenced in the Penn State College of Medicine Core Facility. The primers used for mutagenesis are listed in Table 1. Double mutants were made by successive rounds of site-directed mutagenesis using the first mutated plasmid as a template for the second.

2.3 Transient transfections in HEK-293 cells

Wild-type and mutant plasmids were transiently expressed in HEK-293 cells using Lipofectamine 2000 transfection reagent (Invitrogen). Briefly, 60 x 15 mm plates of HEK-293 cells were grown to 90% confluency in DMEM with 10% FBS but without antibiotics/antimycotics. The meprin plasmids (8 μg) were diluted into 0.5 ml OptiMEM reduced serum medium (Gibco). The Lipofectamine 2000 reagent (20 μl) was also diluted into 0.5 ml OptiMEM and incubated at 25°C for 5 min. The DNA and Lipofectamine solutions were mixed, incubated at 25°C for an additional 25 min, then added to the plates of HEK-293 cells. The plates were incubated at 37°C for 4-6 hr, and the media was replaced with DMEM lacking FBS. The medium from each plate was
Table 1

**Primers for Site-directed Mutagenesis**

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Primers</th>
</tr>
</thead>
<tbody>
<tr>
<td>N152Q</td>
<td>5'-GAT CAA CAG GTG GGA CAG CAA ATT TCC ATT GGT GAG GGA TG-3' 5'-CAT CCC TCA CCA ATG GAA ATT TGC TGT CCC ACC TGT TGA TC-3'</td>
</tr>
<tr>
<td>N270Q</td>
<td>5'-ATA AGA CTG AAT CGA ATG TAC CAG TGC ACC GCA ACA CAT ACT CTG-3' 5'-CAG AGT ATG TGT TGC GGT GCA CTG GTA CAT TCG ATT CAG TCT TAT-3'</td>
</tr>
<tr>
<td>K352G</td>
<td>5'-CGA ATC CTT TAT CCC AAG AGG GGC CAG CAG TGT TTG CAG-3' 5'-CTG CAA ACA CTG CTG GCC CCT CTT GGG ATA AAG GAT TCG-3'</td>
</tr>
<tr>
<td>K361Y</td>
<td>5'-CAG TGT TTG CAG TTT TTT TAT TAC ATG ACT GGA AGC CCT GCA GAC-3' 5'-GTC TGC AGG GCT TCC AGT CAT GTA ATA AAA AAA CTG CAA ACA CTG-3'</td>
</tr>
<tr>
<td>R369Q</td>
<td>5'-CTG GAA GCC CTG CAG ACC AGT TTG AAG TCT GGG TGA G-3' 5'-CTC ACC CAG ACT TCA AAC TGG TCT GCA GGG CTG GGA G-3'</td>
</tr>
<tr>
<td>R376E</td>
<td>5'-GAT TTG AAG TCT GGG TGA GAG AGG AAC ACG CGG AGC AG-3' 5'-CTT GCC CGC GTT GTC GTC CTC TCT CAC CCA GAC TTC CTT C-3'</td>
</tr>
<tr>
<td>D377Y</td>
<td>5'-GAA GTC TGG GTG AGA AGG TAC GAC AAG GCG GCC AG-3' 5'-CTT GCC CGC GTT GTC GTC CTC TCT CAC CCA GAC TTC-3'</td>
</tr>
<tr>
<td>R378T</td>
<td>5'-AGA TCT GGG TGA GAA GGG ACA CCA ACG CGG GCA AG-3' 5'-CTT GCC CGC GTT GTC GTC CTC TCT CAC CCA GAC TT-3'</td>
</tr>
<tr>
<td>R384G</td>
<td>5'-GAC AAC GCG GCC AAG TGT CCT GGC CAG CTG AGC ATC CAG-3' 5'-CTG GAT CTT GGC CAG CTG ACC CAC CTT GCC CGC GTT GTC-3'</td>
</tr>
<tr>
<td>K388L</td>
<td>5'-GGT GCG ACA GCT GCC CTT GAT GTA CCT CCA GAC TTG GAC-3' 5'-GTC ACA TGG TCC TCA TTG TCC GCA GCT GTC GCA CC-3'</td>
</tr>
<tr>
<td>R384A</td>
<td>5'-GAC AAC GCG GCC AAG TGT CCT GTC CAG CTG GCC AAG ATC CAG-3' 5'-CTG GAT CTT GGC CAG CTG ACC ATC CAG GC-3'</td>
</tr>
<tr>
<td>R384E</td>
<td>5'-GAC AAC GCG GCC AAG TGT GGT GCA CAG CTG TGC GCT CAG-3' 5'-CTG GAT CTT GGC CAG CTG TTT CAC CTT GCC CGC GTT GTC-3'</td>
</tr>
<tr>
<td>R384K</td>
<td>5'-GAC AAC GCG GCC AAG TGT GGA CAG CTG TGC GCC AAG ATC CAG-3' 5'-CTG GAT CTT GGC CAG CTG TTT CAC CTT GCC CGC GTT GTC-3'</td>
</tr>
<tr>
<td>E138N</td>
<td>5'-CAT TCA CGC TGG GAA GCA AAA TTT GTC CAT CGG GAC AAA CT-3' 5'-AGT TTG TCC CGA TGG ACA AAT TGT GCT TCC CAG CGT GAA TG-3'</td>
</tr>
</tbody>
</table>
collected 24 hr posttransfection and analyzed for protein expression by SDS-PAGE and Western blotting. For preparation of cellular lysates, the transiently transfected cells were washed twice and resuspended in cold phosphate buffered saline (PBS), centrifuged, and resuspended in 1 ml PBS with an EDTA-free Complete Mini protease inhibitor cocktail (Roche). Cells were lysed by sonication and analyzed by SDS-PAGE. For oligomeric size, stability, and activity assays, the 24 hr posttransfection media samples were concentrated four-fold using YM-50 Centricon concentrators (Amicon), and the EDTA-free Complete Mini inhibitor cocktail was added after concentrating. The media samples were aliquoted and stored at -80°C until further use.

All mutants were transiently transfected with the exception of E138N rat meprin B. In this case, the cells were transfected with Lipofectamine 2000. At 24 hr posttransfection, the cells were split 1:5 into DMEM with 10% FBS. After another 24 hr, the cells were placed in DMEM containing 0.5 mg/ml Geneticin (Gibco) for stable line selection.

2.4 Gel electrophoresis and Western blotting

For sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), samples were separated on 7.5% Tris-HCl Ready gels (Biorad) or 8% polyacrylamide gels at 40-45 mA in 25 mM Tris, 192 mM glycine, pH 8.5 with 0.1% SDS. For native PAGE, samples were separated on NuPAGE 3-8% Tris-acetate gels (Invitrogen) in running buffer described above, except without SDS. Gels were run at 150 V for 130 min. All gels were transferred onto nitrocellulose membrane (BioRad) at 16 V using a
BioRad semi-dry blotting apparatus with transfer buffer containing 25 mM Tris, 192 mM glycine, 0.04% SDS, and 20% methanol. Blots were blocked in 10% nonfat dry milk in 20 mM Tris, 138 mM NaCl, with 0.1% Tween-20 (TBS-T) then incubated with an appropriate anti-meprin antibody at a dilution of 1:3300 (HMC14 for meprin A; PSU57 for meprin B). Anti-rabbit or anti-mouse secondary antibody coupled to horseradish peroxidase (HRP, Amersham) was added after washing in TBS-T, and blots were developed with the appropriate chemiluminescent substrate (SuperSignal West Pico or West Dura, Pierce).

2.5 Activity assays

The peptidase activity of meprin A was measured using the fluorogenic substrate 2-aminobenzoyl-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg-Lys(Dnp)-Gly-OH (BK+), where Dnp is dinitrophenyl, as described previously (50). Assays were carried out at 25°C or 30°C, as indicated, after activation of the samples by limited trypsin digestion (1:20 trypsin:meprin for purified samples or 5-10 ng/µl trypsin for crude media samples) for 30-60 min at 37°C in 20 mM Tris, 150 mM NaCl, pH 7.5 to remove the prosequence. Trypsin was inhibited with a 3-10 fold excess of soybean trypsin inhibitor (Sigma) for 15-20 min at 25°C before assaying activity in 50 mM ethanolamine buffer, pH 8.7 in the presence of 10 μM BK+ (final). The fluorescence was monitored at 320 nm excitation and 417 nm emission using a Hitachi-F2000 fluorimeter. The substrate OCK+ (2-aminobenzoic acid-Met-Gly-Trp-Met-Asp-Glu-Ile(Dnp)-Ser-Gly-OH) was used to measure meprin B activity in 20 mM HEPES, 100 mM NaCl, pH 6.8, at an excitation
wavelength of 326 nm and an emission wavelength of 418 nm (33). For crude samples, the specific activities of each protein were calculated by Western blot and densitometry. The concentration of wild-type protein was measured from its BK activity compared to the fluorescence/μg of purified protein.

2.6 Total carbohydrate analysis

The molar amounts of each neutral (fucose, mannose, glucose, galactose) and amino sugars (glucosamine, galactosamine) in purified, recombinant meprins were determined by acid hydrolysis and separation by high-pH-anion-exchange HPLC (HPAE-HPLC) as previously described (122,123). Samples were analyzed by Dr. Arivalagan Muthusamy at the Penn State College of Medicine. For mouse meprin A, samples from two different purifications of the protein were concentrated to smaller volumes with YM-30 Microcon concentrators (Amicon). The protein concentrations were determined using the Micro-BCA total protein assay (Pierce). A 20 μg sample from each purification was concentrated to dryness in a very clean acid hydrolysis tube using a Speed-vac. The dried samples were hydrolyzed in 2.5 M trifluoroacetic acid (TFA) for 6 hr at 100°C. After concentrating to dryness, the samples were resuspended in an appropriate amount of sterile water. Monosaccharides were analyzed on a CarboPac10 high-pH anion-exchange column (4 x 250mm, Dionex) with 20 mM sodium hydroxide for elution and pulse-amperometric detection. The response factor for each sugar was determined by using a set of monosaccharide standards of a known amount. Rat meprin A and rat meprin B
samples were prepared as above, except that they were not concentrated before drying in the acid hydrolysis tubes.

2.7 Full and partial deglycosylation with PNGaseF and EndoH

Complete deglycosylation of meprins was achieved by first boiling the sample in 1% β-mercaptoethanol (BME) and 0.5% SDS, then incubating the proteins for 1 hr at 37°C with either PNGaseF or EndoH (New England Biolabs) in 50 mM sodium phosphate buffer, pH 7.5 and 1% NP-40 for PNGaseF and 50 mM sodium citrate buffer, pH 5.5 for EndoH. For partial deglycosylation, the same reactions buffers were used, leaving out the BME, SDS, and NP-40. Samples were incubated at 37°C for 2 hr, and then used for further experiments. A typical deglycosylation reaction (complete or partial) contained at least 500 units of deglycosidase to 20 μg of meprin.

2.8 Labeling of meprin A with biotin hydrazide

Purified meprin A was oxidized with 10 mM sodium periodate (Sigma, NaIO₄) in 0.1 M sodium acetate buffer, pH 5.0 for 2 hr in the dark. Excess NaIO₄ was removed with G-25 Sephadex resin packed into a 1ml spin column (Sigma). The oxidized, desalted meprin was fully reduced and denatured, then incubated in the presence or absence of PNGaseF (New England Biolabs) for 1 hr at 37°C as described above. Carbohydrates were labeled for 2 hr in the dark at pH 5.0 with 10 μM biotin hydrazide (Pierce) dissolved in DMSO (dimethylsulfoxide) at a final concentration of 10%. The
samples were separated by SDS-PAGE and transferred to nitrocellulose membrane. The nitrocellulose membrane was blocked for 30 min in 3% bovine serum albumin (BSA) in TBS-T, then incubated with streptavidin-HRP (1:5000, Amersham) in 3% BSA/TBS-T for 45 min. The blot was washed with TBS-T twice for 10 min each, then treated with chemiluminescent substrate (Supersignal WestDura, Pierce) and exposed to film.

2.9 MALDI of intact protein

Matrix-assisted laser desorption ionization/time of flight (MALDI/TOF) mass spectrometry was used to determine the subunit molecular mass of mouse meprin α, with and without glycosylation. Purified, His-tagged protein (20 μg) was boiled in 10 mM DTT (MS grade, Sigma) for 10 min in the absence of SDS and NP-40. The samples were then incubated with PNGaseF (glycerol-free) or EndoH in the appropriate deglycosylation buffer for 2 hr at 37°C. A 2 μg sample was removed for SDS-PAGE analysis, and the rest of the samples were dialyzed against 0.1% TFA. After dialysis, another 2 μg sample was removed for the gel and the rest of the samples were concentrated to dryness in a Speed-vac and resuspended in 10 μl of 5% formic acid. Each sample was serially diluted by mixing the sample 1:1 with 20 mg/ml sinapinic acid (Sigma) dissolved in 60% acetonitrile/0.1% TFA and spotted onto a stainless steel plate. Spectra were acquired on a Voyager-DE RP/PRO Biospectometry workstation (PerSeptive Biosystems) in linear, positive mode with a delayed extraction of 600 ns. The accelerating voltage was 25000 V, the grid voltage was 90%, and the guide wire voltage was 0.15%. Resulting spectra were analyzed using the DataExplorer software (Applied
Biosystems) and calibrated using a Sequazyme IgG protein standard (PerSeptive
Biosystems).

2.10 Glycosylation site mapping using TFMS and ESI/MS

In order to facilitate glycosylation site mapping, a mass marker was created at
each glycosylation site using a chemical deglycosylation method adapted from previously
published protocols (124,125). Lyophilized fetuin (500 μg, Sigma) was resuspended
directly in 75 μl trifluoromethane sulfonic acid (TFMS, Sigma). Purified mouse meprin
A (500 μg) was concentrated to dryness in a Speed-vac and also resuspended in 75 μl
TFMS. Samples were incubated on ice for 2 hr, and quenched by the slow addition of
pre-chilled 60% pyridine (200 μl, aq). A light colored precipitate formed upon addition
of the pyridine, but dissolved upon pipetting. The samples were dialyzed against water
for 4 hr, using SpectraPor dialysis tubing (10mm, 3500 MWCO). The deglycosylated
proteins were concentrated to dryness, resuspended in 100 μl of 100 mM ammonium
bicarbonate, pH 8.0, and digested 1:20 with trypsin (Sigma) for 24 hr at 37°C. Disulfide
bonds were reduced with 10 mM dithiothreitol (DTT, Sigma) for fetuin and 50 mM DTT
for meprin immediately prior to injection onto the HPLC column. Rat meprins A and B
(500 μg each) were deglycosylated and digested as described for fetuin and mouse
meprin A, except that the proteins were desalted first with a G-25 Sephadex spin column
and the final tryptic digest was reduced with 100 mM DTT.

One half of the fetuin tryptic digest was injected onto a ThermoHypersil
BioBasic4 C4 column (5 μm, 50 x 1 mm) coupled to a Mariner electrospray
ionization/time-of-flight mass spectrometer (ESI/TOF, Perseptive Biosystems). The split flow rate was 0.05 ml/min, and a gradient program was used to partially separate the peptides. Positive ion mode was used for detection, and data were collected between $m/z$ 200 and 2500. Peptides were loaded onto the column under the starting conditions of 100% Buffer A (0.1% formic acid/H$_2$O) for 6 min, diverting the flow to prevent salt interference. The peptides were eluted under a gradient to 95% Buffer B (0.1% formic acid/acetonitrile) over 26 min. Following elution of the peptides, the conditions were held for 5 min, then changed to 95% Buffer C (0.1% formic acid/isopropanol) over 5 min. The column was flushed for the remaining 5 min of the run and re-equilibrated under the starting conditions.

For meprin A N-linked glycosylation site mapping, a BetaBasic C$_{18}$ column (5 μm, 150 x 1 mm) was used, and the column was also coupled to the ESI/TOF instrument. The split flow rate was 0.05 ml/min, and a gradient program was used to partially separate the peptides. Positive ion mode was used for detection, and data were collected between $m/z$ 200 and 2500. Peptides were loaded onto the column under the starting conditions of 100% Buffer A (0.1% formic acid/H$_2$O) for 6 min, diverting the flow to prevent salt interference. The peptides were eluted under a gradient to 95% Buffer B (0.1% formic acid/acetonitrile) over 44 min. Following elution of the peptides, the conditions were held for 5 min, then changed to 90% Buffer C (0.1% formic acid/isopropanol) over 5 min. The column was flushed for the remaining 5 min of the run and re-equilibrated under the starting conditions.
MS data were analyzed using the accompanying DataExplorer 4.0.0.1 software (Applied Biosystems), utilizing the Extracted Ion Chromatogram feature to search for the predicted m/z values of glycopeptides.

2.11 Carbohydrate crosslinking of the noncovalent dimer

The carbohydrate moieties of the meprin A homooligomer were oxidized in the presence of 10 mM sodium periodate (Sigma) in 100 mM sodium acetate, pH 5.5 for 2 hr at 23°C in the dark. Excess sodium periodate was removed with a 1 ml desalting column packed with G-25 Sephadex resin (Sigma) and equilibrated with water. For the crosslinking reaction, triethanolamine, pH 7.5 was added to the sample at a final concentration of 40 mM, and reduction of the intersubunit disulfide bond was carried out in the presence of 5 mM Tris-(2-carboxyethyl)phosphine HCl (TCEP), pH 8.0. After approximately 15 min, the carbohydrate and cysteine reactive crosslinker N-[β-maleimidopropionic acid] hydrazide·TFA (BMPH, Pierce) was added to a final concentration of 10 mM in 10% dimethylformamide (DMF, Fisher). The crosslinking reaction proceeded for 2 hr at 23°C. The samples were analyzed by nonreducing SDS-PAGE and Western blotting as described above.

2.12 Mapping of sites removed by PNGaseF

To determine which glycans were removed in the partially deglycosylated protein, 900 μl of 4.6 μM meprin (330 μg total) was treated with 20 μl glycerol-free PNGaseF
(New England Biolabs) under non-denaturing and non-reducing conditions (-SDS, -BME, but +NP-40) and prepared for ESI/MS as described above, except that half of the sample was digested with trypsin (1:20 in 20 mM Tris, 150 mM NaCl, pH 7.5) and the other half with endoproteinase Glu-C (V8 protease, Sigma; 1:20 in 100 mM ammonium bicarbonate, pH 8.0). The data sets collected from each digest were searched using the Extracted Ion Chromatogram tool of the DataExplorer software, looking for the predicted tryptic or Glu-C fragment plus one mass unit, which resulted from the conversion of Asn to Asp by PNGaseF.

2.13 Circular dichroism and fluorescence spectroscopy

The far-ultraviolet (UV) circular dichroism (CD) spectra of the wild-type and partially deglycosylated proteins in the absence of urea were obtained using a Jasco J-710 spectropolarimeter fitted with a xenon lamp. Samples were scanned from 190-250 nm at a scan rate of 50 nm/min at 25°C using quartz cuvettes with a pathlength of 1 mm. Spectra are an average of three scans. Samples of latent meprin A were prepared as described above, then diluted to 3.2 μM with Nanopure water (8 mM sodium phosphate, final) and incubated at 23°C for 15 min before analysis.

Fluorescence spectra were obtained with a PTI QuantaMaster luminescence spectrometer. Wild-type and partially deglycosylated samples were diluted to 10 μg/ml (118 nM) in Nanopure water. Tris and NaCl were added to a final concentration of 20 mM and 150 mM, respectively at pH 7.5. Samples were incubated at 23°C for 30 min before analysis. Quartz cuvettes with pathlengths of 1 cm x 2 mm were used, and the
cuvette holder was kept at 25°C. Excitation slit width was 0.5 nm and emission slit widths was 2.0 nm. Excitation wavelength was 280 nm to measure total protein fluorescence (from tryptophan and tyrosine), and the emission spectra were recorded from 300-400 nm.

2.14 Size exclusion chromatography

The oligomeric states of purified and crude meprin A samples were analyzed by size exclusion chromatography on a Superose 12 column (Amersham) equilibrated in 20 mM Tris, 150 mM NaCl, pH 7.5. A 200 μl sample was injected at a flow rate of 0.25 ml/min, and 1 ml fractions were collected. A small portion of each fraction was analyzed by SDS-PAGE and Western blotting with the appropriate meprin antibody.

2.15 Urea induced unfolding of meprin A and test for reversibility

The urea induced unfolding of the meprin oligomer was followed by monitoring the change in ellipticity by CD spectroscopy as well as the change in intrinsic tryptophan fluorescence. For CD, the samples were diluted to 4.6 μM and incubated with an appropriate amount of 9 M urea for 1-2 hr at 23°C, then spectra were collected as described above. The ellipticity at 225 nm was used to monitor the unfolding of the wild type protein. Since the intrinsic tryptophan fluorescence required less protein (118 nM vs 4.6 μM), the remaining unfolding experiments were performed using the fluorimeter. Samples were incubated at 25°C for 30 min, then analyzed. A urea unfolding curve was
obtained by plotting the wavelength of maximal emission ($\lambda_{\text{max}}$) for the wild-type protein versus urea concentration.

The stability of meprin A over time and the reversibility of the unfolding reaction were tested by measuring the BK+ activity of the sample at given time points. Wild-type meprin (0.8 $\mu$M) was activated with 1 ng/$\mu$l trypsin for 40 min at 37°C. The trypsin was inhibited with 10-fold excess soybean trypsin inhibitor for 20 min at 23°C. The activated protein was kept at 23°C, then assayed for BK+ activity at $t = 0, 4, 21, 27,$ and 70 hr. At each timepoint, a sample of freshly thawed and activated protein was used as a positive control. To test the reversibility of the unfolding reaction, activated meprin (350 nM) unfolded in the presence of 7 M urea for 24 hr at 23°C was diluted with water 12-fold so that the final concentration of urea was 0.57 M. This sample was incubated at 23°C and assayed for activity at $t = 0, 2, 4, 6,$ and 24 hr post-dilution.

### 2.16 Heat stability assays

For the stability of partially deglycosylated meprin A, activated samples were incubated in the presence and absence of PNGaseF under native conditions, as described above. The samples were then incubated at 55°C for 0, 5, 10, 15, 30, and 45 min. At each timepoint, a small aliquot of the protein was removed, cooled to 23°C, diluted into assay buffer, and assayed for BK+ activity in triplicate at 23°C. The percent of initial activity over time was plotted for each treatment.
For crude media samples of meprin A mutants, the samples were activated as indicated, then incubated at 55°C for 0, 5, 15, 30, and 45 min and were processed as described for partially deglycosylated protein except that the samples were diluted into assay buffer and allowed to equilibrate at 23°C for at least 15 min. The samples were then equilibrated to 30°C and BK+ activity was measured at this temperature. Due to the low concentration of meprin in the 24 hr posttransfection samples, measuring BK+ activity at this temperature gave a better signal.

2.17 Tryptic stability assays

To test the activation and stability of the meprin mutants, crude media samples were incubated with increasing amounts of trypsin (0, 5, 40, 80 ng/μl for the glycosylation site mutants; 0, 10, 40, 80 ng/μl for the MAM mutants) for 45 min at 37°C. Trypsin was inhibited for 15 min at 23°C by a three-fold excess of soybean trypsin inhibitor. Samples were mixed with SDS-PAGE sample buffer (+BME) and immediately boiled. The entire set of samples was then boiled for an additional 5 min before analysis by SDS-PAGE and Western blotting.
Chapter 3

RESULTS

3.1 The identity and role of glycans in the formation and stability of the meprin A homooligomer

3.1.1 Rationale and overview of glycosylation studies

In order to better understand the role of glycans in the quaternary structure of meprin A, the presence or absence of a glycan at each potential glycosylation site was first determined. Two approaches were used to investigate the effect that glycosylation has on the oligomeric structure of mouse meprin A. First, enzymatic deglycosylation of the secreted, purified protein was used to study how glycans influence the structure of the oligomer once it is formed, i.e. their effect on the native conformation. Second, single as well as multiple glycosylation site mutants (with the consensus sequence removed by the change of Asn to Gln) were used to investigate the role of glycans on the formation of the oligomer during biosynthesis. In both instances, the presence or absence of a particular glycan or group of glycans may affect the distribution of oligomers (octamers/decamers vs tetramers vs dimers) that are observed, as well as the protein’s stability.
3.1.2 Structural role for glycans in the latent homooligomer

In previous studies, a crosslinking approach was employed to demonstrate that carbohydrate moieties were proximal to the noncovalent interface of activated meprin A (106). These sugars may form direct contacts between the subunits or influence the equilibrium of subunit associations. Since there is a detectable conformational change upon activation, the crosslinking experiment was performed here on latent meprin A to determine if sugars were at or near the subunit interface of this species. Meprin was reduced with TCEP to yield only noncovalently linked dimers, as previously reported (44). Addition of the sugar and cysteine reactive crosslinker $N$-[β-maleimidopropioinic acid]hydrazide•TFA (BMPH, Figure 8a) resulted in crosslinking of the carbohydrate residues of one subunit to cysteine residues of a different subunit (cysteine residues were activated upon reduction of one disulfide bond from the action of TCEP). After treatment with BMPH, the samples were analyzed by nonreducing SDS-PAGE, and a dimer band was observed for the sample incubated with TCEP and BMPH (Figure 8b, lane 3). In the absence of crosslinker and reducing agent, the protein migrated as a disulfide linked dimer (lane 1), while reduction of the protein in the absence of crosslinker produced a monomer (lane 2). Thus, the ability of the meprin noncovalent dimer to be crosslinked via carbohydrates indicates that carbohydrate groups are close to or within the subunit interface and can potentially influence the oligomeric structure in both the latent and active forms of the protease. A reciprocal experiment in which the glycosylation is partially removed and crosslinking is attempted could not be performed, because the noncovalent dimer is completely deglycosylated (Figure 8c).
Figure 8. The noncovalent interface can be crosslinked via carbohydrates. The carbohydrate moieties of latent meprin A were oxidized with 10 mM sodium periodate (NaIO₄), pH 5.5. After removal of the excess sodium periodate, the intersubunit disulfide bonds were reduced with 5 mM TCEP, pH 8.0 to produce a noncovalently associated dimer. Crosslinking was initiated with the addition of 10 mM BMPH.  

a) Structure of the carbohydrate and cysteine reactive crosslinker BMPH.  

b) After oxidation, reduction, and crosslinking as indicated, samples were separated by SDS-PAGE under nonreducing conditions and visualized by Western blot with an anti-meprin A antibody.  

c) Meprin was incubated in the presence or absence of 5 mM TCEP for 30 min at 23°C, then excess TCEP was removed with a 1 ml G-25 Sephadex spin column. After reduction, the samples were incubated with PNGaseF under non-denaturing conditions for 2 hr at 37°C and analyzed by reducing SDS-PAGE and silver staining.  

- lane 1, (-) TCEP, (-) PNGaseF; lane 2, (+) TCEP, (-) PNGaseF; lane 3, (+) TCEP, (+) PNGaseF. MW standards are indicated on the left.
a) dimer
b) monomer

c) Spacer arm: 8.1Å

TCEP
BMPH

+ +
+ -
-

200 kDa
130 kDa
80 kDa

1 2 3
3.1.3 Characterization of glycans in mouse meprin A

3.1.3.1 Total carbohydrate analysis of mouse meprin A

To determine if meprin A contained any O-linked glycosylation, as characterized by the presence of N-acetylgalactosamine (GalNac), the molar ratios of individual monosaccharides from recombinantly expressed and purified mouse meprin A were determined by acid hydrolysis and separation of monosaccharides by high-pH-anion-exchange chromatography (HPAE-HPLC). As shown in Table 2, there were no detectable levels of GalNAc from two different protein preparations. The majority of the glycans are composed of N-acetylglucosamine (GlcNAc, 56%), mannose (24%), and galactose (13%), characteristic of complex or hybrid oligosaccharides. A small but reproducible amount of fucose was also detected, accounting for 7% of the total carbohydrate analyzed in this sample.

To confirm the absence of O-linked glycosylation and to show that the glycosidase PNGaseF completely removed the glycans from the protein, mouse meprin A was oxidized with sodium periodate, fully deglycosylated with PNGaseF, and labeled with the carbohydrate reactive label biotin-hydrazide. As shown in Figure 9a, the lack of reactivity with streptavidin-HRP for the deglycosylated sample (lane 4) indicates that all of the glycans were removed by PNGaseF and confirms that there is no significant
Table 2

*Total carbohydrate analysis of homooligomeric mouse meprin A*

Purified recombinant mouse meprin A was hydrolyzed by trifluoroacetic acid, separated by HPLC, and the total molar amount of each monosaccharide was determined for each sample using known amounts of monosaccharides as standards. The results reflect the average ratio for each monosaccharide from two different protein purifications, with each sample run in duplicate. *GalNAc, n-acetylgalactosamine; GlcNAc, n-acetylglucosamine.*

<table>
<thead>
<tr>
<th>monosaccharide</th>
<th>nmole carbohydrate: nmole protein</th>
<th>% total carbohydrate&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>fucose</td>
<td>2.87 ± 0.54</td>
<td>7%</td>
</tr>
<tr>
<td>GalNAc</td>
<td>ND&lt;sup&gt;b&lt;/sup&gt;</td>
<td>ND&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>GlcNAc</td>
<td>22.9 ± 1.00</td>
<td>56%</td>
</tr>
<tr>
<td>galactose</td>
<td>5.39 ± 0.30</td>
<td>13%</td>
</tr>
<tr>
<td>mannose</td>
<td>9.95 ± 0.52</td>
<td>24%</td>
</tr>
</tbody>
</table>

<sup>a</sup> The total nmoles of monosaccharide divided by the total amount of carbohydrate detected in each sample.

<sup>b</sup> No significant amounts were detected.
**Figure 9. Labeling of meprin A carbohydrates in the presence and absence of PNGaseF.**  
*a*) After oxidation of the carbohydrates with sodium periodate (NaIO₄), meprin A was incubated in the presence (+) or absence (-) of PNGaseF under reducing and denaturing conditions, and labeled with 10 μM biotin hydrazide. The samples were separated by SDS-PAGE and probed with streptavidin-HRP.  
*b*) Western blot of the labeled samples probed with anti-meprin A antibody.
a) NaIO₄    +    +    +    +
PNGaseF    -    -    +    +
Biotin-hydrazide    -    +    -    +

b) NaIO₄    +    +    +    +
PNGaseF    -    -    +    +
Biotin-hydrazide    -    +    -    +
O-linked glycosylation. All samples were still detected by an anti-meprin A antibody (Figure 9b).

### 3.1.3.2 MALDI of intact protein, (+/-) glycosylation

Secreted meprin A is only slightly sensitive to Endo H, indicating a small amount of high mannose sugars. However, it has not been determined exactly how much of the molecular mass is attributed to Endo H-sensitive glycans or if the estimate of total carbohydrate from SDS-PAGE gels is accurate. To more accurately determine the total percentage of carbohydrate on His-tagged, recombinant mouse meprin A, the monomeric molecular masses of the wild-type, PNGaseF-treated, and Endo H-treated proteins were determined using MALDI/TOF.

Optimal deglycosylation and dialysis conditions were determined to recover the majority of protein after deglycosylation and to remove buffers and substances interfering with MALDI. Since samples have to be denatured for full enzymatic deglycosylation but SDS and NP-40 (needed for PNGaseF activity in the presence of SDS) interfere with MALDI, the proteins were reduced and denatured by boiling in MS-grade 10 mM DTT. Dialysis of the deglycosylated samples into 0.1% TFA was necessary to prevent oxidation of reduced cysteines. Aliquots of the samples before dialysis against 0.1% TFA (Figure 10a, lanes 1, 3, 5) were compared to the amount of protein left after dialysis (Figure 10b, lanes 2, 4, 6), and approximately half of the protein was recovered, as determined by densitometry of the gel.
Figure 10. Initial molecular mass determination of wild-type and deglycosylated mouse meprin A by MALDI. Purified, recombinant mouse meprin A was denatured by boiling in the presence of 10 mM DTT and then incubated in the presence or absence of deglycosidases for 2 hr at 37°C. After dialysis against 0.1% TFA, the samples were dried under vacuum and resuspended in 5% formic acid. For MALDI analysis, serial dilutions of the samples were made by mixing the protein 1:1 with 20 mg/ml sinapinic acid in 60% acetonitrile/0.1% TFA. a) Reducing SDS-PAGE separation and silver staining of the wild-type (lanes 1, 2), Endo H-treated (lanes 3,4), and PNGaseF-treated (lanes 5,6) meprin A before (lanes 1, 3, 5) and after (lanes 2, 4, 6) dialysis against 0.1% TFA. MW markers are indicated on the left. The MALDI spectra of b) wild-type, c) Endo H-treated, and d) PNGaseF-treated protein were then acquired in positive, linear mode. The spectra were acquired once for each sample.
The secreted protein is only slightly EndoH sensitive (lanes 3, 4), and maintains the heterogeneity of the wild-type species (lanes 1, 2). Likewise, a broad, heterogenous peak is observed in the mass spectrum of wild-type protein (Figure 10b). The mass of the wild-type species was determined to be 85 kDa, while the mass of the EndoH treated protein was 82 kDa. After full deglycosylation with PNGaseF, the mass of meprin is decreased to 70 kDa, indicating that approximately 18% of the subunit molecular mass is due to carbohydrate, of which 4% is due to high mannose sugars. However, there is a discrepancy between the molecular mass determined for fully deglycosylated protein (70 kDa) and the molecular mass estimated from the amino acid sequence of His-tagged protein (67 kDa, reflecting the prosequence through R615, with the addition of a six-histidine tag). The peak for the fully deglycosylated protein is also rather broad for a protein without sugars. The quality of these spectra was too low (counts < 1000-5000) for processing beyond molecular calibration against an IgG standard. The differences from the expected values may be due to measurement error or they may reflect further posttranslational modifications in addition to N-linked glycosylation.

3.1.3.3 Test of glycosylation site mapping by chemical deglycosylation and ESI/MS

Chemical deglycosylation with trifluoromethane sulfonic acid (TFMS) removes the carbohydrate residues from glycoproteins with the exception of the innermost GlcNAc or GalNAc, creating a mass marker of +203 mass units and eliminating any heterogeneity in the glycans, and has been used to map the glycosylation sites of carcinoembryonic antigen and nonspecific crossreacting antigen proteins (126,127). In
these studies, gas-phase microsequencing and fast-atom bombardment mass spectrometry (FAB/MS) were used to detect the glycopeptides. To determine if the glycopeptides produced from a proteolytic digest could be detected by electrospray ionization mass spectrometry, a well characterized glycoprotein, fetuin, was tested first. Although fetuin contains O-linked glycosylation the glycans are not in any of the tryptic peptides containing the known N-linked glycosylation sites. Thus, each glycosylation site could be isolated into one proteolytic fragment. After fetuin was deglycosylated with TFMS, it was digested with trypsin and disulfides were reduced with dithiothreitol (DTT). Individual peptides were separated by reverse-phase HPLC and analyzed by ESI/MS. The entire tryptic digest in the total ion chromatogram (TIC, Figure 11a) was then searched using the extracted ion chromatogram tool to pinpoint areas of the chromatogram where there were ions corresponding to predicted glycopeptides. Figure 11b shows the extracted ion chromatogram for the peptide containing Asn99. The predicted glycosylated mass for this peptide was 3759.80, and masses corresponding to the +3 ($m/z$ 1254.03) and +4 ($m/z$ 940.73) ions were found in the mass spectrum for the region at $t = 19.1$ min. Overall, this method was successful in detecting two of the three N-linked glycosylation sites in fetuin (Table 3). No peptides were found in this dataset that matched either the unmodified or glycosylated peptide mass for N156. Thus, this method is capable of detecting glycopeptides.
Figure 11. Reverse phase HPLC and ESI/MS analysis of fetuin glycopeptides after TFMS deglycosylation.  
a) The total ion chromatogram of the tryptic digest eluted from a C$_{18}$ reverse phase column.  
b) The extracted ion chromatogram for Asn99 based on the expected glycopeptide ions of $m/z$ 3760.80, 1880.90, 1254.27, and 940.95.  
c) The mass spectrum of the extracted ion peak at $t = 19.1$ min. Ions corresponding to the +3 ($m/z$ 1254.03) and +4 ($m/z$ 940.73) charge states of the Asn99 glycopeptide are indicated by arrows.
Table 3

*N-linked glycosylation mapping of fetuin using electrospray mass spectrometry*

Fetuin was chemically deglycosylated with TFMS, digested with trypsin, and analyzed by ESI/MS as described in the Materials and Methods. The expected nonglycosylated (NG) and glycosylated (G) tryptic masses are listed, as well as the calculated m/z values of each peptide ion. Glycopeptides were distinguished from unmodified peptides by the presence of one N-acetylglucosamine residue (GlcNAc, +203 mass units).

<table>
<thead>
<tr>
<th>Asn</th>
<th>Mass</th>
<th>+1</th>
<th>+2</th>
<th>+3</th>
<th>+4</th>
<th>Observed ions</th>
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<tbody>
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<td>3557.72</td>
<td>1779.36</td>
<td>1186.57</td>
<td>890.18</td>
<td>G: 1254.03(z3),</td>
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<tr>
<td></td>
<td>G: 3759.80</td>
<td>3760.80</td>
<td>1880.90</td>
<td>1254.27</td>
<td>940.95</td>
<td>G: 940.73 (z4)</td>
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<tr>
<td>156</td>
<td>NG: 1625.79</td>
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<td>813.90</td>
<td>542.93</td>
<td>407.45</td>
<td>N/Ab</td>
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<tr>
<td></td>
<td>G: 1828.87</td>
<td>1829.87</td>
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<td>1006.19</td>
<td>754.89</td>
<td>G: 1073.88 (z3)</td>
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<td></td>
<td>G: 3218.65</td>
<td>3219.65</td>
<td>1610.32</td>
<td>1073.88</td>
<td>805.66</td>
<td></td>
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</table>

*a* Numbers in parentheses indicate the charge of the observed ion.

*b* Not available. The glycosylation of this site could not be established from the data set in this experiment.
3.1.3.4 Glycosylation site mapping of recombinant mouse meprin A

The chemical deglycosylation method and ESI/MS analysis were used to identify which of the ten potential N-linked glycosylation sites are occupied in recombinant mouse meprin A. By generating extracted ion chromatograms in the total ion chromatogram (Figure 12a) based on the predicted tryptic fragments, seven glycopeptides were identified corresponding to N41, N152, N234, N270, N452, N546, and N553 (Table 4). A tryptic peptide corresponding to N614 was identified, indicating that this site at the putative C-terminus was not glycosylated. N546 and N553 were contained in one peptide due to lack of proteolytic cleavage sites between the consensus sequences. Masses corresponding to both one and two GlcNAc residues in this peptide were observed in the digest, suggesting that there is heterogeneity in this region. Two sites, N330 and N426, could not be found in this particular digest. Results from the glycosylation site mapping are summarized schematically in Figure 12b.
Figure 12. Glycosylation site mapping in recombinant mouse meprin A.  

a) Total ion chromatogram of the meprin A tryptic digest eluted from a C$_{18}$ reverse phase column and detected by ESI/MS.  
b) Scheme illustrating the meprin A glycopeptides identified by ESI/MS. The numbers correspond to the Asn of each consensus sequence, and the occupancy of each glycosylation site by a glycan is designated by the symbols listed in the legend.
a) 

Retention Time (Min) 

% Intensity 

b) 

N-linked glycosylated site  

? Inconclusive from this experiment  

N Non-glycosylated site  

N Heterogeneity
Mouse meprin A was chemically deglycosylated with TFMS, digested with trypsin, and analyzed by ESI/MS as described in the Materials and Methods. The expected nonglycosylated (NG) and glycosylated (G) tryptic masses are listed, as well as the calculated m/z values for the peptide ions. Glycopeptides were distinguished from unmodified peptides by the presence of one N-acetylglucosamine residue (GlcNAc, +203 mass units).

<table>
<thead>
<tr>
<th>Asn</th>
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<td>882.91</td>
<td>588.94</td>
<td>441.95 G: 984.52(z2),</td>
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<td>G: 1966.89</td>
<td>1967.89</td>
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<td>G: 656.64(z3), G: 492.73(z4)</td>
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<td></td>
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<td>1386.62</td>
<td>924.75</td>
<td>693.81 G: 1488.29(z2),</td>
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<td>NG</td>
<td>G: 2974.31</td>
<td>2975.31</td>
<td>1488.16</td>
<td>992.44</td>
<td>744.59 G: 992.54(z3)</td>
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<td>1102.59</td>
<td>1103.59</td>
<td>552.30</td>
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<td>276.65 G: 635.84(z2)</td>
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<td></td>
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<td>2140.90</td>
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<td>1071.45</td>
<td>714.63</td>
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<td>G: 2343.98</td>
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<td>782.33</td>
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<td>1958.98</td>
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<td>980.49</td>
<td>653.99</td>
<td>490.75 G: 1082.06(z2),</td>
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<tr>
<td>546</td>
<td>NG</td>
<td>2162.06 (1 GlcNAc)</td>
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<td>1082.03</td>
<td>721.69</td>
<td>541.51 G: 789.42(z3)</td>
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<tr>
<td></td>
<td></td>
<td>2365.14 (2 GlcNAc)</td>
<td>2366.14</td>
<td>1183.57</td>
<td>789.38</td>
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<tr>
<td></td>
<td>553</td>
<td>NG: 867.46</td>
<td>868.46</td>
<td>434.73</td>
<td>290.15</td>
<td>217.87 NG: 868.47(z1),</td>
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<td>536.27</td>
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<td>268.63 NG: 434.72(z2),</td>
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<td>NG: 290.15(z3)</td>
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</table>

a Numbers in parentheses indicate the charge of the observed ion.
b Due to miscleavage, masses reflect a larger fragment of residues 265-285.
c Not available. The glycosylation of these two sites could not be established from the data set in this experiment.
3.1.4 The effect of glycosylation on meprin A oligomeric structure and stability

3.1.4.1 Enzymatic deglycosylation of meprin A

3.1.4.1.1 Glycosylation sites removed by PNGaseF under native conditions

Meprin A can be partially deglycosylated by treatment with PNGaseF for 2 hr at 37°C under nonreducing and nondenaturing conditions (106). PNGaseF removes the entire carbohydrate moiety, converting the Asn to Asp in the process of breaking the bond between the innermost GlcNAc residue and the protein. The conversion of Asn to Asp is useful in identifying the deglycosylated peptides since there is a difference of +1 mass unit. Thus, in order to identify the PNGaseF sensitive glycosylation sites, latent meprin A was partially deglycosylated, digested with trypsin, and deglycosylated sites were mapped using ESI/MS. To ensure that PNGaseF did not continue to remove glycosylation sites during tryptic digestion, the partially deglycosylated sample was boiled to inactivate the deglycosidase. Analysis of meprin treated with PNGaseF under several conditions demonstrated that boiling alone was sufficient to prevent further removal of sugars for the duration of time needed for tryptic digestion (Figure 13a, lane 4; Figure 13b, lanes 5,6). Analysis of the tryptic digest of partially deglycosylated meprin A showed that two sites in the protease domain (N234, N270) and one site in the TRAF domain (N452) were removed by PNGaseF in the latent homooligomer (Table 5).
**Figure 13. Inactivation of PNGaseF for mapping experiments.** Meprin was incubated in the presence or absence of PNGaseF, with or without reducing/denaturing buffer as indicated.  

*a* Meprin was incubated for 2 hr at 37°C with PNGaseF then immediately mixed with reducing SDS-PAGE sample buffer (lanes 1-3) or boiled and incubated at room temperature for an additional 1 hr (lane 4). Meprin was also fully reduced and denatured, mixed with PNGaseF, then immediately boiled before a 15 min (lane 5) or 60 min (lane 6) incubation at 23°C.  

*b* Meprin was incubated with or without PNGaseF for 1 hr (lanes 1,2,4,5) or 2 hr (lanes 3,6) under the conditions indicated. A portion of each partially deglycosylated sample (lanes 2,3) was boiled for 15 min, then incubated overnight at 23°C to determine if there was further PNGaseF activity (lanes 5,6). Protein was visualized by Western blotting with an anti-meprin A antibody.
a) SDS/BME ---+        --
   PNGaseF - +     +      +     +

1 hr  2 hr  1 hr  2 hr

1     2     3      4      5      6

b)  

SDS/BME - - - + - - - 
PNGaseF - + + + + + + 

1 2 3 4 5 6
Table 5

Mapping of the glycosylation sites removed by PNGaseF in latent meprin A

Latent, His-tagged mouse meprin A was deglycosylated with PNGaseF under nonreducing and nondenaturing conditions, digested with trypsin, and analyzed by ESI/MS as described in the Materials and Methods section. The expected nonglycosylated (NG) and deglycosylated (DG) tryptic masses are listed, as well as the calculated m/z values for the peptide ions. Deglycosylated peptides are indicated by a change of +1 mass unit, due to the conversion of Asn to Asp when the glycan is removed by PNGaseF.

<table>
<thead>
<tr>
<th>Asn</th>
<th>Mass</th>
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<th>+2</th>
<th>+3</th>
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<td>1103.59</td>
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<td>368.53</td>
<td>276.65</td>
<td>(DG) 552.92(z2)</td>
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<tr>
<td></td>
<td>(DG) 1103.59</td>
<td>1104.59</td>
<td>552.80</td>
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<td>270</td>
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<td></td>
<td>(DG) 536.62(z4)</td>
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<tr>
<td>452</td>
<td>(NG) 1257.69</td>
<td>1258.69</td>
<td>629.85</td>
<td>420.23</td>
<td>315.42</td>
<td>(DG) 1260.02(z1),</td>
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<tr>
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<td>(DG) 1258.69</td>
<td>1259.59</td>
<td>630.35</td>
<td>420.56</td>
<td>315.67</td>
<td>(DG) 630.49(z2)</td>
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</table>

aN Numbers in parentheses indicate the charge of the observed ion.
These data complemented the glycosylation site mapping data obtained after chemical
deglycosylation, confirming the presence of glycans at these sites. The mass difference
of +1 mass unit is subtle, but the m/z values observed and reported in Table 5 are within
approximately 0.2 m/z units of the calculated m/z values for the deglycosylated peptides,
which is acceptable for time-of-flight instruments. There was no evidence of the
unmodified peptides for these glycosylation sites in this particular digest.

3.1.4.1.2 Structure and activity of partially deglycosylated meprin A

The effect of partial deglycosylation on the secondary and tertiary structures of
meprin A was determined using circular dichroism (CD) and intrinsic tryptophan
fluorescence. The far-UV CD spectrum of a protein is a measure of the protein’s
secondary structure, while the fluorescence spectrum detects changes in the local
environment of tryptophans as well as changes in tertiary structure (128). Small
differences are observed in the CD spectra after deglycosylation of the latent protein
(Figure 14a); the greatest change in ellipticity is observed around 212 and 225 nm. The
CD spectra of the activated samples were not obtained since these experiments require
much more protein than the fluorescence experiments, and the supply of protein was
limited at this time. The fluorescence spectra of the latent wild-type and partially
deglycosylated samples show only subtle differences (Figure 14b). The wild-type protein
has a \( \lambda_{\text{max}} \) of emission at 331 nm while the wavelength for the partially deglycosylated
protein changes to 334 nm (Figure 14b, Table 6). A slight change in the fluorescence
intensity is also observed. The \( \lambda_{\text{max}} \) of the wild-type protein changes to 335 nm upon
Figure 14. Secondary and tertiary structure of partially deglycosylated meprin A.

a) The far-UV CD spectra of latent, wild-type and partially deglycosylated meprin A after 15 min at 23°C. Final protein concentration was 3.2 μM. (▲) wild type; (●), partially deglycosylated. b) The intrinsic fluorescence spectra of wild-type and partially deglycosylated meprin A were obtained in the absence of urea after 30 min at 23°C with excitation at 280nm. Final protein concentration was 118 nM. (▲) latent, wild type; (●) latent, partially deglycosylated; (♦) active, wild type; (×) active, partially deglycosylated.
a) Ellipticity (mdeg) vs. Wavelength (nm)

b) Intrinsic Fluorescence (counts) vs. Wavelength (nm)
The wavelength of maximal emission ($\lambda_{\text{max}}$) was measured for latent and active protein, with or without treatment with PNGaseF under non-denaturing conditions (partially deglycosylated). The values for native proteins were taken in the absence of urea; the values for unfolded proteins were taken in the presence of 8 M urea, after 30 min at 23°C.

<table>
<thead>
<tr>
<th></th>
<th>$\lambda_{\text{max}}$, native</th>
<th>$\lambda_{\text{max}}$, unfolded</th>
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</thead>
<tbody>
<tr>
<td>Latent, wild-type</td>
<td>331</td>
<td>351</td>
</tr>
<tr>
<td>Latent, partially deglycosylated</td>
<td>334</td>
<td>352</td>
</tr>
<tr>
<td>Active, wild-type</td>
<td>335</td>
<td>352</td>
</tr>
<tr>
<td>Active, partially deglycosylated</td>
<td>338</td>
<td>352</td>
</tr>
</tbody>
</table>
activation, consistent with a conformational change observed previously for meprin A (40). Partial deglycosylation of the active protein changes the $\lambda_{\text{max}}$ to 338 nm and there is more of a change in fluorescence intensity. All proteins have a $\lambda_{\text{max}}$ of 351-352 nm in the presence of 8 M urea, which is within the range of $\lambda_{\text{max}}$ values for unfolded proteins and indicates that the unfolded state is similar for all samples (128).

The BK+ activity of meprin A after partial deglycosylation was also measured. As shown in Figure 15a, both the latent and active proteins that were partially deglycosylated with PNGaseF retained 86% and 74% of the BK+ activity, respectively. For latent samples, meprin was first deglycosylated then activated, while the active samples were activated first then deglycosylated. The data obtained from the deglycosylated protein in Figure 15a are representative of the average percent activity; there was some variability between independent deglycosylation reactions so that the activity ranged from 70-97% of wild type. For comparison, the small percentage of high mannose sugars removed by EndoH did not have any effect on BK+ activity (Figure 15b). Taken together, the CD, fluorescence, and activity data indicate that some minor changes occur upon deglycosylation, which may or may not affect the oligomeric state of the protein.

3.1.4.1.3 Oligomeric state of partially deglycosylated meprin A

To determine if removal of the three glycans in partially deglycosylated meprin A affects the oligomeric state, or distribution of states, the wild-type and partially deglycosylated proteins were analyzed by size exclusion chromatography. The
Figure 15. Activity of partially deglycosylated meprin A. The BK+ activity of meprin A after partial deglycosylation with either PNGaseF or EndoH was compared to that of untreated, wild-type protein. a) PNGaseF treatment  b) EndoH treatment. The specific activity of each sample was measured in triplicate. The data are representative of ten individual experiments for latent, PNGaseF-treated protein, five individual experiments for active, PNGaseF-treated protein, and two individual experiments for latent, EndoH-treated protein.
82

a)

![Graph a)

b)

![Graph b)
elution of the protein was detected by the absorbance at 280 nm as well as by Western blotting of the fractions. Both latent and active proteins were deglycosylated under nonreducing and nondenaturing conditions since there may be a different effect on each form of the protein. As shown in Figure 16a (top panel), the latent protein mainly elutes as an octamer in Fraction 8, based on calibration of the column with protein standards, and accounts for 68% of the total immunoreactivity. The partially deglycosylated protein is also predominantly in Fraction 8, corresponding to 78% of the protein. The chromatograms (Figure 16a, lower panel) show a small shoulder for the wild-type protein that is not as noticeable for the deglycosylated protein. Thus, there appears to be a slight change in the distribution of species, either resulting from smaller oligomers forming the large molecular mass species or a certain amount of aggregation. Similar behavior was observed for activated protein (Figure 16b), although wild-type and partially deglycosylated proteins both demonstrated a small amount of trailing in the Western blots and chromatograms. The protein in Fraction 8 accounted for 39% of the wild-type protein and 47% of the partially deglycosylated protein. Overall, removing the glycans at N234, N270, and N452 did not dramatically change the distribution of the meprin A oligomers, since the amount of partially deglycosylated protein eluting in Fraction 8 was only 8-10% higher than wild type, and there was no increase in the amount of smaller oligomeric species.
Figure 16. Oligomeric state of wild-type and partially deglycosylated meprin A.

The oligomeric state of wild-type (-PNGaseF) and partially deglycosylated (+PNGaseF) meprin A was assessed by size exclusion chromatography. *a*) latent meprin A; *b*) active meprin A. *Top panels*, Western blots of each 1 ml Superose fraction; *bottom panels*, chromatogram of the absorbance at 280 nm.
3.1.4.1.4 Unfolding of the meprin A homooligomer

Although the oligomeric state of meprin A is not affected by the removal of glycans, these glycans may contribute to the overall stability of the wild-type homooligomer. The stability of proteins can be compared by their susceptibility to denaturing agents, such as chaotropic agents and heat. The unfolding reaction of wild-type meprin A was followed by CD and intrinsic fluorescence, plotting the change of signal at a given wavelength. As shown in Figure 17a, the CD spectrum of wild-type, latent meprin A changes in the presence of increasing amounts of urea. In 5 M urea, the protein is completely unfolded with a spectrum characteristic of a random coil. When the ellipticity at 225 nm is plotted vs urea concentration, an unfolding transition is observed at 3 M urea. Since meprin is a large, multidomain protein it is likely that unfolding of the meprin subunits is not a simple two state process. The absence of a cooperative folding transition, as well as the lack of an isodichroic point in the CD spectra, is consistent with a multistep unfolding process. Like the latent protein, the unfolding of the activated oligomer demonstrates a broad transition, although in this case the midpoint is higher than for the latent protein, indicating a more stable conformation (Figure 18). In this particular experiment, an increase in the fluorescence at 1 M urea was observed. This was seen is several experiments, and is also indicative of a multistate unfolding process.
Figure 17. Unfolding of meprin A monitored by CD. The unfolding of wild-type, latent meprin A by urea was monitored by the change in ellipticity at 225 nm at a final protein concentration of 4.6 μM. 

a) CD spectra at various urea concentrations, as indicated. 

b) Unfolding curve created by plotting the ellipticity at 225 nm vs urea concentration after 1.5 hr at 23°C. Spectra were not corrected for the contribution of the denaturant in this experiment.
Figure 18. Unfolding of meprin A monitored by intrinsic tryptophan fluorescence.

The unfolding of wild-type, active meprin A by urea was monitored by the change in intrinsic tryptophan fluorescence. Excitation wavelength was 280 nm. 

a) Intrinsic fluorescence spectra at various urea concentrations, as indicated.  
b) Unfolding curve created by plotting the fluorescence at 335 nm ($\lambda_{\text{max}}$) vs urea concentration after 30 min at 23°C. Samples were not corrected for the contribution of denaturant in this experiment.
Intrinsic fluorescence (counts s\(^{-1}\))

wavelength (nm)

urea (M)

Intrinsic fluorescence at 335 nm (counts s\(^{-1}\))
3.1.4.1.5 Reversibility of the meprin A unfolding reaction

In order for a thermodynamic value to be calculated for the contribution of glycans to oligomer stability, the unfolding transition of the protein must be reversible, that is 90% of the signal must be recovered once the denaturant is removed (128). Meprin A is a very stable protein, maintaining almost wild-type activity over a period of 70 hr at 23°C (Figure 19a), compared to its initial activity at t = 0 hr and a positive control freshly activated at each time point. The unfolding of meprin A in the presence of 7 M urea is nearly complete in 4 hr, as assessed by loss of enzymatic activity. However, when the sample is diluted so that the final urea concentration is 0.5 M, there is no recovery of BK+ activity over a 48 hr period, indicating that the unfolding process is not readily reversible (Figure 19b). Thus, a thermodynamic value cannot be assigned to the contribution of glycans to oligomer stability. Instead, only a relative comparison can be made that will be sensitive to experimental conditions.

3.1.4.1.6 Stability of partially deglycosylated meprin A

Urea induced unfolding and heat stability assays were used to assess the stability of meprin A after partial deglycosylation. The active, partially deglycosylated protein begins to unfold at lower urea concentrations than the wild type when monitored by intrinsic tryptophan fluorescence (Figure 20a). As stated above, a thermodynamic value could not be assigned to this difference since the reaction is irreversible. Based on data from several experiments, there appeared to be less of a difference in stability for the latent protein. A difference in stability was also observed by incubating meprin at 55°C
Figure 19. Stability of meprin A over time and reversibility of the urea induced unfolding.  

a) The BK+ activity of meprin A was measured after incubation at 23°C for the times indicated and was compared to a control sample that was freshly thawed and activated at each timepoint.  

b) Activated meprin A was incubated in the presence ( ■ ) or absence ( ♦ ) of 7 M urea for 24 hr, and BK+ activity was measured at the times indicated. For renaturation, the remaining sample was diluted to a final urea concentration of 0.57 M in the presence of 20 mM Tris, pH 7.5 and 10 μM ZnCl₂. Samples were then incubated at 23°C for an additional 24 hr with monitoring of activity by BK+ (assayed in triplicate). Arrows indicate the point where the samples were diluted.
a) 

![Bar chart showing average specific activity of meprin at different times.]

- Freshly activated meprin
- Meprin at 23°C

b) 

![Line graph showing average specific activity over time.]

- Fluorescence units s⁻¹ μg⁻¹
Figure 20. Stability of the partially deglycosylated meprin A oligomer.  

a) Active meprin A (+/- PNGase F under nondenaturing conditions) was incubated with increasing concentrations of urea for 30 min at 23°C at a final protein concentration of 118 nM. The intrinsic tryptophan fluorescence at 335 nm was used to monitor the unfolding transition. (♦) wild type; (■) partially deglycosylated.  

b) Heat stability of wild-type and partially deglycosylated meprin A. Activated samples (+ soybean trypsin inhibitor) were incubated in the presence and absence of PNGaseF under nondenaturing conditions, then preincubated at 55°C for the times indicated. Samples were cooled to 23°C and residual BK+ activity was measured in triplicate for each time point. (♦) wild type; (■) partially deglycosylated.
a) Intrinsic fluorescence at 335 nm (counts)

b) Average specific activity vs. preincubation (min)
for different periods of time and measuring residual BK+ activity. In this assay, the partially deglycosylated protein loses activity about five times faster than wild type (Figure 20b). Therefore, although glycosylation does not affect the oligomeric state of the wild-type meprin protein, it contributes to oligomer stability.

3.1.4.2 Role of glycosylation during homooligomer synthesis

Since glycosylation does not dramatically affect the structure of the meprin in the folded state, studies were undertaken to investigate the role glycans play in protein folding and oligomer formation.

3.1.4.2.1 Oligomeric state of single N-linked glycosylation mutants

To determine if any one glycan affected the oligomeric state, the single N-linked glycosylation mutations described by Kadowaki et al. were analyzed for their ability to form disulfide-bonded dimers and high molecular mass oligomers (14). These mutants had only been analyzed for their expression (under reducing and denaturing conditions) and apical targeting. It is possible that the high degree of heat inactivation observed for the N152Q, N234Q, and N270Q mutants is due to lack of higher order oligomer formation observed for monomeric forms of meprin A (14,50,51). N614Q also demonstrated a similar degree of instability; however, this site was found to be unmodified in the mapping experiment. While this effect is not likely due to absence of a glycan at this position, the Asn to Gln mutation may have perturbed the quaternary
structure in some other manner. N330 is only 10 a.a. away from an intersubunit disulfide bond; it is possible that this glycan influences the rate of disulfide bond formation in this region (129). Thus, each single glycosylation site mutant was transiently transfected into HEK-293 cells, the media was collected 24 hr posttransfection, concentrated four-fold, and analyzed by nonreducing SDS-PAGE and native PAGE.

In contrast to the present studies, the single glycosylation mutants had previously been stably transfected into polarized Madin-Darby canine kidney (MDCK) cells for targeting studies. Unlike the MDCK cell expression, N41Q (in the prosequence) was not as highly expressed as wild-type during transient transfections in HEK-293 cells (Figure 21a). Three plates of HEK-293 cells were simultaneously transfected with the N41Q plasmid, and 24 hr posttransfection expression was assessed by SDS-PAGE and immunoblotting of media collected directly from the cells. All other single N-linked glycosylation site mutants were expressed at levels similar to wild type, and all ten glycosylation site mutants could form the disulfide-bonded dimer (Figure 21b). The amount of media loaded for the N41Q sample was increased in Figure 21b so that it could be detected in the range of the other mutants.

The ability of each glycosylation site mutant to form the high molecular mass oligomers was assessed by native PAGE with Western blotting of the crude media samples. Each of the mutants is capable of forming the large oligomeric species observed for wild-type protein (Figure 22). Meprins do not run as distinct bands in native gel systems because of the high level of glycosylation and heterogeneity of the oligomers, but the distribution of immunoreactivity throughout the lane is consistently observed
Figure 21. Transient transfections and nonreducing SDS-PAGE of single N-linked glycosylation site mutants.  

a) Expression and secretion of N41Q.  HEK-293 cells were transiently transfected with wild-type or N41Q cDNA, and media samples were collected and analyzed by reducing SDS-PAGE and Western blotting 24 hr posttransfection. Plates 1-3 represent three different transfections of the N41Q mutant.  

b) All single N-linked glycosylation site mutants were transiently transfected in HEK-293 cells, media samples were collected and concentrated four-fold 24 hr posttransfection, and the samples were analyzed by nonreducing SDS-PAGE and Western blotting.  The volume of N41Q loaded was increased relative to the remaining mutants to load a proportional amount of meprin.
Figure 22. Oligomeric state of single N-linked glycosylation site mutants. Media samples from transiently transfected HEK-293 cells were collected, concentrated four-fold, and analyzed by native PAGE on a 3-8% gradient gel with Western blotting. The arrow indicates the large molecular mass oligomers that migrate near the top portion of the gel. N41Q was run on the same gel as the other mutants, but a longer exposure was required to visualize the protein.
null
with meprins capable of forming higher order oligomers (44). Several of these mutants were confirmed to be oligomeric by size exclusion chromatography (see below). Thus, no one glycosylation site dramatically affects the oligomeric state of the meprin homooligomer, either through prevention of disulfide-bonded dimers or higher order oligomers. It is possible that there is a change in the distribution of the size of the oligomers. For example, there may be a larger population of intermediately sized species in N234Q where there is increased immunoreactivity, or this effect may simply be an artifact of the gel system.

3.1.4.2.2 Oligomeric state of multiple glycosylation site mutants

Since no one glycosylation site affected the formation of the homooligomer, double and triple mutants were made, focusing on the protease and MAM domains. The protease domain glycosylation sites are the most destabilizing individually, and the MAM domain glycosylation sites may direct oligomerization by influencing the protein structure or regulating intersubunit disulfide bond formation. A scheme of the double and triple glycosylation site mutants is shown in Figure 23. These mutants were transiently expressed in HEK-293 cells and analyzed for their ability to form disulfide-bonded dimers and high molecular mass oligomers. The protease domain mutants were all expressed (Figure 24a). In a separate set of transfections, the N330Q/N426Q mutant was also expressed (data not shown). The mobilities of the N152Q/N234Q and N234Q/N270Q mutants were consistent with the removal of the two glycans. However, the N152Q/N270Q and N152Q/N234Q/N270Q mutants appeared to have more
Figure 23. Scheme of multiple glycosylation site mutants. Site-directed mutagenesis was used to create multiple glycosylation site mutants in the protease and MAM domains of mouse meprin α. Glycosylation sites are indicated by lines and numbers, with an X designating that the site has been removed by mutation of Asn to Gln.
Figure 24. Expression of multiple glycosylation site mutants. HEK-293 cells were transiently transfected with the multiple glycosylation site mutants and expression was analyzed 24 hr posttransfection by \textit{a}) reducing SDS-PAGE and \textit{b}) nonreducing SDS-PAGE. Meprin was visualized by Western blotting with an anti-meprin A antibody. For reducing SDS-PAGE, media samples from two separate transfections performed simultaneously were analyzed.
heterogeneity and a mobility that was slower than expected. The expression of mutants relative to wild type was assessed by densitometry of the two individual transfections. As shown in Figure 25, the N152Q/N234Q and N234Q/N270Q mutants were secreted at wild-type levels (109% and 83%) while the N152Q/N270Q and the triple mutants were expressed at 60% and 75% of wild type. Under nonreducing conditions the N152Q/N270Q and the N152Q/N234Q/N270Q mutants do not appear to form the disulfide-bonded dimers to the same extent as wild-type (Figure 24b). On average, the latter two mutants formed approximately one-third the amount of dimer as compared to wild type (25% ± 6% for N152Q/N270Q and 25% ± 18% for N152Q/N234Q/N270Q versus 75% ± 4% for wild type). The amount of dimer formed may be influenced by cellular conditions at the time of transfection, or it may result from oxidation of the sample after secretion. For example, in this particular transfection, the triple mutant does not form any dimer, but in two other transfections the media contained 15 and 60% dimer.

Although they do not form disulfide-bonded dimers, it is still possible that these mutants are capable of noncovalent associations, leading to formation of a dimer or larger oligomer, as observed for intersubunit cysteine mutants of rat meprin B (39). Size exclusion chromatography was used to assess oligomeric state instead of native gels since it provides a clearer picture of the distribution of species and the samples can be analyzed under normal solution conditions (20 mM Tris, 150 mM NaCl, pH 7.5). The presence of meprin in the fractions from a Superose 12 column were visualized by reducing SDS-PAGE and Western blotting with an anti-meprin A antibody since the samples were not pure. The largest species formed by the wild-type protein eluted in Fraction 8, and this
Figure 25. *Several multiple glycosylation site mutants decrease secretion of meprin A into the media.* The expression of the glycosylation mutants was quantified by densitometry of the bands from the Western blot in Figure 24, expressed as a percentage of wild-type protein. The percentages reflect an average of two individual transfections performed simultaneously and are consistent with two other sets of transfections performed previously.
Relative expression of meprin mutants in the media (average % wild-type)
Figure 26. Oligomeric state of multiple glycosylation site mutants. Media samples from transiently transfected cells were collected, concentrated four-fold, and analyzed by size exclusion chromatography on a Superose 12 column. One-ml fractions were collected and meprin was visualized in the fractions by reducing SDS-PAGE and Western blotting. An asterisk (*) indicates the fraction with the highest level of meprin as determined by densitometry. a) wild type, b) N152Q, c) N234Q, d) N270Q, e) N152Q/N234Q, f) N152Q/N270Q, g) N234Q/N270Q, h) N152Q/N234Q/N270Q, and i) N330Q/N426Q.
reflects a molecular mass of an octamer based on a subunit molecular mass of 85 kDa (Figure 26a). The N152Q, N234Q, and N270Q single glycosylation mutants also formed large molecular mass species, consistent with native PAGE (Figure 26b-d). In addition, the N152Q/N234Q, N234/N270Q, and N330Q/N426Q double mutants were all capable of oligomerization (Figure 26e,g,i). There was no difference in the distribution of the oligomeric species based on densitometry of the bands on the Western blot (data not shown). However, the N152Q/N270Q and N152Q/N234Q/N270Q mutants did not form the high molecular mass species, with the majority of the protein eluting in Fractions 11 and 12 (Figure 26f,h). When the Superose 12 fractions of these mutants were analyzed under nonreducing conditions (Figure 27), the dimeric species in Fractions 8-13 accounted for only 6% of the total immunoreactivity for N152Q/N270Q and 22% for the triple mutant. The appearance of disulfide-bonded dimers in Fractions 8, 9, and 10 may reflect a small portion of the population that has achieved a native fold, or it may be due to nonspecific oxidation and aggregation of the protein during synthesis or after secretion. Overall, the N152Q/N270Q and triple mutants are predominantly monomeric species.

3.1.4.2.3 Characterization of the multiple glycosylation site mutants

The multiple glycosylation mutants in the protease domain were further characterized for their stability and protease activity. The oligomeric mutants are resistant to degradation by trypsin (Figure 28), but the monomeric mutants are mostly degraded in the presence of 40 ng/μl of trypsin. This may be due to a combination of
Figure 27. Nonreducing SDS-PAGE of N152Q/N270Q and N152Q/N234Q/N270Q Superose 12 fractions. Disulfide bonds in the size exclusion chromatography fractions of the a) wild type, b) N152Q/N270Q, and c) N152Q/N234Q/N270Q mutants were detected by nonreducing SDS-PAGE and Western blotting.
<table>
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<th>Fraction</th>
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Figure 28. Tryptic activation and stability of the multiple glycosylation site mutants. The concentrated media collected for each mutant was incubated at 37°C for 45 min with trypsin at a final concentration of 0, 5, 40, and 80 ng/μl. A three-fold excess (relative to the highest concentration of trypsin) of soybean trypsin inhibitor was added and the samples were separated and analyzed by reducing SDS-PAGE with Western blotting.
overall misfolding as well exposure of both subunit interfaces. Since all of mutants were stable at 5 ng/μl of trypsin, the mutants were activated at this concentration and their peptidase activity was measured using BK+ as the substrate. Again, only the oligomeric mutants maintained wild-type or near wild-type activity (Table 7). There was no detectable protease activity for the N152Q/N270Q and N152Q/N234Q/N270Q mutants, even though there was a similar amount of protein present in the sample as determined by densitometry. The corresponding single glycosylation site mutants were included in this experiment for comparison. As shown in Table 7, these mutants maintained wild-type or near wild-type activity. This is in contrast to activity measurements reported by Kadowaki et al. (14). In that study, the N152Q mutant maintained only 28% of the wild-type activity (compared to 73% in the present study). The N234Q mutant had 75% of wild-type activity (versus 100%) and the N270Q mutant had 63% activity (versus 89%). This discrepancy may be due to differences in cellular conditions or other modifications that in turn affect the population of active meprin molecules. In the previous study, the samples were activated at 1 ng/μl trypsin for 30 min at 25°C versus 5 ng/μl trypsin for 45 min at 37°C. Thus, it is not likely that the previously measured activities are due to degradation of the protein. The discrepancies may be due, however, to incomplete activation at the lower trypsin concentration.

The stability of each multiple glycosylation site mutant was assessed by susceptibility to heat inactivation. Samples were activated as described above, then preincubated at 55°C for different periods of time. Residual BK+ activity was used as an indication of resistance to heat induced unfolding. The N152Q/N270Q and
Table 7

*BK+ activity of the multiple glycosylation site mutants*

The peptidase activities of the multiple glycosylation site mutants in crude media samples were measured with the fluorogenic substrate BK+ after activation with 5 ng/μl trypsin for 45 min at 37°C. Specific activities are expressed in fluorescence units s⁻¹ μg⁻¹. The amount of meprin in each sample was based on the densitometry measurements from Western blots of the crude media, relative to the wt sample. The absolute concentration of meprin in the wt sample was measured by BK+ activity compared to the fluorescence from a known amount of pure protein. The values listed are an average of one activated sample assayed in triplicate with the corresponding standard error.

<table>
<thead>
<tr>
<th>Mutant Combination</th>
<th>Specific activity</th>
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<tbody>
<tr>
<td>wild type</td>
<td>138 ± 9</td>
</tr>
<tr>
<td>N152Q/N234Q</td>
<td>113 ± 3</td>
</tr>
<tr>
<td>N152Q/N270Q</td>
<td>ND&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>N234Q/N270Q</td>
<td>137 ± 9</td>
</tr>
<tr>
<td>N152Q/N234Q/N270Q</td>
<td>ND&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>N152Q</td>
<td>101 ± 5</td>
</tr>
<tr>
<td>N234Q</td>
<td>142 ± 2</td>
</tr>
<tr>
<td>N270Q</td>
<td>123 ± 6</td>
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</tbody>
</table>

<sup>a</sup> Not detected.
N152Q/N234Q/N270Q mutants were not included since they did not have any detectable activity. All glycosylation site mutants (single and double) were less stable than wild type (Figure 29). The N152Q and N234Q mutants were the most stable of the group, losing activity only 3-times faster than wild type. The other single glycosylation site mutant, N270Q, lost activity 5-times faster. The double mutants lost activity roughly additively based on the single mutations (6-times faster for N152Q/N234Q and 10-times faster for the N234Q/N270Q). It is interesting that the N234Q/N270Q mutant was the least stable of all. This mutant corresponds to the partially deglycosylated oligomer of pure meprin A, where these glycans are removed by PNGaseF and decrease the overall stability of the protein (see Figure 20). In partially deglycosylated meprin A, PNGaseF also removed the glycan at N452. Since a single meprin A mutation at this position had wild-type heat stability, it is reasonable to conclude that the protease domain glycosylation sites of N234 and N270 are mainly responsible for stability (14).

3.1.5 Glycosylation of other meprin isoforms

Glycosylation has been shown herein to be involved in meprin A oligomer formation and stability. The hypothesis that differences in glycosylation patterns lead to differences between the meprin isoforms requires characterization of both the meprin A and meprin B glycans. Thus, an initial characterization of the glycans from rat meprin A and rat meprin B was undertaken.
Figure 29. Heat stability of the multiple glycosylation site mutants. Concentrated media samples of each mutant were activated with trypsin at a final concentration of 5 ng/μl as described above, incubated at 55°C for 0, 5, 15, 30, and 45 min, cooled to 23°C, and the residual BK+ activity was measured at each timepoint. (◆) wild type; (■) N152Q/N234Q; (▲) N234Q/N270Q; (×) N152Q; (★) N234Q; (●) N270Q.
Preincubation at 55°C (min)
3.1.5.1 Quantitative carbohydrate analysis of rat meprins A and B

To determine if rat meprins A and B had a similar distribution of monosaccharides as compared to mouse meprin A, a total carbohydrate analysis was performed. As shown in Table 8, the amounts of GlcNAc, galactose, and mannose in rat meprin A are similar to those in mouse meprin A (Table 2). However, rat meprin A has no detectable fucose. This is in contrast to mouse meprin A which contains significant amounts of fucose (7% of the total carbohydrate). Surprisingly, both rat meprin A and B had detectable amounts of GalNAc, accounting for 8% and 6% of the total carbohydrate, respectively. These results reflect only one total carbohydrate analysis from a single purification of the protein, and the experiment would thus need to be repeated to determine if the amount of fucose and GalNAc detected are reproducible for these isoforms.

3.1.5.2 Glycosylation site mapping of rat meprins

The glycosylation sites of rat meprins A and B were mapped using the chemical deglycosylation method and ESI/MS. Rat meprin A contains only six potential N-linked glycosylation sites, and there was evidence that N223, N259, N441, and N542 are glycosylated (Table 9). However, there was also evidence of the unmodified tryptic peptide in the digest for N223 and N441, and this indicates heterogeneity at these
Table 8

*Total carbohydrate analysis of homooligomeric rat meprins A and B*

Purified recombinant rat meprin A and rat meprin B were hydrolyzed by trifluoroacetic acid, separated by HPLC, and the total molar amount of each monosaccharide was determined for each sample using known amounts of monosaccharides as standards. The results reflect the average ratio for each monosaccharide from one protein purification, with each sample injected once. *GalNAc, N-acetylgalactosamine; GlcNAc, N-acetylglucosamine.*

<table>
<thead>
<tr>
<th>Monosaccharide</th>
<th>Rat meprin A</th>
<th>Rat meprin B</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nmole carbohydrate: nmole protein</td>
<td>% total carbohydrate&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>fucose</td>
<td>ND&lt;sup&gt;b&lt;/sup&gt;</td>
<td>ND&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>GalNAc</td>
<td>7.7</td>
<td>8%</td>
</tr>
<tr>
<td>GlcNAc</td>
<td>50.7</td>
<td>52%</td>
</tr>
<tr>
<td>galactose</td>
<td>10.4</td>
<td>11%</td>
</tr>
<tr>
<td>mannose</td>
<td>28.8</td>
<td>30%</td>
</tr>
</tbody>
</table>

<sup>a</sup> The total nmoles of monosaccharide divided by the total amount of carbohydrate detected in each sample.

<sup>b</sup> No significant amounts were detected.
Rat meprin A was chemically deglycosylated with TFMS, digested with trypsin, and analyzed by ESI/MS as described in the Materials and Methods. The expected nonglycosylated (NG) and glycosylated (G) tryptic masses are listed, as well as the calculated \( m/z \) values for the peptide ions. Glycopeptides were distinguished from unmodified peptides by the presence of one \( N \)-acetylglucosamine residue (GlcNAc, +203 mass units).

<table>
<thead>
<tr>
<th>Asn</th>
<th>Mass</th>
<th>+1</th>
<th>+2</th>
<th>+3</th>
<th>Observed ions(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>141</td>
<td>NG: 5037.203</td>
<td>5038.211</td>
<td>2519.610</td>
<td>1680.412</td>
<td>N/A(^b)</td>
</tr>
<tr>
<td></td>
<td>G: 5240.282</td>
<td>5241.290</td>
<td>2621.149</td>
<td>1748.105</td>
<td></td>
</tr>
<tr>
<td>223</td>
<td>NG: 1116.630</td>
<td>1117.638</td>
<td>559.323</td>
<td>373.554</td>
<td>NG: 559.381 (z2), G: 660.913 (z3), G: 441.240 (z3)</td>
</tr>
<tr>
<td></td>
<td>G: 1319.709</td>
<td>1320.717</td>
<td>660.863</td>
<td>441.247</td>
<td></td>
</tr>
<tr>
<td>259</td>
<td>NG: 786.315</td>
<td>787.323</td>
<td>394.166</td>
<td>263.449</td>
<td>G: 990.551 (z1), G: 495.755 (z2)</td>
</tr>
<tr>
<td></td>
<td>G: 989.394</td>
<td>990.402</td>
<td>495.705</td>
<td>331.142</td>
<td></td>
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<tr>
<td>319</td>
<td>NG: 2569.216</td>
<td>2570.224</td>
<td>1285.616</td>
<td>857.749</td>
<td>N/A(^b)</td>
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<tr>
<td></td>
<td>G: 2772.295</td>
<td>2773.303</td>
<td>1387.156</td>
<td>925.442</td>
<td></td>
</tr>
<tr>
<td>441</td>
<td>NG: 1243.677</td>
<td>1244.685</td>
<td>622.847</td>
<td>415.903</td>
<td>NG: 622.923 (z2)</td>
</tr>
<tr>
<td></td>
<td>G: 1446.756</td>
<td>1447.764</td>
<td>724.386</td>
<td>483.596</td>
<td>G: 724.480 (z2)</td>
</tr>
<tr>
<td>542</td>
<td>NG: 2022.017</td>
<td>2023.025</td>
<td>1012.017</td>
<td>675.350</td>
<td>G: 743.134 (z3)</td>
</tr>
<tr>
<td></td>
<td>G: 2225.096</td>
<td>2226.104</td>
<td>1113.556</td>
<td>743.043</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) Numbers in parentheses indicate the charge of the observed ion.

\(^b\) Not available. The glycosylation of these two sites could not be established from the data set in this experiment.
glycosylation sites. No peptide ions were observed for two glycosylation sites, N141 and N319; thus the modification at these sites is unknown.

Rat meprin B contains eight potential glycosylation sites in the prosequence through the TRAF domain. Glycosylation site mapping of recombinant, secreted meprin B indicated that N422, in the MAM domain, is not glycosylated (Table 10), while N219 (protease domain) and N547 (TRAF domain) are glycosylated. The five other glycosylation sites were not accounted for in this experiment, and thus further analysis is needed to determine the status of these potential glycosylation sites.

### 3.1.5.3 Partial deglycosylation of rat meprin B

The sensitivity of rat meprin B to PNGaseF and EndoH under nonreducing and nondenaturing (native) conditions was investigated to determine if, like mouse meprin A, the glycans have a stabilizing effect on the homodimeric structure. Similar to mouse meprin A, the rat meprin B dimer is not very susceptible to deglycosylation by EndoH under both native and denaturing conditions (Figure 30a). Thus, only a small portion of the glycans are high mannose oligosaccharides. Like mouse meprin A, PNGaseF deglycosylates rat meprin B more efficiently than EndoH under native conditions (Figure 30a). When latent and active meprin B were treated with PNGaseF under similar conditions, prolonged incubation was needed for latent protein to see a shift in the mobility by SDS-PAGE (Figure 30b, lanes 2, 3). Interestingly, the activated form of meprin B produces three species upon partial deglycosylation, and their mobilities correspond roughly to fully glycosylated, partially deglycosylated, and fully
**Table 10**

*N-linked glycosylation mapping of rat meprin B using electrospray mass spectrometry*

Rat meprin B was chemically deglycosylated with TFMS, digested with trypsin, and analyzed by ESI/MS as described in the Materials and Methods. The expected nonglycosylated (NG) and glycosylated (G) tryptic masses are listed, as well as the calculated \( m/z \) values for the peptide ions. Glycopeptides were distinguished from unmodified peptides by the presence of one \( N \)-acetylglucosamine residue (GlcNAc, +203 mass units).

<table>
<thead>
<tr>
<th>Asn</th>
<th>Mass</th>
<th>+1</th>
<th>+2</th>
<th>+3</th>
<th>Observed ions&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
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<tr>
<td>193</td>
<td>NG: 3437.515</td>
<td>3438.523</td>
<td>1719.766</td>
<td>1147.182</td>
<td>N/A&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td></td>
<td>G: 3640.594</td>
<td>3641.602</td>
<td>1821.305</td>
<td>1214.845</td>
<td></td>
</tr>
<tr>
<td>219</td>
<td>NG: 1509.767</td>
<td>1510.775</td>
<td>755.892</td>
<td>504.600</td>
<td>G: 857.571 (z2)</td>
</tr>
<tr>
<td></td>
<td>G: 1712.846</td>
<td>1713.854</td>
<td>857.431</td>
<td>572.293</td>
<td></td>
</tr>
<tr>
<td>316</td>
<td>NG: 2576.142</td>
<td>2577.150</td>
<td>1289.079</td>
<td>860.058</td>
<td>N/A&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>G: 2779.221</td>
<td>2780.229</td>
<td>1390.619</td>
<td>572.293</td>
<td></td>
</tr>
<tr>
<td>422</td>
<td>NG: 2632.298</td>
<td>2633.306</td>
<td>1317.157</td>
<td>878.777</td>
<td>NG: 1317.390 (z2)</td>
</tr>
<tr>
<td></td>
<td>G: 2835.377</td>
<td>2836.385</td>
<td>1418.697</td>
<td>946.470</td>
<td>NG: 878.909 (z3)</td>
</tr>
<tr>
<td>437</td>
<td>NG: 3485.698</td>
<td>3486.706</td>
<td>1743.857</td>
<td>1163.243</td>
<td>N/A&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td></td>
<td>G: 3688.777</td>
<td>3689.785</td>
<td>1845.397</td>
<td>1230.936</td>
<td></td>
</tr>
<tr>
<td>528</td>
<td>NG: 1783.783</td>
<td>1784.791</td>
<td>892.900</td>
<td>595.938</td>
<td>N/A&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>547</td>
<td>NG: 1654.810</td>
<td>1655.818</td>
<td>828.413</td>
<td>552.947</td>
<td>G: 930.604 (z3)</td>
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<tr>
<td></td>
<td>G: 1857.889</td>
<td>1858.897</td>
<td>929.953</td>
<td>620.640</td>
<td></td>
</tr>
<tr>
<td>592</td>
<td>NG: 5509.607</td>
<td>5510.615</td>
<td>2755.812</td>
<td>1837.880</td>
<td>N/A&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>G: 5712.686</td>
<td>5713.694</td>
<td>2857.351</td>
<td>1905.573</td>
<td></td>
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</tbody>
</table>

<sup>a</sup> Numbers in parentheses indicate the charge of the observed ion.

<sup>b</sup> Not available. The glycosylation of these five sites could not be established from the data set in this experiment.
Figure 30. Partial deglycosylation of rat meprin B. Secreted rat meprin B was incubated with deglycosidases for 1 hr at 37°C under nonreducing and non-denaturing (native) conditions, then analyzed by reducing SDS-PAGE and Western blotting. 

a) Latent meprin B (lane 1) was incubated with either EndoH at pH 5.5 (lanes 2-4) or PNGaseF at pH 7.5 (lanes 5-7) after full reduction and denaturation (lanes 2, 5), native conditions (lanes 3, 6), or first under non-denaturing conditions, then reduced, denatured, and reincubated with the deglycosidase (lanes 4, 7). 

b) Samples of latent and active meprin B were partially deglycosylated under native conditions with PNGaseF for the times indicated. Arrows indicate the species that appear to correspond to a fully glycosylated, partially deglycosylated, and fully deglycosylated protein.
(a) 

Latent active

1hr 2hr 1hr 2hr

Native Native denaturing denaturing

wt wt denaturing

EndoH PNGaseF

(b) 

Latent active

1hr 2hr 1hr 2hr

Native Native denaturing denaturing

wt wt denaturing
deglycosylated species (Figure 30b, lanes 6,7). Partial deglycosylation decreased the OCK+ activity of meprin B to 83% of wild type after a two hour incubation, while a sample deglycosylated for one hour maintained 90% (Figure 31). Future studies would be needed to address the stability of the meprin B homooligomer by urea induced unfolding or heat stability.

3.1.5.4 Addition of a glycosylation site to rat meprin B

As shown in Figure 7, there are several glycosylation consensus sequences that are completely conserved in one meprin subunit and not the other. For instance, the glycosylation site homologous to N152 in mouse meprin A is conserved in all α subunits but is not conserved in the β subunits. The β subunits have conserved sequences at the sites homologous to N422 and N547 in rat meprin B, which are lacking in the α subunits. Since the N422 peptide was not found to be glycosylated in the ESI/MS mapping experiment, this site may have less influence on the oligomeric structure of the isoforms. Since N152 was implicated, in concert with N270, in the oligomerization of mouse meprin A, the N152 glycosylation site was introduced into the rat meprin B sequence by site-directed mutagenesis of Glu to Asn. The remaining consensus sequence was already present in rat meprin B so a single amino acid change was sufficient to create a complete consensus sequence. The E138N mutant was expressed in HEK-293 cells at a level similar to wild type, and could form disulfide-bonded dimers (Figure 32a,b). However, this mutation did not affect the oligomeric state of rat meprin B (Figure 32c). Thus, a
Figure 31. Activity of partially deglycosylated rat meprin B. The OCK+ activity of meprin B was measured in triplicate after partial deglycosylation of the activated protein for 1 hr and 2 hr at 37°C.
Average specific activity (Fluorescence units s$^{-1}$ ug$^{-1}$)

- Untreated
- 1 hr
- 2 hr
Figure 32. Addition of a glycosylation site to rat meprin B. The E138N mutant was used to introduce a consensus sequence into the rat meprin B protease domain, and both wild-type and mutant proteins were stably expressed in HEK-293 cells. 

a) Reducing SDS-PAGE of media samples, in the presence of serum. 
b) Nonreducing SDS-PAGE of media samples, in the presence of serum. 
c) 3-8% native PAGE of media samples. 

Samples were visualized by Western blotting with an anti-meprin B antibody.
single glycosylation site introduced in the rat meprin B sequence does not promote higher order oligomer formation, although it was not determined if this site was actually glycosylated in the mutant protein.

3.2 The contribution of the MAM domain to meprin A oligomerization

3.2.1 The MAM truncation mutant forms nonnative disulfides

Previous work indicated that truncating the meprin α subunit after the MAM domain decreased the stability and activity of the protein and that homooligomeric meprin A does not form the large oligomeric species unless the entire TRAF domain is present (26,27). However, the TRAF truncation mutants appeared to form additional disulfide-bonded species on nonreducing SDS-PAGE. The MAM truncation mutant (1-445) also forms species of slower mobility under nonreducing conditions (Figure 33a), which are larger than the expected mobility after truncation. It is possible that the MAM domain can oligomerize to a certain degree on its own, or the larger species may be due to nonspecific intersubunit disulfide bonding. To evaluate this, the MAM truncation mutant (1-445) was analyzed by size exclusion chromatography and the fractions collected were analyzed by reducing and nonreducing chromatography. As shown in Figure 33b (top), the mutant elutes in Fractions 8-12. As shown for the glycosylation site mutants, wild-type protein that mainly forms large oligomeric species elutes in Fraction 8 with a small portion eluting as smaller oligomeric species. For the MAM truncation mutant, the distribution is shifted to the smaller oligomeric species, with the majority of
**Figure 33. Oligomeric state of the MAM truncation mutant.** 

*a)* Wild-type and MAM truncation (1-445) proteins were transiently expressed in HEK-293 cells and the culture media were analyzed by reducing (lanes 1, 2) and nonreducing (lanes 3,4) SDS-PAGE. The asterisk (*) indicates the expected disulfide-bonded dimer based on mobility of the monomer. The arrows indicate higher MW species that have a slower mobility than the expected dimer.  

*b)* Fractions from size exclusion chromatography of the MAM truncation mutant under reducing (top panel) and nonreducing (bottom panel) conditions. Arrows indicate the nonnative disulfide-bonded species. The asterisk indicates the expected disulfide-bonded dimer. The arrowhead indicates the monomer.
a) Reducing Nonreducing

wt 1-445

200 kDa
130 kDa
80 kDa

b) Fraction

 wt 1-445

200 kDa
130 kDa
80 kDa

*
the immunoreactivity observed in Fractions 11 and 12. The immunoreactivity below the 
MAM truncation band is due to crossreactivity with residual serum in the samples, which 
obeys the monomer band to a certain extent. However, the distribution of species 
observed in the size exclusion fractions was consistent between two independent 
transfections. As shown in Figure 33b (bottom), the larger “oligomeric” species in 
Fractions 8-11 are formed from the presence of multiple intersubunit disulfide bonds 
instead of the one covalent interface of the “normal” disulfide-bonded dimer (marked by 
an asterisk). Thus, the absence of the TRAF domain in the MAM truncation mutant leads 
to formation of nonnative disulfide-bonded species.

3.2.2 Rationale for the MAM domain mutants

The MAM domain has been implicated in both meprin A and meprin B 
oligomerization because of the presence of intersubunit disulfide bonds. Furthermore, 
this domain is critical for the synthesis and secretion of the meprin A protein. It is 
hypothesized that differences in the sequence of the MAM domains between the α and β 
subunits leads to differences in the oligomerization of the subunits. A comparison of the 
MAM domains from mouse, rat, and human meprin α and β subunits reveals that there is 
a much higher occurrence of charged residues in the α MAM domain than in the β MAM 
domain (Figure 34). Due to the salt dependence of meprin A oligomerization, it is 
possible that charged interactions in the MAM domain govern oligomerization of meprin 
A to a certain extent. Furthermore, there are more instances where the charges are 
conserved in α subunits but not β subunits (Figure 34, arrows). A subset of these 19
**Figure 34. Sequence alignment of meprin α and β MAM domains.** The MAM domains of mouse (mmep), rat (rmep), and human (hmep) meprins were compared. Positively charged amino acids are blue, negatively charged amino acids are red. Completely conserved amino acids are in bold, and potential N-linked glycosylation sites are in italic. Cysteine residues are highlighted in green. Arrows indicate charged residues that are conserved in α subunits but not β subunits. The solid lines beneath the sequence indicate charged residues that are conserved in β subunits but not α subunits. Numbering is based on the mouse meprin α sequence.
amino acids were mutated to the corresponding residue in meprin B. The analysis was first focused on amino acids between K352 and K388. Charged amino acids in the first third of the MAM domain were not analyzed at this time, since the two intersubunit disulfide bonds are in this region and these amino acids would more than likely be at or near the covalent interface, not the noncovalent interface. Cys355 was previously found to be a free cysteine in mouse meprin A, which could be used to crosslink the noncovalent dimer interface (44). K388 lies directly before a region that is not charged in the α subunit, but that represents a patch of charged residues in the β subunit. Thus, the mutants K352G, K361Y, R369Q, R376E, D377Y, D378T, R384G, and K388L were made using site-directed mutagenesis and analyzed for their effect on the oligomeric state of meprin A.

3.2.3 Effect of single point mutations in the MAM domain

3.2.3.1 Expression of MAM mutants

HEK-293 cells were transiently transfected with the MAM domain mutants and their secretion into the culture media was assessed 24 hr posttransfection by SDS-PAGE and Western blotting. All of the mutants were expressed except K352G and K388L (Figure 35a). In this transfection, it appears that R384G is expressed at a slightly higher level than wild type. The mutants that are secreted are capable of forming disulfide-bonded dimers (Figure 35b). To determine if the K352G and K388L mutants are retained
Figure 35. Expression of the MAM domain single charged residue mutants. HEK-293 cells were transiently transfected with the MAM mutants and samples of the media were analyzed by SDS-PAGE under a) reducing and b) nonreducing conditions 24 hr posttransfection. c) Reducing SDS-PAGE of media (lanes 1, 3, 5) and lysate (lanes 2, 4, 6) samples from cells not secreting the K352G and K388L mutants. The asterisk (*) indicates the wild-type meprin species in the media and lysates. The arrows indicate the lack of these bands in the MAM mutant lysates. All samples were visualized by Western blotting.
a) wt K352G K361Y R369Q R376E D377Y D378T R384G K388L

b) wt K361Y R369Q R376E D377Y D378T R384G

c) wt K352G K388L

200 kDa 130 kDa 80 kDa

monomer

dimer

200 kDa 130 kDa 80 kDa

wt K352G K361Y R369Q R376E D377Y D378T R384G K388L

200 kDa 130 kDa 80 kDa

* **
in the cell instead of secreted, lysates of the transiently transfected cells were prepared and compared to the unconcentrated cell culture media. The two predominant meprin bands, indicated by asterisks and detected during pulse-chase analysis of meprin A, are detected in wild-type lysate, but the K352G and K388L mutants do not accumulate to any significant levels in the cells or in the media (Figure 35c), indicating that these mutations lead to degradation of the protein intracellularly.

3.2.3.2 Oligomeric state of MAM mutants

The secreted MAM mutants were screened by native PAGE for their ability to oligomerize. As shown in Figure 36, all mutants form smaller oligomeric species than wild type, except K361Y. For this mutant, there was some faint immunoreactivity near the top of the gel with the same mobility as that of the wild-type oligomer. The majority of the immunoreactivity of the remaining mutants is consistent with a dimer, based on the mobility of standards and the meprin B homooligomer. However, size exclusion chromatography was used to confirm the oligomeric state of each mutant. The K361Y mutant did in fact form oligomeric species similar to wild type (Figure 37b). In this particular analysis using wild type, most of the immunoreactivity was seen in Fraction 9, but not Fraction 8. This likely resulted from uneven loading of samples. A change in oligomeric state is unlikely because wild type consistently eluted in Fraction 8 in size exclusion analysis from at least four or more transfections. The other MAM mutants eluted mainly in Fractions 11 and 12 (Figure 37c-g), consistent with the formation of dimers. A small amount of protein was detected in Fraction 10 for R376E (Figure 37d).
Figure 36. Oligomeric state of the MAM mutants by native PAGE. Media from cells expressing the MAM mutants was concentrated four-fold and analyzed by 3-8% native PAGE and Western blotting. The high molecular weight oligomer, dimer, and monomer species are indicated.
145 oligomer

wt K361Y R369Q R376E D377Y D378T R384G

oligomer →
dimer →
monomer →
Figure 37. Oligomeric state of the MAM mutants by size exclusion chromatography. Media samples from transiently transfected cells were collected, concentrated four-fold, and analyzed by size exclusion chromatography on a Superose 12 column. One-ml fractions were collected and meprin was visualized in the fractions by reducing SDS-PAGE and Western blotting.  

- a) wild type,  
- b) K361Y,  
- c) R369Q,  
- d) R376E,  
- e) D377Y,  
- f) D378T, and  
- g) R384G.
Overall, this series of MAM mutants limit oligomer formation to a disulfide-bonded dimer.

3.2.3.3 Characterization of the MAM mutants

The MAM mutants were further characterized for their stability and activity. Incubation of the mutants with increasing amounts of trypsin demonstrated that the MAM mutants K369Q, R376E, D377Y, and D378T were less stable than wild type (Figure 38). The oligomeric mutant, K361Y, and the dimeric mutant R384G were as stable as the wild type. It is interesting that the R384G mutant is significantly less susceptible to trypsin compared to the other dimeric mutants. This implies a different conformation of the R384G mutant, and increased trypsin susceptibility is not solely due to loss of the noncovalent interface. Since all mutants were stable at 10 ng/μl trypsin, their peptidase activity was measured with the substrate BK+. All mutants displayed at least 70% of wild-type BK+ activity (Table 11) indicating that the protease domain was folded into an active conformation despite the lack of higher oligomer formation. The lack of higher oligomer formation also led to a decreased heat stability for all dimeric mutants (Figure 39). Overall, these MAM domain mutations have identified residues involved in meprin A oligomerization that affect stability while maintaining activity.
Figure 38. Tryptic activation and stability of the MAM mutants. The concentrated media collected for each mutant was incubated at 37°C for 45 min with trypsin at a final concentration of 0, 10, 40, and 80 ng/μL. A three-fold excess (relative to the highest concentration of trypsin) of soybean trypsin inhibitor was added and the samples were separated and analyzed by reducing SDS-PAGE with Western blotting.
wt  K361Y  R369Q

R376E  D377Y

D378T  R384G

ng/μl trypsin  0  10  40  80  0  10  40  80  0  10  40  80  0  10  40  80
Table 11

*K* activity of the MAM domain mutants

The peptidase activities of the MAM domain mutants in crude media samples were measured with the fluorogenic substrate *K* after activation with 10 ng/μl trypsin for 45 min at 37°C. Specific activities are expressed in fluorescence units s⁻¹ μg⁻¹. The amount of meprin in each sample was based on the densitometry measurements from Western blots of the crude media, relative to the wt sample. The absolute concentration of meprin in the wt sample was measured by *K* activity compared to the fluorescence from a known amount of pure protein. The values listed are an average of one activated sample assayed in triplicate with the corresponding standard error.

<table>
<thead>
<tr>
<th></th>
<th>Specific activity</th>
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<tbody>
<tr>
<td>wild type</td>
<td>128 ± 3</td>
</tr>
<tr>
<td>K361Y</td>
<td>98 ± 2</td>
</tr>
<tr>
<td>R369Q</td>
<td>89 ± 7</td>
</tr>
<tr>
<td>R376E</td>
<td>120 ± 3</td>
</tr>
<tr>
<td>D377Y</td>
<td>123 ± 0.8</td>
</tr>
<tr>
<td>D378T</td>
<td>91 ± 3</td>
</tr>
<tr>
<td>R384G</td>
<td>94 ± 2</td>
</tr>
</tbody>
</table>
Figure 39. Heat stability of the MAM mutants. Concentrated media samples of each mutant were activated with trypsin at a final concentration of 10 ng/μl as described above, incubated at 55°C for 0, 5, 15, 30, and 45 min, cooled to 23°C, and the residual BK+ activity was measured at each timepoint. (♦) wild type; (■) K361Y; (▲) R369Q; (×) R376E; (★) D377Y; (●) D378T; (+') R384G.
Preincubation at 55°C (min)

Percent of initial activity

Preincubation at $55^\circ$C (min)
3.2.4 Variants at R384 in the MAM domain

The MAM domain mutants described above were prepared based on the corresponding meprin β subunit sequence. In all cases, the charged residue in α was changed to a noncharged residue. However, in two cases, K352 and R384, the charged residue was replaced with glycine. It is known that the presence of glycine in protein sequences has the tendency to change secondary structure, so this mutation may have had an effect based solely on inducing a different secondary structure in α rather than simply eliminating a charged residue. Since the R384G mutant was expressed and the K352G mutant was not, the next series of experiments focuses on variants at R384. Three additional mutants were made: R384A, R384E, and R384K. If the absence of a charged residue is the reason that the higher oligomer is not formed, the R384A mutation would be expected to produce the same effect as R384G without as much potential to change secondary structure. If the R384 residue was involved in some type of ionic, salt bridge interaction, the R384E mutant would be expected to disrupt oligomerization, while the R384K mutant would be expected to have the wild-type phenotype since it would preserve the positive charge of R384. These mutants were created by site-directed mutagenesis and their expression and oligomeric state were analyzed.

3.2.4.1 Expression of the R384 variants

The cell culture media from transient transfections of the R384 variants were analyzed as described above for the MAM mutants. Interestingly, the R384A mutant was not secreted into the medium, while the R384K mutant was secreted to a lesser extent
than wild type (Figure 40). The R384E mutant was secreted at levels similar to wild type and the original mutant, R384G (Figure 40a). The secreted mutants were also capable of forming intersubunit disulfide bonds (Figure 40b). Analysis of the lysate from cells transiently transfected with R384A revealed that, like K352G and K388L, this mutant did not accumulate in the cell (Figure 40c). Thus, contrary to the original prediction, the R384A mutant was more deleterious to the synthesis of the meprin A homooligomer than R384G.

3.2.4.2 Oligomeric state and characterization of the R384 variants

The oligomeric states of the R384 variants were also assessed by size exclusion chromatography and Western blotting of the fractions. As shown in Figure 41, both the R384E and the R384K mutants are dimeric like R384G, eluting in Fractions 11 and 12 (panels b, c). Unlike the original hypothesis, the conservation of charge in the R384K mutant does not preserve the oligomeric state of the wild-type protein, suggesting that more than the charge at this position is important in promoting higher order oligomer formation.
**Figure 40. Expression of variants at position R384 in meprin A.** Media samples of mutants at R384 were analyzed 24 hr posttransfection by *a*) reducing and *b*) nonreducing SDS-PAGE. *c*) Reducing SDS-PAGE of media (lanes 1, 2, 5, 6) and lysates (lanes 3, 4, 7, 8) samples from cells not secreting the R384A mutant. Samples were loaded based on total protein (20 µg in lanes 1, 3, 5, 7; 40 µg in lanes 2, 4, 6, 8). The asterisks (*) indicate meprin species. Arrows indicate the absence of meprin species in the R384A lysate. All samples were visualized by Western blotting.
a) wt R384A R384E R384G R384K

200 kDa
130 kDa
80 kDa

monomer

b) wt R384A R384E R384G R384K

200 kDa
130 kDa
80 kDa

monomer

dimer

c) wt R384A

200 kDa
130 kDa
80 kDa

*
Figure 41. Oligomeric state of the R384 mutants by size exclusion chromatography.

Media samples from transiently transfected cells were collected, concentrated four-fold, and analyzed by size exclusion chromatography on a Superose 12 column. One-ml fractions were collected and meprin was visualized in the fractions by reducing SDS-PAGE and Western blotting. An asterisk (*) indicates the fraction with the highest level of meprin as determined by densitometry.  

a) wt;  b) R384E;  c) R384K.
The tryptic stability of the R384E and R384K mutants differed from each other. While R384E had wild-type tryptic stability, the R384K mutant was completely degraded with $40\text{ ng/\mu l}$ trypsin (Figure 42). However, both variants have full enzymatic activity (Table 12). In this experiment, the R384 mutants have slightly higher activity than wild type, which may be due to errors in quantification of protein by densitometry or differences in the population of active meprin oligomers between transfections (compare the specific activities of the two wild-type transfections). Overall, like the other MAM mutants, the R384 variants that are expressed maintain an intact protease domain.
Figure 42. **Tryptic activation and stability of the R384 mutants.** The concentrated media collected for each mutant was incubated at 37°C for 45 min with trypsin at a final concentration of 0, 10, 40, and 80 ng/μl. A three-fold excess (relative to the highest concentration of trypsin) of soybean trypsin inhibitor was added and the samples were separated and analyzed by reducing SDS-PAGE with Western blotting.
wt R384E R384G

130 kDa

80 kDa

ng/µl trypsin 0 10 40 80 0 10 40 80 0 10 40 80

wt R384K

130 kDa

80 kDa

ng/µl trypsin 0 10 40 80 0 10 40 80
Table 12

**BK+ activity of the R384 mutants**

The peptidase activities of the R384 mutants in crude media samples were measured with the fluorogenic substrate BK+ after activation with 10 ng/μl trypsin for 45 min at 37°C. Specific activities are expressed in fluorescence units s⁻¹ μg⁻¹. The amount of meprin in each sample was based on the densitometry measurements from Western blots of the crude media, relative to the wt sample. The absolute concentration of meprin in the wt sample was measured by BK+ activity compared to the fluorescence from a known amount of pure protein. The values listed are an average of one activated sample assayed in triplicate with the corresponding standard error.

<table>
<thead>
<tr>
<th></th>
<th>Specific activity</th>
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<tbody>
<tr>
<td>wild type&lt;sup&gt;a&lt;/sup&gt;</td>
<td>186 ± 3</td>
</tr>
<tr>
<td>R384E</td>
<td>239 ± 5</td>
</tr>
<tr>
<td>R384G</td>
<td>233 ± 3</td>
</tr>
<tr>
<td>wild type&lt;sup&gt;a&lt;/sup&gt;</td>
<td>347 ± 12</td>
</tr>
<tr>
<td>R384K</td>
<td>450 ± 6</td>
</tr>
</tbody>
</table>

<sup>a</sup> Two wild type samples were measured since the mutant media samples were collected from two different transfections.
Chapter 4
DISCUSSION

The work herein demonstrates the importance of N-linked glycosylation and charged residues of the MAM domain in promoting oligomerization of the meprin A homooligomer. Glycosylation of the protease domain was implicated in both oligomer formation and stability. The combined presence of N152 and N270 is required to form the disulfide-linked dimer as well as a functional active site and the noncovalent interface, while the glycans at N234 and N270 have an overall stabilizing effect in the native conformation. Unlike the glycosylation site mutants, mutations at single charged residues of the MAM domain only affect the noncovalent interface while maintaining activity. These residues define a region within the domain that is critical to oligomer formation.

4.1 Characterization of glycans and implications for meprin structure and function

The compositions of N-linked glycans from recombinantly expressed mouse meprin A, rat meprin A, and rat meprin B are characteristic of complex glycosylation, with significant amounts of GlcNAc, mannose, and galactose (see example of complex glycosylation in Figure 4). The percentages of GlcNAc, mannose, and galactose in the recombinantly expressed samples were similar to the percentage of these sugars in heterooligomeric meprin A isolated from kidneys, even though this reflects glycosylation
of both the \( \alpha \) and \( \beta \) subunits (J. Bond, personal communication). High mannose or hybrid glycans account for a small percentage (4\%) of the total carbohydrate on mouse meprin A, as determined by MALDI analysis of the EndoH-treated protein. Given that a typical high mannose structure (\( \text{Man}_9\text{GlcNAc}_2 \)) is 1884 Da, this indicates that there are likely two high mannose or hybrid glycans on mouse meprin A (based on the difference in mass between wild-type and EndoH-treated proteins, Figure 10). These sites can be mapped by ESI/MS as the other glycosylation sites were determined. Like TFMS, EndoH leaves the innermost GlcNAc as a mass marker.

The glycans isolated from the purified recombinant proteins reflect the glycosylation machinery and cellular status of the HEK-293 cells in which they were expressed. There may be differences in the composition and branching of the antennae depending on the cell type used, in addition to meprins expressed in different organs (i.e. the intestine vs the kidney). Previous experiments with purified kidney meprins, both heterooligomeric meprin A and homooligomeric meprin B, indicate differences in the types of complex glycans on these isoforms, but there is no detectable sialic acid in either (100). Differences are also observed between male and female mice, which differ in the extent of glycosylation as well as in composition (130). These differences are not observed in young mice, and are hormonally regulated. Recently, kidney meprins isolated from C57BL/6 mice were shown to have a non-sulfated form of the human natural killer-1 (HNK-1) carbohydrate, an N-acetyllactosamine modified by sulfoglucuronic acid (104). The sulfated HNK-1 epitope is mainly expressed in neuronal cells on molecules that mediate cell adhesion (105). The functional significance of the nonsulfated epitope, as well as its effect on meprin function, has yet to be determined.
Given that the HNK-1 carbohydrate is normally expressed on cell adhesion molecules, this carbohydrate may mediate the interaction of meprins with other cell surface kidney molecules or with other cells that arrive in the proximal tubules, such as immune cells after renal injury or bacteria during kidney infections.

The absence of GalNAc in the total carbohydrate analysis of mouse meprin A and the inability of PNGaseF-treated meprin A to be labeled by biotin hydrazide indicates that this isoform lacks O-linked glycosylation. Human meprin A has also been shown to lack O-linked glycosylation when expressed in MDCK cells (102). The detection of GalNAc in the samples of rat meprin A and rat meprin B may be due to the presence of O-linked glycosylation, or it may reflect the addition of GalNAc to the antennae of complex glycans chains. Human meprin β subunits bear O-linked glycans in a 13 a.a. sequence near the end of the TRAF domain; this amino acid region is also where meprin β can be cleaved under certain conditions in order to release it from the cell membrane (29,102). If the detection of GalNAc is reproducible for the rat meprins, the labeling experiment using biotin hydrazide can be used to distinguish between the presence of GalNAc in the antennae of complex or hybrid structures and the presence of O-linked glycosylation of rat meprin A and B.

A small amount of fucose was detected for mouse meprin A and rat meprin B (7% and 5%, respectively, of the total carbohydrate). Fucose can be added to the antennae of complex glycans, or it can be attached to the innermost GlcNAc residue in an α-1,6 linkage or α-1,3 linkage (core fucosylation). Determining the location of the fucose within the meprin glycans will need to be addressed, since fucosylation is involved in
many important processes such as host-microbe interactions, cell adhesion events
mediated via the selectin family of cell adhesion receptors, fertilization and development,
and pathological processes such as cancer and atherosclerosis (58,131). Fucosylation of
surface molecules in the intestinal epithelium, a location where meprin expression is
high, is regulated by the interaction of bacteria with the host gut (132). Fucose is also a
binding determinant for the calcium dependent (C-type) serum lectins, such as mannose-
binding protein (MBP) (133). Meprin A and B activities have been shown to be
decreased by the binding of MBP, and it is possible that the fucose on meprins is
involved in a specific recognition event, which is different from the binding of MBP to
the high mannose glycans on meprin β (134). Although MBP is mainly involved in the
innate immune response against pathogens and does not usually recognize normal human
cells, it has recently been shown to recognize fucose-containing epitopes on SW1116
human colorectal carcinoma cells (135). Selectins, lectin-type cell adhesion receptors,
bind to fucosylated leukocyte surface molecules, facilitating leukocyte extravasation, and
meprins were shown to be expressed on leukocytes (17,58). Direct fucosylation of
Ser/Thr residues can also occur in EGF domains of multimodular proteins, and affects
signaling pathways (53,58). Thus, the presence of fucose in the glycans of meprins has
the potential to regulate meprin function in several pathways.

In addition to differences and heterogeneity in the composition of glycans, there
may also be variability in the utilization of potential glycosylation sites. Occupancy of a
glycosylation site depends on several factors, including availability of the dolichol-linked
core oligosaccharide, composition and activity of the oligosaccharide transferase (OST)
complex, the accessibility and flanking sequence of the consensus site, and the extent of
protein folding in that region (136). None of the consensus sequences in meprins have a proline in the X position of the sequence N/X/(S/T), which would prevent glycosylation (137,138). It is possible that the heterogeneity observed in the TRAF domain of mouse meprin A is due to the effects of protein structure since much of the protein has been synthesized and if this region is partially folded, these sites may have a lower affinity or restricted binding to OST as they emerge from the translocon. However, in rat meprin A there is some heterogeneity observed at N223 (N234 in mouse), a protease domain glycosylation site, as well as in the TRAF domain glycosylation N441 (N452 in mouse). It is interesting that in this particular set of mapping experiments there was no heterogeneity observed for N270 in mouse meprin A or N259 in rat meprin A, since this site was implicated in meprin A oligomerization. If surrounding protein structure dictates glycosylation site usage, the structure of this region may be such that it is effectively exposed to the glycosylation machinery. Thus, this site may always be glycosylated in order to promote efficient disulfide bond formation, protein folding, and oligomerization. However, although lack of heterogeneity might correlate with importance in protein structure, it is possible that heterogeneity exists at several or all of the glycosylation sites, and it simply was not detected by the mass spectrometer due to extremely low abundance or alteration of the peptides with another modification. This may be the case for mouse meprin A, in which no heterogeneity was detected for any of the glycosylation sites. The composition of the glycan chains attached to meprin A did not affect meprin activity directly (14). However, heterogeneity in both site occupancy and the composition of the glycans can lead to populations of meprins with different affinities for molecules that regulate meprin’s activity, as in the case of MBP. Carbohydrate heterogeneity is thus a
way to fine tune protein function without further processing of the protein backbone and will also be sensitive to cellular conditions.

The rationale for examining the effect of glycosylation on oligomeric structure was based on the conservation of consensus sequences in one meprin subunit and not the other. N152 in mouse meprin A, the first glycosylation site of the protease domain, was shown to be involved in meprin A oligomerization. The glycosylation status of this site was undetermined for rat meprin A, however, due to the importance of this glycosylation site in mouse, it is likely that it is also glycosylated in rat and human. Two meprin β subunit glycosylation sites were also noted, N422 and N547 in rat, since they were completely conserved in β subunits and absent in α subunits. N422 in the MAM domain was clearly not glycosylated in rat meprin B; thus, it is unlikely to contribute to differences in oligomeric structure. The consensus sequence for N422 is NLS. It has been reported that the presence of aspartic acid, glutamic acid, tryptophan, and leucine in the X position also decreases glycosylation efficiency, and may be the reason this site is not occupied (139). The other site conserved in β subunits, N547 in the TRAF domain, was glycosylated, and it is possible that this glycan influences the folding and oligomerization of meprin B. The corresponding region of meprin α subunits contains two closely spaced cysteine residues, CXC; the presence of glycosylation versus a potential intrasubunit disulfide bond may cause the subunits to adopt two very different structures in this region.

Overall, the method of chemical deglycosylation with TFMS and detection with ESI/MS was successful in determining the status of 8/10 potential glycosylation sites in
mouse meprin A, 4/6 in rat meprin A, and 3/8 for rat meprin B. The first MAM domain glycosylation site (equivalent to N330 in mouse) was not detected in any of the meprin isoforms examined, although this site was shown be glycosylated in heterooligomeric kidney meprin A (101). This glycosylation site is near an intersubunit disulfide bond, and might not have been detected if this region was incompletely digested by trypsin. Likewise, the N156 glycopeptide in fetuin, which was also not detected in the test of the chemical deglycosylation and electrospray method, is also part of a disulfide linkage. In retrospect, the proteins should have been reduced and cysteines alkylated before tryptic digestion, not after. Trypsin may have been unable to digest the MAM domain properly, considering the number of cysteines and disulfide bonds in this region. Also, there may have been some protein degradation during the highly exothermic quenching step that involved the addition of aqueous pyridine, thus leading to absence of particular peptides in the spectra or low abundance despite starting with several hundred μg of protein.

Methylation of free carboxylates (+14 mass units) or succinimide ring formation on Asp residues (-18 mass units) has also been reported for TFMS-treated proteins, which would not be accounted for when searching for the expected glycopeptide masses (140). In future experiments using this method, the temperature of the quenching step should be more carefully controlled, and the protein should be reduced and alkylated prior to tryptic digestion to obtain data on the glycans missed in the first set of mapping experiments.
4.2 Glycosylation directs folding of the meprin A homooligomer

The original hypothesis that particular glycosylation sites in the mouse meprin α subunit could affect the formation of the meprin A homooligomer was supported by the study of multiple glycosylation site mutants. An interplay between two particular glycosylation sites of the protease domain, N152 and N270, was demonstrated since the absence of these glycans caused secretion of an unstable, inactive monomer. It is clear that the effect of N152 and N270 on oligomerization is due to this specific combination of glycans since single mutations at these glycosylation sites did not prevent oligomerization. This effect is also not simply due to the absence of glycosylation at two sites, because the N152Q/N234Q and the N234Q/N270Q mutants were oligomeric with near wild-type activity. Thus, it is not simply a global destabilization of the protease domain that prevents oligomerization, but a more specific effect on structure or protein-protein interactions. Surprisingly, the effects on glycosylation on oligomeric state involved only protease domain glycosylation sites. The MAM double mutant, N330Q/N426Q, as well as the corresponding single mutants, were oligomeric, as were the single TRAF domain mutants. Multiple TRAF glycosylation site mutants were not investigated due to time constraints. It is possible that the MAM and TRAF domain glycosylation sites could affect oligomerization, since these domains have been implicated in oligomerization in previous studies. Multiple glycosylation site mutants involving combinations of sites from different domains (protease-MAM, protease-TRAF) may also have an effect.
The decreased level of secretion for the N152Q/N270Q and the N152Q/N234Q/N270Q mutants may be due to increased retention of these mutants in the ER by CNX or CRT since they are misfolded, intracellular degradation of a population of the protein that is terminally misfolded, or slower movement through the Golgi compartment. The presence of these mutants in the lysates of the transfected cells was not determined. There is an increased heterogeneity in these mutants, observed as a diffuse band in SDS-PAGE despite the removal of two and three glycans. This probably reflects an altered glycosylation pattern resulting from altered interactions with the Golgi glycosyltransferases. C-terminal processing and oligomerization of meprin A occurs before complex glycosylation, i.e. it is an ER localized event (121,141). Lack of the wild-type oligomeric structure may make the mutants accessible to glycosyltransferases that meprin would not normally interact with or extend the interactions, leading to hyperglycosylation. In this way, oligomerization may contribute to meprin’s function in determining the type and extent of glycosylation that is added by the Golgi enzymes; as discussed in the previous section, glycan composition may be a determinant for the function of meprins in several disease processes.

The N152 and N270 glycans may affect the folding and oligomerization of meprin A in several ways. First, they may recruit chaperones to critical regions of the protein in order to direct proper folding, including proper disulfide bond formation (80-82). The lectin chaperones, CNX and CRT, have been shown to interact with meprin A (74). In turn, CNX and CRT recruit the oxidoreductase, ERp57, which catalyzes the formation, reduction, and rearrangement of disulfide bonds in glycoproteins through the formation of mixed disulfides (142-144). Disulfide bond formation is an early event in
the synthesis of both meprin A and meprin B; thus it has the potential to influence later folding steps which lead to oligomerization (13,74,145,146). One way to explain the effect of the N152 and N270 glycans on folding and disulfide bond formation is that these glycans recruit CNX and CRT to this region of the protein, which then allows ERP57 to bind to meprin. If the interaction with CNX and CRT is absent in this region, ERP57 cannot bind and catalyze native disulfide bonds necessary for proper folding and stability. However, several pieces of evidence argue against this exact mechanism. First, meprin A can fold into an active conformation in the absence of CNX and CRT interactions, although under normal conditions the folding of meprin A is accelerated by the two chaperones (14,74). In addition, the rate of disulfide bond formation is not affected when CNX and CRT interactions are prevented. This would seem to rule out ERP57 as the primary oxidoreductase involved in meprin A disulfide bond formation under normal conditions, since ERP57 does not bind to its substrates without the substrates first binding to CNX and CRT (144). Although, it may also suggest usage of an alternate oxidoreductase, such as protein disulfide isomerase (PDI), which has both oxidoreductase and foldase activities (147-149). CNX and CRT have been shown to be involved in a large network of ER resident proteins that direct protein folding, and a CNX/CRT independent network that includes the chaperone BiP has been observed (77,150). Tyrosinase-related protein-1 (TRP-1) has been shown to utilize an alternate chaperone pathway that allows the protein to be transported correctly, but leads to a folded structure that is less stable (75). Similar effects were seen with thyroglobulin (151). Although meprin A synthesized in the absence of CNX and CRT interactions is active, the stability and oligomeric state of the protein were not determined.
Proper intersubunit disulfide bonding is required to form the noncovalent interface of meprin A. Mutants that prevent the two intersubunit disulfides in meprin A are monomeric, but retain full activity (50). Formation of the disulfide-bonded interface may position two subunits in such a way that they are then able to interact with another disulfide-bonded dimer to form the noncovalent interface. In the absence of the intersubunit disulfide bonds, the tertiary structure of the monomer can form, but it does not allow formation of the noncovalent interactions. However, the double and triple glycosylation mutants seem to have an improperly folded protease domain, which indicates that the tertiary structure did not form properly.

It would be of interest to determine whether the glycosylation mutants form other nonnative disulfide bonds, in addition to a lack of intersubunit disulfide bonds. If the protease domain glycans influence the folding pathway of this domain, they may also affect the proper intrasubunit disulfide bonds by altering the conformation so that the cysteines are improperly aligned or the conformation is such that the oxidoreductase involved cannot catalyze the correct disulfide formation. The fact that this in turn affects intersubunit disulfide bonds in another domain, namely the MAM domain, would be a result of the sequential, hierarchical nature of disulfide bond formation in which nonnative pairings form first, then are reshuffled by oxidoreductases as the protein folds (152,153). If one cysteine is missing, this will affect the formation of other disulfide bonds, as in the case of meprin A and meprin B cysteine mutants (39,49,50). In the multiple glycosylation site mutants, the cysteines are present, but nonnative structures in the protease domain may lead to aberrant oxidoreductase-meprin interactions in either the protease or MAM domains. This correlation between glycosylation and disulfide bond
formation has also been demonstrated for influenza hemagglutinin and tripeptidylpeptidase. The lack of N81 in influenza hemagglutinin and N286 in tripeptidylpeptidase leads to incorrect folding and nonnative disulfide bond pairing, and, the case of hemagglutinin, lack of oligomerization (80,154). Glycans have also been shown to regulate the rate of intersubunit disulfide bond formation in intestinal mucin 2; if the glycan is absent, the disulfides form too quickly and the protein misfolds (129).

A model of the meprin A protease domain, based on the crystal structure of astacin, shows that N152 and N270 are located in the “top” portion of the domain where there is more well defined secondary structure (Figure 43). The consensus sequence for N270 contains C271, which is part of an intradomain disulfide bond with C119. This disulfide bond brings the C-terminus of the domain in contact with the N-terminal portions. N152 is located 7 a.a. away from C159, which is involved in the other intradomain disulfide bond between it and C140. It is possible that the glycans at these positions influence the formation of the disulfide bond, either by stabilizing this region of the protein so that a conformation favoring disulfide bond formation is achieved or keeping this region from aggregating with other portions of the protein. For example, disulfide bond formation was favored in a peptide from the nicotinic acetylcholine receptor when the peptide’s potential N-linked glycosylation site was occupied (155,156). In the absence of one glycan, as in the single mutants, the folding and dynamics of the protease domain may be such that the disulfides can form correctly. However, the absence of both glycans distorts the domain enough so that the disulfides are not formed,
Figure 43. The location of glycosylation sites in the meprin A protease domain. The meprin A protease domain was modeled from the crystal structure of astacin (157). The β sheets are illustrated by blue arrows, and the α helices are drawn as red tubes. The Asn residues of the glycosylation sites are in green ball and stick. The two intradomain disulfide bonds are shown in yellow ball and stick. The zinc atom in the active site cleft is shown in purple.
or perhaps formed improperly. As discussed above, this may lead to improper disulfide bond formation in other domains of the protein. It is possible, however, that the protease domain intrasubunit disulfide bonds do form correctly and the protease domain is misfolded in some other manner that prevents catalysis, intersubunit disulfide bond formation, and oligomerization. If meprins can fold in the absence of a chaperone such as CNX or CRT, the presence of all critical glycans and an oxidoreductase interaction for proper disulfide bond formation may be the minimum requirements for directing the correct folding and oligomerization of meprin.

Regardless of the mechanism by which glycans influence meprin A folding, the data presented herein point to the protease domain as a critical folding unit of the meprin A homooligomer, perhaps serving as a folding nucleus for the rest of the protein (71). Previous studies have also shown that an improperly folded active site due to absence of zinc binding leads to secretion of a monomeric, inactive protease (51). In rat meprin B, the protease domain has been shown to make contacts with the protease domain in the other half of the dimer, in addition to the TRAF and EGF domains (39). Interdomain contacts have not been mapped for meprin A, but deletion and truncation mutants in the MAM and TRAF domains indicate an interaction between these domains and the protease domain either by direct contact or long range effects (26). Meprins are the only oligomeric members of the astacin family. The crayfish astacin is monomeric, containing only the prosequence and protease domain, and lacking any potential glycosylation sites (4). Bone morphogenic protein-1 (BMP-1) is glycosylated at its single protease domain glycosylation site, although this is not homologous to any of meprin’s glycosylation sites. Interestingly, the site homologous to N152 in mouse meprin α (NIS) is changed to AIS in
BMP-1, thus preventing glycosylation. BMP-1 is glycosylated in its noncatalytic domains, but it is not oligomeric. Although the astacin and meprin protease domains are 32-40% identical, meprins contain insertions and deletions in addition to glycosylation in this domain (157). The additional sequence may create an interface for contacts with other meprin domains, and it has been demonstrated herein that glycosylation of protease domain directs oligomerization.

4.3 Glycosylation and the native state

In addition to directing the folding and oligomerization of meprin A, glycans also stabilize the native folded structure of the protease. The removal of N234 and N270 in the protease domain and N452 in the TRAF domain by PNGaseF under non-denaturing conditions made the oligomer more susceptible to urea and heat induced denaturation. It can be speculated that the protease domain sites are mainly responsible for this stability. Individually, each of the single protease domain glycosylation site mutants were shown to be less stable than the wild-type protein, while the mutant N452Q had wild-type stability (14). The double glycosylation site mutant N234Q/N270Q was the least stable of the double glycosylation site mutants tested in the current studies. If the TRAF domain glycosylation site contributed little to oligomer stability, a triple mutant, N234Q/N270Q/N452Q, would be expected to have a similar rate of heat inactivation compared to N234Q/N270Q. Given the central nature of the protease domain in oligomer formation, the contribution of the N452 site might be small compared to the other two, and destabilization of the protease domain leads to destabilization of the
overall protein structure. As discussed above, the N270 glycan is located in the “top” portion of the protease domain, adjacent to an intrasubunit disulfide bond. N234 is located in the “bottom” portion of the active site cleft, where there is less well defined secondary structure (Figure 43). The glycan at N234 may stabilize this loop region by decreasing the dynamics of the peptide backbone.

The difference in oligomer stability upon partial deglycosylation appeared to be more evident when active meprin A was partially deglycosylated, compared to latent meprin A. If oligomerization of meprin A serves to concentrate proteolytic activity in regions distal from the site of secretion, it is necessary to keep the oligomer stable until it reaches its target site (45). In this sense, it might be more advantageous to stabilize the latent oligomer, as opposed to the active form, so that it can reach its target and then be activated. It is possible that the glycans also stabilize the latent state to a certain degree, but the irreversibility of the unfolding process made the assays very condition dependent and this effect may not have been evident under the conditions tested. It is also possible that other glycans serve to stabilize the oligomer, but are inaccessible to PNGaseF and thus their effects were not measured. In either case, stabilization of the oligomer in the harsh, digestive environment of the intestinal and kidney lumen, for example, may be critical in preserving meprin activity. Depending on the orientation of the glycans with respect to the protein surface, they may also serve to shield vulnerable regions of the oligomer from proteases in the extracellular milieu.

Subtle differences were observed in the CD and fluorescence spectra of wild-type and partially deglycosylated meprin A, indicating some minor changes in secondary and tertiary structure, as well as the local environment of tryptophan residues. N452 is near a
tryptophan residue; it is possible that part of the changes in the fluorescence spectra arise from exposing a tryptophan that is normally shielded by the glycan. The activity of partially deglycosylated meprin A was also near wild-type levels, which also indicates that critical regions of tertiary structure are still intact. Despite the differences in structure, the partially deglycosylated oligomers were still predominantly large molecular mass oligomers, indicating that the changes are not sufficient enough to change the association between the subunits and alter the quaternary structure. A small difference was seen in partially deglycosylated, latent meprin A. In this case, the small shoulder of smaller oligomeric species disappeared, and the fraction containing the largest oligomers increased in intensity. This is probably due to aggregation of the protein; aggregation was also seen for partially deglycosylated meprin by electron microscopy (F. Ishmael, personal communication). Thus, another function of meprin glycosylation may be to prevent aggregation.

The urea induced unfolding of meprin A was shown to be irreversible under the conditions tested, leading to complete denaturation of the protein even though the disulfide bonds were maintained. The fact that the unfolding reaction is not readily reversible could argue for the necessity of a chaperone in the folding process, perhaps one that can resolve misfolded structures instead of simply sequestering them like CNX or CRT would do (71). It is also possible that certain modifications of the protein occurred while denatured, such as oxidation and modification of amino groups by cyanate, a byproduct of urea (128). Furthermore, the unfolding of the meprin A homooligomer did not appear to have any stable, detectable intermediates (Figures 17 and 18). A disulfide-bonded dimer might have been expected as an intermediate if the
noncovalent interface was first disrupted, followed by unfolding of the secondary and tertiary structure of each subunit. Absence of a stable dimeric intermediate suggests that unfolding of the tertiary structure and disassociation of the noncovalent interface may occur concomitantly. This is also observed for the family of oligomeric legume lectins, as well as other oligomeric proteins (158). Formation of the covalent interface through intersubunit disulfide bonds may be an initial folding event that tethers the subunits together so that the rest of the tertiary structure and the noncovalent interface can form. When the intersubunit disulfide bonds are selectively reduced, only the noncovalent interface remains (44). Thus, once the noncovalent interface is formed, it is stable and involves a highly intricate folding pattern. As previously suggested, it is possible that the formation of the meprin oligomers occurs via domain swapping, in which a domain of one subunit is switched with the identical domain of another subunit during the process of oligomerization (106).

Crosslinking of the noncovalent dimer of meprin A via carbohydrates implicates meprin A carbohydrates in a structural role as well. Glycans may be in proximity to this interface without being absolutely required for subunit-subunit interactions or structural integrity. However, the glycans may also function in a manner similar to the glycan in the Fc region of the IgG molecule (Figure 6) by making critical contacts or acting as a spacer between the meprin subunits. Identifying the glycosylation sites involved in these crosslinks will be challenging, but would further characterize the location and role of the glycans of meprin A. An affinity label like biotin would be needed to isolate the crosslinks, and the crosslinks would have to be cleaved and deglycosylated in order to identify the meprin peptides to which they were attached. PNGaseF does recognize and
remove oxidized carbohydrates (Figure 8), so the crosslinks could be deglycosylated. No such crosslinker (biotin labeled, carbohydrate and cysteine reactive) is currently commercially available, so this would also have to be synthesized. If the N234, N270, and N452 glycosylation sites are PNGaseF sensitive in the native folded structure, then they would not be expected to be involved in the crosslinking of the noncovalent interface. N614 is not glycosylated, so it would not be involved in a carbohydrate crosslink. There is an indication that N41 in the prosequence is also removed by PNGaseF under nondenaturing conditions, so it may not be part of the crosslink either (deglycosylation of the N41 peptide was seen by ESI/MS in a Endoproteinase Glu-C digest of partially deglycosylated meprin A, data not shown). This would leave five other glycosylation sites on the protein (N152, N330, N426, N546, and N553). One or more of these glycans can be involved in the crosslinking of the noncovalent interface. The influence of N152 on oligomer formation has been demonstrated herein; it is possible that in addition to directing folding and formation of the noncovalent interface, the glycan is located in a region that forms the dimer interface. The MAM (N330, N426) and TRAF (N546, N553) glycosylation sites are attractive candidates since these domains have been implicated in meprin oligomerization. The double MAM mutant, N330Q/N426Q, was oligomeric, and perhaps the ability of this mutant to be crosslinked can be tested, as long as it forms a stable noncovalent dimer like wild type upon reduction with TCEP.

Identifying the glycans at or near the noncovalent interface would complement a study to map the interdomain contacts of meprin A, in the absence of a crystal structure. It would be interesting to know whether the meprin A homooligomer folds back upon itself so that
the TRAF domain makes contact with the protease domain, as in meprin B, or has a completely different topology (39).

### 4.4 Contribution of MAM domain single charged residues to oligomerization

The contribution of the MAM domain to the formation of the meprin A homooligomer was further elucidated with a series of mutants that eliminated charged residues and introduced the corresponding β subunit sequences into the putative noncovalent interface. Of the eight mutants created, five of them are limited to a disulfide-bonded dimer, two are not secreted, and one is oligomeric. The two mutants that are not secreted into the medium to any appreciable amount, K352G and K388L, do not accumulate in the cell either. These mutations must create terminally misfolded proteins, which are then degraded intracellularly. The Lys to Gly mutation is drastic, and may be the reason that this mutation caused misfolding. The remaining mutants were all secreted, but were not oligomeric with one exception, K361Y. The non-oligomeric mutants maintained wild-type activity, but were less stable. Although the R384G mutant had decreased heat stability, it was not as susceptible to tryptic degradation, indicating that it creates a misfold different from the other mutants. Despite the lack of higher order oligomer formation, the MAM mutants retain wild-type levels of peptidase activity, suggesting that the tertiary structure of the protease domain is intact. Initial characterization of the azocaseinase activity of R384G indicates that this mutant also retains the ability to hydrolyze protein substrates as well. Previous mutants of meprin A that were not oligomeric did not maintain azocasein activity (26,50).
It is remarkable that a single amino acid change at any of several positions in the MAM domain will prevent the noncovalent interface from forming despite the presence of the correct flanking sequence. Each mutation may disrupt or alter a critical region of secondary structure that either directly forms the interface or influences the local structure enough to prevent contacts in other regions of the protein. Other single mutations in oligomeric proteins also disrupt or alter the distribution of oligomers, but in these instances the lack of oligomerization also results in a loss of activity (159-162). The members of the legume lectin family have very similar primary, secondary, and tertiary structures, yet they differ in their quaternary structure as a result of subtle changes in sequence and posttranslational modifications (158). This is similar to the meprin isoforms, which also show a fairly high homology yet form very different oligomeric structures. Thus, these subtle differences in sequence have evolved to optimize the interactions which drive oligomerization.

The original hypothesis for making this series of MAM mutants was based on the importance of charge at key positions in the domain, which would likely be involved in ionic interactions in the interface. It was proposed that removing those charges would alter the association of the subunits, at least changing the distribution of oligomers seen if not entirely disrupting the interface. However, as the R384 variants demonstrate, introduction of a conservative mutation (R384K), an oppositely charged mutation (R384E), and a mutation that eliminates the bulkier side chain (R384A) all lead to disruption of the noncovalent associations. Thus, it is not simply the presence of charge at this residue that is important for oligomerization and may reflect a strict sequence requirement. The region defined by the single charge mutants may contain other residues
critical for oligomerization. Of the single charge mutants, variants can also be made at these positions to determine whether it is the charge or the identity of the amino acid that is critical. Other noncharged residues that are conserved in α but not β subunits may also contribute to formation of the noncovalent interface since there appears to be a structural element defined at least between K352 and K388. There are eight instances of other distinct differences in this region: Q353F, F359Y, Y360L, M362N, P366G, W374Y, V375T, A387T (Figure 34). It is also possible that there are other regions of the MAM domain that direct the formation of the noncovalent interface. Even though residues near the intersubunit disulfides were not tested based on the reasoning that they would be at or near the covalent interface, they may still have a more distant effect on the noncovalent interface by promoting correct MAM domain structure. If the covalent interface forms first, proper alignment of the subunit in this interface may allow for proper alignment of the noncovalent interface as well.

The exact effect these single charge mutants have on the secondary and tertiary structure is only speculative in the absence of a meprin crystal structure. No other MAM domain containing proteins have been crystallized, thus making modeling of this domain difficult as well. The MAM domain from PTPμ can be expressed independently of the rest of the protein’s ectodomain and can associate to form a homodimer (117). However, given the complex set of interactions that occur between meprin’s protease, MAM, and TRAF domains, it is unlikely that the MAM or TRAF domain expressed independently of the other meprin domains would adopt a conformation and oligomerize in a manner similar to wild-type protein.
Truncation mutants have shown that both the MAM and TRAF domains are needed for a protein that is oligomeric, stable, and active (26,27). When the meprin α TRAF domain cysteines are removed by truncation, all of the mutants have nonnative disulfide bonds which lead to larger disulfide-bonded species, as seen in the size exclusion chromatography of the MAM truncation mutant under nonreducing conditions. The TRAF domain cysteines are thus likely to be involved in the cysteine shuffling process discussed above. The incorrect formation of intersubunit disulfide bonds in the MAM and TRAF truncations may lead to nonnative pairings elsewhere, such as the protease domain, which in turn abolishes activity. Homology modeling of the meprin α TRAF domain based on the crystal structure of intracellular TRAFs shows that there are 11 residues in the meprin α TRAF domains that correspond to points of intersubunit contact in the TRAF structure. Distinct differences in charge occur between α and β subunits at these points, and they may have effects similar to the MAM domain mutants. However, two of the other proposed points of contact involve differences in noncharged residues and may also contribute to the oligomeric differences between meprin A and meprin B.

The oligomeric state of the MAM single charge mutants is reminiscent of the meprin B homooligomer, a disulfide-bonded dimer. At first glance, it is tempting to speculate that the reason the mutants are more “β-like” is due solely to the introduction of amino acids from the β sequence. Overall, each of the β amino acids introduced into the α subunit appear to disrupt a critical region of structure, and it is easy to create a loss of function. This does not necessarily mean that a single amino acid introduced into the
meprin β subunit will induce that critical structure and allow oligomerization of meprin B to higher order oligomers. It is likely that the combined effect of several “oligomerization factors” is what distinguishes homooligomeric meprin A from meprin B. Introducing a protease domain glycosylation site, equivalent to N152, did not induce formation of higher order oligomers even though the subunit already had the other glycosylation site found to be critical for oligomerization (the site homologous to N270). A 9 a.a. region in the TRAF domain, found to be important for formation of the meprin A homooligomer, would not induce oligomerization of meprin B when introduced in a swap mutant (27). The presence of an extra intersubunit disulfide bond in meprin B, in addition to several other conserved glycosylation sites exclusive to the β subunit, may alter the fold of the protein enough to prevent oligomerization even in the presence of these other oligomerization factors. As a result, each of the meprin subunits may be optimized to form their unique oligomeric state.

4.5 Overview and Future Directions

Overall, as several studies have shown, the folding pathway of meprin A is directed by features in the protease, MAM, and TRAF domains. Critical regions of secondary and tertiary structure, glycosylation, zinc binding, and disulfide bonds are all factors in oligomerization. In addition to the features that have evolved to optimize oligomerization, the sequences of the meprin α and β subunits have probably also been optimized for unique protein-protein interactions at the cell surface and in the extracellular space. The meprin β subunit has recently been shown to bind and alter the
activity of an epithelial sodium channel, ENaC (Garcia-Caballero et al., submitted).

Interaction of the meprin β cytoplasmic tail with an intracellular portion of ENaC positions the two proteins so that cleavage of ENaC occurs in the extracellular domain. Mapping of the binding surface between the extracellular domains of the two proteins will likely elucidate specific features of meprin β that are conducive to these interactions. The particular folding pattern and oligomeric state of each meprin isoform may also create unique binding surfaces for their respective binding partners.

Many of these structural studies would be facilitated by a crystal structure of one or more of the meprin isoforms. Crystal structures of glycoproteins are often obtained after partial or full deglycosylation of the protein to reduce heterogeneity. Meprin B is a better target for crystallization than meprin A since it exists exclusively as a homodimer; the variations in the size of the meprin A homooligomer are not very amenable to crystallization. In both instances, the heterogeneity imparted by the glycosylation also hampers crystallization. Partial deglycosylation of meprin A with PNGaseF would also not be amenable to crystallization since this oligomer is less stable, and may result in increased protein dynamics in the protease domain. Mutagenesis to eliminate certain nonessential glycosylation sites is a possibility, and the studies herein have elucidated which glycosylation sites are dispensable to meprin A folding and oligomerization. An alternative way to produce a homogenous sample for crystallization is to produce the protein in glucosidase-deficient cells, which would lead to retention of high mannose glycans at all positions, eliminating the heterogeneity inherent of complex glycans (163).

Defining the composition and structure of individual glycans of meprins A and B will be of importance as the interactions of these molecules with other cell surface and
secreted proteins are elucidated. The fine structure of the glycans will most likely
determine the avidity and binding specificity of these molecules. Populations
constituting different glycoforms of meprins A and B most likely exist, and these
glycoforms probably fine tune the interactions and activities of the proteases. Due to the
implication of meprins in several disease processes that are affected by fucosylation, it
would be necessary to define the location and function of the fucose detected in a total
carbohydrate analysis. A more complete map of the glycosylation site occupancy in
meprin B is also needed, since this form of meprin has been shown to interact with ENaC
and MBP (134). MBP is the first endogenous inhibitor of meprins to be identified, and
may be important in the regulation of meprins if meprins are found to play a role in
innate immunity.

The biochemical and structural information collected thus far on meprins has been
useful in predicting new functions of meprins in both healthy and diseased states. It is
likely that future studies of the structure of meprins will be focused on determining the
details that fine tune meprin interactions in cellular processes.
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