Protease Domain Glycans Affect Oligomerization, Disulfide Bond Formation, and Stability of the Meprin A Metalloprotease Homo-oligomer*

Received for publication, March 23, 2006, and in revised form, September 8, 2006 Published, JBC Papers in Press, October 13, 2006, DOI 10.1074/jbc.M602769200

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The meprin A homo-oligomer is a highly glycosylated, secreted zinc metalloprotease of the astacin family and metzincin superfamily. This isoform of meprin is composed of disulfide-bonded dimers of α subunits that further associate to form large, secreted megadalton complexes of 10 or more subunits. The aim of this study was to determine the sites of glycans attachment and to assess their ability to affect the formation and stability of the homo-oligomer. Nine of the ten potential N-linked glycosylation sites (Asn-41, Asn-152, Asn-234, Asn-270, Asn-330, Asn-426, Asn-452, Asn-546, and Asn-553) were found to be glycosylated in recombinant mouse meprin A using chemical and enzymatic deglycosylation methods and electrospray ionization mass spectrometry. Chemical cross-linking demonstrated that carbohydrates are at or near the noncovalent subunit interface. The removal of two glycans in the protease domain at Asn-234 and Asn-270, as well as one in the tumor necrosis factor receptor-associated factor domain at Asn-452, by a deglycosidase under nondenaturing conditions decreased the chemical and thermal stability of the homo-oligomer without affecting quaternary structure. Site-directed mutagenesis demonstrated that no single glycan was essential for oligomer formation; however, the combined absence of the glycans at Asn-152 and Asn-270 in the protease domain hindered inter-subunit disulfide bond formation, prevented noncovalent associations, and abolished enzymatic activity. These studies provide insights into the role of glycans in the biosynthesis, activity, and stability of this extracellular protease.

With a molecular mass of 1–8 MDa, the meprin A homo-oligomer is the largest known secreted protease (1, 2). It forms unique crescent, ring, barrel, and spiral structures through both disulfide bonds and noncovalent associations, and it is composed of α subunits that have a monomeric molecular mass of 78–85 kDa depending on the species (1, 3). Meprin A is secreted as an oligomer from the brush border epithelial cells of the kidneys and the intestine, as well as certain leukocytes and cancer cells (4–8). Oligomerization of meprin A serves to concentrate the proteolytic activity of the α subunit in the extracellular space for potential delivery to downstream targets in the kidney and intestinal lumen or for degradation of extracellular matrix proteins during leukocyte migration and cancer metastasis (2, 6, 7, 9). In addition, mutants that are not oligomeric are much less stable than the wild type, and this may serve to protect the protein from the harsh extracellular milieu (10, 11).

The folding and oligomerization process of the meprin A homo-oligomer is influenced by several factors within the α subunit. Mutagenesis of His-167, a zinc ligand in the protease domain, leads to a monomeric, inactive protein (11). The MAM4 (meprin, A5 protein, protein-tyrosine phosphatase μ) and tumor necrosis factor receptor-associated factor (TRAF) domains are noncatalytic, but their presence is required for the secretion of a stable and active oligomer (12). The MAM domain has also been implicated in oligomerization through the presence of intersubunit disulfide bonds, in addition to forming part of the noncovalent interface (3, 10, 13). However, the influence of protein modifications such as glycosylation on the formation and structure of the homo-oligomer has not been investigated.

Glycosylation accounts for ~18–20% of the molecular mass of the meprin α subunit (14). Based on the cDNA-deduced amino acid sequence, the secreted α subunit from mouse contains 10 potential N-linked glycosylation sites with the consensus sequence of NX(S/T), where X is any amino acid except proline (Fig. 1) (15). Glycosylation can influence the structure and function of a protein in several ways. During biosynthesis in the endoplasmic reticulum (ER), protein folding is monitored by the glycan-mediated interaction of the protein with ER-resident chaperones such as calnexin and calreticulin (16). The

*This work was supported by National Institutes of Health Grant DK19691 (to J. S. B.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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4 The abbreviations used are: MAM, meprin, A-5 protein, protein-tyrosine phosphate μ; TRAF, tumor necrosis factor receptor-associated factor; ER, endoplasmic reticulum; MBP, mannan-binding protein; HEK-293 cells, human embryonic kidney 293 cells; DMEM, Dulbecco’s modified Eagle’s media; ESI/MS, electrospray ionization/mass spectrometry; TFMS, trifluoroacetic acid; TCEP, Tris-(2-carboxyethyl)phosphine HCl; BMPH, N-(β-maleimidopropionic acid) hydrazide-trifluoroacetic acid; PNGase F, peptide-N-glycanase F; BME, β-mercaptoethanol; BK+, fluoro- genic bradykinin analog 2-aminobenzoyl-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg-Lys(Dnp)-Gly-OH; NEM, N-ethylmaleimide; HPLC, high pressure liquid chromatography; DTT, dithiothreitol.
expression of the wild-type, full-length meprin mouse oligomeric meprin A was purified from the media of human

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EXPERIMENTAL PROCEDURES

FIGURE 1. Domain organization and processing of the mouse meprin α subunit showing potential N-linked glycosylation sites. The prosequence, protease, MAM, and TRAF domains are present in the secreted meprin α subunit. The signal sequence and the C-terminal domains (I, EGF, TM, and C) are removed during biosynthesis. Lines above the domains indicate the Asn residue in the N-linked glycosylation consensus sequence, and lines below the domains indicate the first amino acid of that domain based on the cDNA deduced amino acid sequence (15). S, signal sequence; Pro, prosequence; Protease, catalytic domain; I, inserted domain; EGF, epidermal growth factor-like domain; TM, transmembrane domain; C, cytosolic domain.

placement of glycosylation sites within critical folding regions of the protein can recruit the chaperones to these regions and determine which set of ER chaperones will bind to the molecule (17–20). Glycosylation also influences the physicochemical properties of a protein, such as conformational stability and protein solubility (21, 22). Some glycans mediate protein-protein interactions at the cell surface by influencing the orientation of the proteins and by serving as recognition and binding determinants between the molecules (23). For instance, glycans are involved in the recognition and binding of meprin to the C-type serum lectin mannan-binding protein (MBP), the first endogenous inhibitor of meprin to be identified (24).

Mutagenesis of each individual glycosylation site in mouse meprin A demonstrated that no one particular glycan was responsible for targeting homo-oligomeric meprin A to the apical membrane of polarized epithelial cells (25). However, several of the glycosylation site mutants decreased peptidase activity as well as the stability of meprin A, as assessed by heat inactivation assays. These changes may result from subtle differences affecting catalysis or an overall change in the tertiary or quaternary structure.

Previous attempts at mapping meprin glycosylation sites by lectin blotting of cyanogen bromide fragments detected 2–3 glycans in the MAM and TRAF domains of the α subunit in kidney-purified meprin A (15). This isoform of meprin A is a membrane-bound hetero-oligomer of α and β subunits. However, the lectin experiments did not account for the other glycosylation sites, and there is no information regarding the glycosylation of the α subunit within the context of the homo-oligomer.

To gain insight into the role of the carbohydrates of meprin, the glycosylation sites of the mouse meprin A homo-oligomer were first mapped using a mass spectrometry approach. To elucidate the effect of glycans on the native, folded protein, the structure and stability of a partially deglycosylated oligomer were then investigated. Finally, the effect of glycans on the formation of the oligomer was assessed using a series of single and multiple glycosylation site mutants.

EXPERIMENTAL PROCEDURES

Expression and Purification of Wild-type Meprin A—Homo-oligomeric meprin A was purified from the media of human embryonic kidney 293 cells (HEK-293; ATCC 1573 CRL) stably expressing the wild-type, full-length meprin mouse α cDNA (26) or the wild-type mouse α protein truncated at Arg-615 and His-tagged at the C terminus (27). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) supplemented with 10% fetal bovine serum and antibiotics/antimycotics (100 units/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B; Invitrogen) at 37 °C with 5% CO2 until confluent. The cells were then maintained in serum-free media for 3–4 days. The protein was purified by anion exchange chromatography for the full-length protein or by a nickel chelating column for the His-tagged protein, as described previously (11, 28).

Expression of Single and Multiple Glycosylation Site Mutants—Full-length cDNAs of the single N-linked glycosylation site mutants of mouse meprin α were made previously (25). The double and triple mutants were generated in successive rounds of mutagenesis using QuickChange site-directed mutagenesis kit (Stratagene) with the N234Q mutant plasmid as a template, according to the manufacturer’s instructions. The N152Q mutation was introduced with the following primers: 5′-GAT CAA CAG GTG GGA CAG CAA ATT TCC ATT GGT GAG GGA TG-3′ and 5′-CAT CCC TCA CCA ATG GAA ATT TGC TGT CCC ACC TGT TGA TC-3′. The N270Q mutation was introduced with the following primers: 5′-ATA AGA CTG AAT CGA ATG TAC CAG TGC TGG ACC GCA ACA CAT ACT CTG-3′ and 5′-CAG AAT GTG TGT TGC TGC GTA GAT CAG ATT CAG TGC TGA TC-3′. All plasmids were sequenced by Joe Bednarczyk at the Molecular Genetics Core Facility of Pennsylvania State College of Medicine.

Wild-type and mutant plasmids were transiently expressed in HEK-293 cells using Lipofectamine 2000 transfection reagent (Invitrogen). Briefly, 60 × 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) and Lipofectamine 2000 reagent were then diluted into Opti-MEM reduced serum medium (Invitrogen), mixed according to the manufacturer’s instructions, and added to the plates of HEK-293 cells. The plates were incubated at 37 °C for 4–6 h and then the media were replaced with DMEM lacking FBS. The medium from each plate was collected 24 h post-transfection and analyzed for protein expression by SDS-PAGE and Western blotting. For oligomeric size, stability, and activity assays, the 24-h post-transfection media samples were concentrated 4-fold using YM-50 Centricron concentrators (Amicon), and the EDTA-free Complete Mini inhibitor mixture (Roche Applied Science) was added after concentrating. The media samples were aliquoted and stored at −80 °C until further use.

Gel Electrophoresis and Western Blotting—For SDS-PAGE, samples were separated on 7.5% Tris-HCl Ready gels (Bio-Rad) or 8% polyacrylamide gels at 40–45 mA in 25 mM Tris, 192 mM glycine, pH 8.5, with 0.1% SDS. Gels were transferred onto nitrocellulose membrane (Bio-Rad) at 16 V using a Bio-Rad 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) and then incubated with an appropriate anti-meprin α antibody. Anti-rabbit secondary antibody coupled to horseradish peroxidase (Amersham Biosciences) was added after washing in TBS-T, and blots were
developed with the appropriate chemiluminescent substrate (SuperSignal West Pico or West Dura, Pierce).

N-Linked Glycosylation Site Mapping—To facilitate glycosylation site mapping, a mass marker was created at each glycosylation site using a chemical deglycosylation method adapted from previously published protocols (29, 30). For detection of peptides by electrospray ionization/mass spectrometry (ESI/MS), a 500-µl aliquot of purified meprin A was concentrated to dryness in a Speedvac and then reconstituted in 75 µl of trifluorooromethanesulfonic acid (TFMS; Sigma). The deglycosylation reaction proceeded for 2 h on ice and was quenched with the slow addition of pre-chilled 60% pyridine (200 µl, aqueous). The sample was dialyzed against Nanopure water for 4 h, using SpectraPor dialysis tubing (10 mm, 3500 molecular weight cut-off). The deglycosylated meprin was again concentrated to dryness, resuspended in 100 µl of 100 mM ammonium bicarbonate, pH 8.0, and digested 1:20 with trypsin (Sigma) for 24 h at 37 °C. Disulfide bonds were reduced with 50 mM dithiothreitol (Sigma) immediately prior to injection onto the HPLC column. One-half of the tryptic digest was injected onto a BetaBasic C18 column (5 µm, 150 × 1 mm) coupled to a Mariner mass spectrometer (PerSeptive Biosystems). The split flow rate was 0.05 ml/min, and a gradient program was used to partially separate the peptides. Peptides were loaded onto the column under the starting conditions of 100% solvent A (0.1% formic acid/H2O) for 6 min, diverting the flow to prevent salt interference. The peptides were eluted under a gradient to 95% solvent B (0.1% formic acid/acetonitrile) over 44 min. Following elution of the peptides, the conditions were held for 5 min, and then the column was flushed with 90% solvent C (0.1% formic acid/isopropanol). Electrospray ionization in positive mode was used for detection, and data were collected between m/z 200 and 2500. MS data were analyzed using the accompanying DataExplorer software (Applied Biosystems), utilizing the extracted ion chromatogram feature to search for the predicted m/z values of the glycopeptides.

A separate sample of meprin A was also incubated in the presence or absence of the deglycosidase PNGaseF (New England Biolabs) to determine the occupancy of the glycosylation site using a chemical deglycosylation method adapted from previously published protocols (29, 30). For detection of peptides by electrospray ionization/mass spectrometry (ESI/MS), a 500-µl aliquot of purified meprin A was concentrated to dryness in a Speedvac and then reconstituted in 75 µl of trifluorooromethanesulfonic acid (TFMS; Sigma). The deglycosylation reaction proceeded for 2 h on ice and was quenched with the slow addition of pre-chilled 60% pyridine (200 µl, aqueous). The sample was dialyzed against Nanopure water for 4 h, using SpectraPor dialysis tubing (10 mm, 3500 molecular weight cut-off). The deglycosylated meprin was again concentrated to dryness, resuspended in 100 µl of 100 mM ammonium bicarbonate, pH 8.0, and digested 1:20 with trypsin (Sigma) for 24 h at 37 °C. Disulfide bonds were reduced with 50 mM dithiothreitol (Sigma) immediately prior to injection onto the HPLC column. One-half of the tryptic digest was injected onto a BetaBasic C18 column (5 µm, 150 × 1 mm) coupled to a Mariner mass spectrometer (PerSeptive Biosystems). The split flow rate was 0.05 ml/min, and a gradient program was used to partially separate the peptides. Peptides were loaded onto the column under the starting conditions of 100% solvent A (0.1% formic acid/H2O) for 6 min, diverting the flow to prevent salt interference. The peptides were eluted under a gradient to 95% solvent B (0.1% formic acid/acetonitrile) over 44 min. Following elution of the peptides, the conditions were held for 5 min, and then the column was flushed with 90% solvent C (0.1% formic acid/isopropanol). Electrospray ionization in positive mode was used for detection, and data were collected between m/z 200 and 2500. MS data were analyzed using the accompanying DataExplorer software (Applied Biosystems), utilizing the extracted ion chromatogram feature to search for the predicted m/z values of the glycopeptides.

A separate sample of meprin A was also incubated in the presence or absence of the deglycosidase PNGaseF (New England Biolabs) to determine the occupancy of the glycosylation sites. A 500-µg sample (500 µl of a 11.8 µM solution) of mouse meprin α was denatured by boiling for 5 min at 100 °C and then the disulfides were reduced in the presence of 20 mM DTT (Sigma) for 30 min at 37 °C. N-Ethylmaleimide (Sigma) dissolved in 100% ethanol (HPLC grade; Sigma) was added to the sample (in the presence of the DTT) to a final concentration of 60 mM, and the sample was incubated for an additional 30 min at 37 °C. After denaturation, reduction, and capping of cysteines, the sample was exhaustively dialyzed into Nanopure water (2 × 2 liters) and then concentrated ~5.5-fold using a Speedvac. The sample was then split into two, and one-half of the sample was incubated in 50 mM sodium phosphate buffer, pH 7.5, at 37 °C for 5 h in the presence of the deglycosidase PNGaseF (glycerol-free; New England Biolabs), and the other half was incubated under the same conditions in the absence of PNGaseF. After deglycosylation, trypsin (Promega Gold in 50 mM acetic acid; Sigma) was added to each sample so that the final trypsin/meprin ratio was 1:20, and the pH was 7.5 (in 50 mM phosphate buffer). The meprin samples were digested with trypsin at 37 °C for 39 h. One-tenth of each sample was analyzed using a Waters 2695 HPLC system with a Discovery Bio C5 column (Supelco, 5 µm, 5 × 2.1 mm) with a gradient similar to the chemically deglycosylated samples. A Waters LCT Premier time-of-flight mass spectrometer in positive ion mode was used to detect the peptide ions, and Waters MassLynx 4.0 software was used to search for the expected peptide ions.

Cross-linking of the Meprin Homo-oligomer Noncovalent Dimer through the Carbohydrate Moieties—The carbohydrate moieties of the meprin A homo-oligomer were oxidized in the presence of 10 mM sodium periodate (Sigma) in 100 mM sodium acetate, pH 5.5, for 2 h at 23 °C in the dark. Excess sodium periodate was removed with a 1-ml desalting column packed with G-25 Sephadex resin (Sigma) equilibrated with water. For the cross-linking reaction, triethanolamine, pH 7.5, was added to the sample at a final concentration of 40 mM, and reduction of the intersubunit disulfide bond to yield the noncovalent dimer was achieved in the presence of 5 mM Tris-(2-carboxyethyl)phosphine HCl (TECP), pH 8.0. After ~15 min, the carbohydrate and cysteine-reactive cross-linker N-[β-maleimidomopropanioic acid] hydrazide-trifluoroacetic acid (BMHP; Pierce) was added to a final concentration of 10 mM in 10% dimethylformamide (Fisher). The cross-linking reaction proceeded for 2 h at 23 °C. The samples were analyzed by nonreducing SDS-PAGE and Western blotting as described above.

Partial Deglycosylation of the Meprin Homo-oligomer—For partially deglycosylated samples, meprin A was incubated in the presence of PNGaseF (New England Biolabs), in 50 mM sodium phosphate buffer, pH 7.5, in the absence of β-mercaptoethanol (BME) and SDS (nonreducing and nondenaturing conditions). Samples were incubated at 37 °C for 2 h and then used for further experiments. A typical deglycosylation reaction contained at least 300–500 units of deglycosidase to 20 µg of meprin.

Mapping Glycans 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 of glycerol-free PNGaseF (New England Biolabs) under nondenaturing and nonreducing conditions (~SDS, ~BME, but +Nonidet P-40) and prepared for ESI/MS as described above. One-half of the sample was digested with trypsin (1:20 in 20 mM Tris, 150 mM NaCl, pH 7.5). The data set collected from the tryptic digest was searched using the extracted ion chromatogram tool of the DataExplorer software, looking for the predicted tryptic fragments plus 1 mass unit, which resulted from the conversion of Asn to Asp by PNGaseF.

Circular Dichroism and Intrinsic Tryptophan Fluorescence Spectroscopy—The far-UV 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 to 250 nm at a scan rate of 50 nm/min at 25 °C using quartz cuvettes with a path length of 1 mm. Spectra are an average of three scans. Samples of latent meprin A were partially deglycosylated 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 par-
tially deglycosylated samples were diluted to 10 μg/ml (118 nm) in Nanopure water. Tris and NaCl were added to a final concentration of 20 and 150 mM, respectively, at pH 7.5. Samples were incubated at 23 °C for 30 min before analysis. Quartz cuvettes with path lengths of 1 cm × 2 mm were used, and the cuvette holder was kept at 25 °C. Excitation slit width was 0.5 nm, and emission slit width 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 to 400 nm.

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 Biosciences) 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 to determine the elution profile of the protein.

Urea-induced Unfolding of the Meprin A Homo-oligomer—The urea-induced unfolding of the meprin oligomer (wild-type and partially deglycosylated) was followed by the change in intrinsic tryptophan fluorescence. Samples were diluted to 10 μg/ml with Nanopure water and increasing amounts of urea (0.6 mM phosphate, final) and then incubated at 25 °C for 30 min. Fluorescence spectra were acquired with an excitation wavelength of 280 nm. The excitation and emission slit widths were 0.25 and 1.75 nm, respectively. The fluorescence intensity at the wavelength of maximal emission (λmax) of the wild-type protein was recorded and plotted versus urea concentration to obtain an unfolding curve.

Activity Assays—The peptidase activity of meprin A was measured using the fluorescent substrate 2-aminobenzoyl-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg-Lys(Dnp)-Gly-OH (BK+), where Dnp is dinitrophenyl, as described previously (10). Assays were carried out after activation of the samples by limited trypsin digestion (1:20 trypsin/meprin for purified samples or 5 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. For crude media samples, the specific activities of each protein were calculated by Western blot and densitometry. The concentration of the wild-type protein in the media sample was measured from its BK+ activity compared with the fluorescence/μg of purified protein.

Tryptic Stability Assays—To test the activation and stability of the meprin glycosylation mutants, crude media samples were incubated with increasing amounts of trypsin (0, 5, 40, and 80 ng/μl) for 45 min at 37 °C. Trypsin was inhibited for 15 min at 23 °C by a 3-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.

Mouse Meprin A Glycosylation

Heat Stability Assays—To measure the heat 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 time point, 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 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.

RESULTS

N-Linked Glycosylation Site Mapping of the Mouse Meprin A Homo-oligomer—Chemical deglycosylation with TFMS removes the carbohydrate moieties from glycoproteins with the exception of the innermost GlcNAc residue. This creates a mass marker of +203 mass units on each glycopeptide that can then be detected by mass spectrometry. Under certain conditions, the innermost GalNAc residue of O-linked sugars also remains on the protein (29, 30). However, there was no detectable GalNAc in a total carbohydrate analysis of meprin A, and the protein was not detected by a carbohydrate-reactive label after the protein was fully deglycosylated by PNGaseF, a deglycosidase that removes all types of N-linked glycans; thus there is no significant O-linked glycosylation on meprin A (data not shown). The predominant monosaccharides in the carbohydrate analysis were GlcNAc, galactose, and mannose, with a small amount of fucose. Further study is needed to accurately quantify these monosaccharides and determine the location of the fucose in the context of the terminal glycosylation pattern.

ESI/MS was used to detect glycopeptides from a tryptic digest of chemically deglycosylated meprin A. The total ion chromatogram from the digest was searched for the presence of glycopeptide ions at their calculated mass-to-charge (m/z) ratios. Peptide ions consistent with glycosylation at Asn-152, Asn-234, Asn-270, Asn-452, Asn-546, and Asn-553 were also detected (Table 1). Because there were no potential proteolytic cleavage sites between Asn-546 and Asn-553, these two glycosylation sites were contained in the same peptide. Masses consistent with both one and two GlcNAc residues were observed in the total ion chromatogram, indicating heterogeneity in this region (Table 1). The only site not glycosylated was Asn-614, near the putative C terminus. There were no ions corresponding to either the unmodified or glycosylated peptides for Asn-330 and Asn-426; therefore, the occupancy of these sites could not be determined from this experiment. Based on mutagenesis and structural studies of meprins and protein-tyrosine phosphatase μ, MAM domains are highly disulfide-bonded (31–33). Incomplete cleavage of the protein in this region because of the disulfide bonds may be one reason why the MAM domain peptides were not observed. However, it is likely that at least Asn-330 is glycosylated, because this site was shown to be glycosylated in
Distinguished from unmodified peptides by the change of expected nonglycosylated (NG) and glycosylated (G) monoisotopic tryptic masses are listed, as well as the calculated glycopeptide. To ensure complete digestion of the protein in the digestion method was used to determine whether the Asn-330 and Asn-426 of kidney-purified meprin A (15). Thus, an alternative deglycosylation method was used to determine whether the Asn-330 and Asn-426 glycosylation sites were occupied by a glycan.

Treatment of glycoproteins with the deglycosidase PNGaseF removes the entire carbohydrate moiety from N-linked sugars, converting Asn to Asp, which also creates a mass marker on the glycopeptide. To ensure complete digestion of the protein in the MAM domain, the disulfide bonds in meprin A were first reduced with DTT and capped with N-ethylmaleimide. With this method, each of the glycosylation sites identified by chemical deglycosylation was confirmed, and masses corresponding to the glycosylated forms of the Asn-330 and Asn-426 peptides were observed (Table 2). In a sample that was incubated in the absence PNGaseF, low abundance peptide ions that correspond to the expected nonglycosylated (NG) and glycosylated (G) monoisotopic 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 GlcNAc residue (+203 mass units).

**Table 1**

### N-Linked glycosylation site mapping of mouse meprin A using chemical deglycosylation

Mouse meprin A was chemically deglycosylated with TFFMS, digested with trypsin, and analyzed by ESI/MS as described under "Experimental Procedures." The expected nonglycosylated (NG) and glycosylated (G) monoisotopic 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 GlcNAc residue (+203 mass units).

| Asn | Mass       | +1     | +2     | +3     | +4     | Observed ions |
|-----|------------|--------|--------|--------|--------|---------------|
| 41  | NG, 1763.81| 1764.81| 882.91 | 588.94 | 441.95 | G, 984.52(2), |
|     | G, 1966.89 | 1967.89| 984.44 | 656.63 | 492.72 | G, 656.64(2), |
| 152 | NG, 2771.24| 2772.24| 1386.62| 653.83 | 413.26 | G, 754.26(2), |
| 234 | NG, 1102.59| 1103.59| 552.30 | 547.27 | 104.01 | G, 655.84(2), |
| 270 | NG, 2524.13b| 2525.13| 1263.06| 631.30 | 315.42 | G, 910.17(2), |
| 330 | NG, 1637.77| 1638.77| 819.86 | 547.27 | 104.01 | G, 682.80(2), |
| 426 | NG, 3104.48| 3105.48| 1553.24| 1035.83| 777.12 | NA |
|     | G, 3307.56| 3308.56| 1654.78| 1103.52| 827.89 | NA |
| 452 | NG, 1257.69| 1258.69| 629.85 | 420.23 | 315.42 | G, 731.39(2), |
|     | G, 1460.77| 1461.77| 731.39 | 487.92 | 366.19 | G, 1082.06(2), |
| 546, 553 | NG, 1958.98| 1959.98| 980.49 | 593.99 | 490.75 | G, 798.82(2), |
|     | 2162.06 (1 GlcNAc)| 2163.06| 1082.03| 721.69 | 541.51 | G, 798.82(2), |
|     | 2365.14 (2 GlcNAc)| 2366.14| 1183.57| 789.38 | 592.26 | G, 798.82(2), |
| 614 | NG, 867.46 | 868.46 | 434.73 | 290.15 | 217.87 | G, 868.47(2), |
|     | G, 1070.54 | 1071.54| 536.27 | 357.85 | 268.63 | G, 1070.54(2), |

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 NA indicates not available. Neither the nonglycosylated nor glycosylated peptides were observed.

**Table 2**

### N-Linked glycosylation site mapping of mouse meprin A using enzymatic deglycosylation

Mouse meprin A was enzymatically deglycosylated with PNGaseF, digested with trypsin, and analyzed by ESI/MS as described under "Experimental Procedures." The expected nonglycosylated (NG) and glycosylated (G) monoisotopic tryptic masses are listed, as well as the calculated m/z values for the peptide ions. Glycopeptides were distinguished from unmodified peptides by the change of +1 mass unit upon deglycosylation, reflecting the conversion of Asn to Asp.

| Asn | Mass       | +1     | +2     | +3     | +4     | Observed ions |
|-----|------------|--------|--------|--------|--------|---------------|
| 41  | NG, 1763.81| 1764.81| 882.91 | 588.94 | 441.95 | G, 1765.80(2), |
|     | G, 1966.89 | 1967.89| 984.44 | 656.63 | 492.72 | G, 656.64(2), |
| 152 | NG, 3021.44| 3022.45| 1511.73| 1008.16| 756.37 | G, 1512.19(2), |
|     | G, 3224.45| 3225.46| 1512.23| 1008.49| 756.62 | G, 1512.19(2), |
| 234 | NG, 1102.59| 1103.60| 552.30 | 368.78 | 276.66 | G, 1512.19(2), |
|     | G, 1103.60| 1104.60| 552.81 | 368.78 | 276.91 | G, 1512.19(2), |
| 270 | NG, 2391.11b| 2392.12| 1197.07| 798.04 | 599.04 | G, 1197.04(2), |
|     | G, 2392.12| 2393.12| 1197.07| 798.38 | 599.04 | G, 1197.04(2), |
| 330 | NG, 1637.77| 1638.77| 819.86 | 546.92 | 410.44 | G, 1639.80(2), |
|     | G, 1638.77| 1639.78| 820.40 | 547.27 | 410.70 | G, 1639.80(2), |
| 426 | NG, 3229.58b| 3230.59| 1615.80| 1077.53| 808.40 | G, 1616.27(2), |
|     | G, 3230.59| 3231.60| 1616.30| 1077.87| 808.66 | G, 1616.27(2), |
| 452 | NG, 1257.69| 1258.69| 629.85 | 420.24 | 315.42 | G, 1258.58(2), |
|     | G, 1258.70| 1259.71| 629.85 | 420.58 | 315.68 | G, 1259.58(2), |
| 546, 553 | NG, 1958.98| 1959.98| 980.49 | 593.99 | 490.75 | 1 site |
|     | G, 1959.99 (1 site)| 1960.99| 981.51 | 491.01 | G, 1961.05(2), |
|     | G, 1960.99 (2 sites)| 1962.01| 981.51 | 491.26 | G, 1961.05(2), |
| 614 | NG, 867.46 | 868.46 | 434.73 | 290.15 | 217.87 | G, 868.47(2), |
|     | G, 868.46 | 869.47 | 435.24 | 290.50 | 218.12 | G, 868.47(2), |

a Numbers in parentheses indicate the charge of the observed ion.
b Peptide masses reflect the capping of cysteines in the tryptic peptide.
cosylated peptide masses were identified for eight glycosylation sites, indicating a small amount of heterogeneity in terms of site occupancy (data not shown). Overall, nine of the ten potential glycosylation sites in mouse meprin A are glycosylated, with the exception of Asn-614 at the C terminus.

Carbohydrates Are at or Near the Noncovalent Subunit Interface—The intersubunit disulfide bonds of the meprin A homo-oligomer can be selectively reduced with TCEP to yield a noncovalently associated dimer that is the functional protomer of the protease (3). To determine whether glycans were located in proximity of the noncovalent interface, where they could influence the association of the subunits, the partially reduced, noncovalent dimer of meprin A was incubated with the carbohydrate and cysteine-reactive cross-linker BMPH (Fig. 2a, structure of the carbohydrate and cysteine reactive cross-linker BMPH). After oxidation, reduction, and cross-linking as indicated, samples were separated by SDS-PAGE under nonreducing conditions and visualized by Western blot with anti-meprin α antibodies.

Identification of Glycans Removed in a Partially Deglycosylated Homo-oligomer—To determine the effect of glycans on the native fold of the protein, glycans were enzymatically removed from the homo-oligomer with PNGaseF under non-denaturing conditions. Full deglycosylation of meprin A requires reducing and denaturing the protein; under non-denaturing conditions, only a portion of the glycans are removed. In previous studies, the partially deglycosylated oligomer retained no peptidase activity, but in these studies the protein was incubated with PNGaseF at 37 °C under non-denaturing conditions for up to 18 h (25). In contrast, when the protein was incubated with PNGaseF for only 2 h at 37 °C, an average of 86% of the peptidase activity was retained (Fig. 3a) even though the same amount of carbohydrate was...
removed compared with a 16-h incubation (Fig. 3b). Thus, all further experiments were carried out using a 2-h deglycosylation under nonreducing conditions.

PNGaseF removes the entire carbohydrate moiety, converting the Asn residue of the consensus sequence to Asp, a mass difference of +1 mass unit that can be detected by ESI/MS. Analysis of a tryptic digest of the partially deglycosylated homooligomer revealed that three glycans are removed from the oligomer under nonreducing conditions. Ions corresponding to deglycosylated peptides containing Asn-234 and Asn-270 in the protease domain and Asn-452 in the TRAF domain were detected (Table 3).

Structure and Stability of the Partially Deglycosylated Oligomer—Even though the removal of three glycans from the protein does not affect the activity of the protease, it is possible that the association of the subunits is altered. The effect of partial deglycosylation on the secondary and tertiary structure of meprin A was assayed by CD and intrinsic tryptophan fluorescence. Slight differences were observed in the CD spectra of wild-type and partially deglycosylated meprin A in the regions of 212 and 225 nm, but there was no marked change in the secondary structure of the protein (Fig. 4a). A shift in the wavelength of maximal emission ($\lambda_{\text{max}}$) was observed in the intrinsic tryptophan fluorescence spectrum of the protein upon partial deglycosylation, from 331 nm for wild type to 334 nm for the partially deglycosylated sample (Fig. 4b). However, these differences did not affect the quaternary structure of the oligomer. The majority of the protein in each sample eluted in fraction 8 of a size exclusion column, consistent with the formation of an octamer (Fig. 4c). The smaller species observed in fractions 9 and 10 for the wild-type protein were less apparent in the partially deglycosylated protein, which may be because of aggregation or precipitation of the protein. Overall, there was no noticeable redistribution of the oligomeric species in the absence of the protease and TRAF domain glycans, based on densitometry of the bands. Consistent with the activity assays, similar results were obtained in the structural characterization of both the latent and active oligomers.

Although there was no change in the oligomeric state of the protein, partially removing glycans decreased the overall stability of the oligomer, as assessed by urea-induced unfolding and heat stability assays (Fig. 5). The partially deglycosylated protein unfolded at a lower urea concentration than the wild type (Fig. 5a). The unfolding transition was not readily reversible and was thus condition-dependent; therefore, a thermodynamic value could not be assigned to the difference in stability.

![Figure 4](image)

**FIGURE 4. Structure and oligomeric state of the partially deglycosylated oligomer.** After partial deglycosylation under nonreducing conditions, the structure and oligomeric state of the partially deglycosylated, latent homooligomer were compared with the wild-type protein using CD, intrinsic tryptophan fluorescence, and size exclusion chromatography. a, far-UV CD spectra of the wild-type (○) and partially deglycosylated (×) proteins at 3.2 μM final protein concentration after a 15-min incubation at 23 °C. Symbols correspond to those in a. Excitation wavelength was 280 nm. c, size exclusion analysis of the wild-type (—PNGaseF) and partially deglycosylated (+PNGaseF) samples. Samples were injected at a concentration of 3.9 μM with a flow rate of 0.25 ml/min. Meprin in each 1-ml fraction was analyzed by reducing SDS-PAGE and Western blotting. This difference was also more apparent in the activated protein; little or no change was detected for the latent protein, under the conditions tested. When the activated protein was incubated at 55 °C for different periods of time, the partially deglycosylated
protein lost enzymatic activity five times faster than the wild-type protein (Fig. 5b). Thus, glycans in the protease and TRAF domains are protective, stabilizing the protein against denaturing conditions.

**Oligomerization of Single N-Linked Glycosylation Mutants**—The contribution of each N-linked glycan to the formation of both the covalent and noncovalent interfaces was evaluated using a series of single N-linked glycosylation site mutants in which the Asn of the consensus sequence was mutated to Gln. Glycans have been shown to influence the rate of disulfide bond formation in intestinal mucin 2 (34). In the meprin α subunit, Asn-330 is only 10 amino acids from an intersubunit disulfide bond, and could potentially influence the extent of dimerization. After transient transfection of the single mutants into HEK-293 cells, the media samples containing the secreted proteins were analyzed by nonreducing SDS-PAGE. Each of the mutants was capable of forming the disulfide-bonded dimer to the same extent as the wild-type protein, including the N330Q mutant (Fig. 6a). Furthermore, when the mutants were screened by native gel electrophoresis and size exclusion chromatography for their ability to form the disulfide-bonded dimer, and the large molecular mass homo-oligomer, all of the mutants behaved similarly to wild type. For example, the N152Q, N234Q, and N270Q mutants in the protease domain primarily eluted in fraction 8 of a size exclusion column, indicating formation of an octamer (Fig. 6b). The expression level of the N152Q mutant appears lower in the size exclusion chromatography sample, but this is because of a lighter exposure of the Western blot. Based on three independent transfections, the expression of N152Q was 78 ± 8% of the wild type. A more disperse distribution of immunoreactivity after size exclusion chromatography of media samples was observed compared with purified protein samples (compare Figs. 4c and 6c), which may be due to the composition of the media sample. Overall, the changes in activity and stability of the single glycosylation site mutants reported by Kadowaki et al. (25) are not because of gross changes in the quaternary structure of the protein.

**Oligomerization of Multiple Glycosylation Site Mutants**—Because no one glycosylation site affected the oligomeric state of the meprin A homo-oligomer, the effect of multiple mutations was investigated. All three of the protease domain glycans are completely conserved in the meprin α subunits of mice, rats, and humans. It was hypothesized that the absence of glycosylation in this domain could destabilize the domain enough to prevent proper folding.

Indeed, the N152Q/N234Q/N270Q mutant was impaired in its ability to form the disulfide-bonded dimer (Fig. 7a). In this particular transfection, no dimer species was observed, but on average, the amount of dimer formed was 27 ± 10% of the total immunoreactivity based on five independent transfections (Fig. 7b). This was approximately one-third the amount of dimer formed by the wild-type protein (79 ± 4%). The larger variation
in dimer formation for the triple mutant may reflect different extents of nonspecific oxidation of cysteine residues after secretion of the protein into the media. Formation of the covalent interface was necessary to promote noncovalent associations of the subunits. Size exclusion analysis of the N152Q/N234Q/N270Q mutant revealed that the majority of the protein eluted in fraction 12, corresponding to a monomer (Fig. 7c). The small amount of protein in fraction 11, which would correspond to a dimer based on the elution of protein standards, was also shown to be predominantly monomeric when the fractions were analyzed under nonreducing conditions (data not shown). In transfections where more of the disulfide-bonded dimer species was observed by nonreducing SDS-PAGE, more immunoreactivity was observed in fractions 8–11 as larger species that formed disulfide-bonded dimers, but these species accounted for 25% or less of the total immunoreactivity. Again, these apparently larger species may be due to nonspecific aggregation and oxidation of the protein, or they may reflect a small portion of the oligomer to achieve a native, or near-native, fold.

To determine whether the effect of the triple mutant on the oligomerization was because of a general destabilization and misfolding of the domain or a more localized effect because of a specific combination of glycans, double mutants were created and analyzed by nonreducing SDS-PAGE and size exclusion analysis. The N234Q/N270Q and N152Q/N234Q mutants formed the disulfide-bonded dimer to the same extent as the wild type and were capable of forming the large molecular mass oligomers (Fig. 7a–c). However, like the triple mutant, the N152Q/N270Q mutant was also impaired in its ability to form the disulfide-bonded dimer (Fig. 7a). On average, the percent-dimer formed in seven independent transfections was 30% of the total immunoreactivity (Fig. 7b), and the protein behaved as a monomer with a small amount of larger species present (Fig. 7c).

These results demonstrate that there is a specific interaction between the glycans at Asn-152 and Asn-270 that promotes oligomerization through both the covalent and noncovalent interface, and the effect on oligomerization is not simply because of the absence of more than one glycan. Furthermore, the glycan itself exerts this effect, and the disruption of oligomerization is not because of the Asn to Gln mutation. This was demonstrated by mutating the Ser or Thr residue in the consensus sequence instead of the Asn. A S154A/T272A mutant behaved similarly to the N152Q/N270Q mutant in terms of disulfide-bond formation and higher order oligomer
importance of protease domain study of the partially deglycosylated protein, demonstrating the lost activity 10 times faster. These results complement the inactivated 6 times faster, whereas the N234Q/N270Q mutant was roughly additive; the N152Q/N234Q mutant was inactivated 5 times faster. The inactivation of the double mutants was inactivated 9 times faster. The inactivation of the double protease domain mutants maintained BK activity compared with 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 4**

| Specific activity | Wild type | N152Q/N234Q | N152Q/N270Q | N234Q/N270Q | N152Q/N234Q/N270Q | N152Q | N234Q | N270Q |
|-------------------|-----------|-------------|-------------|-------------|-------------------|-------|-------|-------|
|                   | 138 ± 9    | 113 ± 3     | ND          | 137 ± 9     | ND                | 101 ± 5     | 142 ± 2 | 123 ± 6 |

*ND indicates not detected.

formation, whereas the corresponding single mutants did not have an effect on the quaternary structure (data not shown).

Activity and Stability of the Multiple Glycosylation Site Mutants—The multiple glycosylation site mutants were further analyzed for their ability to be activated by trypsin, their level of BK+ activity, and their heat stability in relation to the wild type and the corresponding single glycosylation site mutants. All of the multiple mutants could be activated by removal of the prosequence at 5 ng/µl trypsin (Fig. 8a). However, the monomeric mutants (N152Q/N270Q and N152Q/N234Q/N270Q) were degraded at lower concentrations of trypsin. These mutants also did not have any detectable peptidase activity (Table 4). These data indicate that there is global misfolding in the protein structure of these mutants. The N152Q/N234Q and the N234Q/N270Q double mutants, which were oligomeric, had wild-type vulnerability to trypsin degradation (Fig. 8a). Furthermore, both the single and double protease domain mutants maintained BK+ activity (Table 4). In contrast to previous studies, the single protease domain glycosylation site mutants displayed wild-type BK+ activity in the current study (25). This discrepancy may reflect different cellular conditions and/or modifications that affect the active population of meprin A molecules. The lower activities previously reported may also be because of incomplete activation at a lower trypsin concentration (1 ng/µl versus 5 ng/µl).

The single and double glycosylation site mutants had different levels of stability, as assessed by a heat inactivation assay (Fig. 8b). The N152Q and N234Q single mutants lost activity ~3 times faster than the wild type, whereas the N270Q mutant was inactivated 5 times faster. The inactivation of the double mutants was roughly additive; the N152Q/N234Q mutant was inactivated 6 times faster, whereas the N234Q/N270Q mutant lost activity 10 times faster. These results complement the study of the partially deglycosylated protein, demonstrating the importance of protease domain N-linked glycans in maintaining the stability of the homo-oligomer.

**DISCUSSION**

In this study, N-linked glycans were identified as factors that contribute to both the oligomerization and stability of the mouse meprin A homo-oligomer. A unique interaction between the glycans at Asn-152 and Asn-270 in the protease domain was identified. Together, these glycans help to direct formation of a stable, active oligomer that can form intersubunit disulfide bonds in the MAM domain. In addition, several glycans were shown to contribute to the conformational stability of the oligomer. The crucial role of the protease domain glycans in particular highlights a new function of the protease domain in the folding, structure, and activity of the protein.

To date, meprins are the only oligomeric members of the astacin family. Crayfish astacin, consisting of a prosequence and a protease domain, is monomeric and does not contain any glycosylation sites (35). BMP-1 (bone morphogenic protein-1) is a multidomain protease and is not oligomeric. Mutating its single protease domain glycosylation site did not have a dramatic effect on activity and stability of the protein (36). However, two of the protease domain glycans of the meprin α subunit are required to form an active, stable oligomer. This effect is not likely because of a general destabilization of the protease domain in the absence of multiple glycans; rather there is a more specific effect on the folding pattern and overall tertiary structure that prevents protein-protein interactions between the subunits.

Glycans can direct the folding pathway of a protein by stabilizing key structural elements, preventing aggregation, and/or mediating crucial chaperone interactions (16). Because the final native fold of meprin A is highly dependent on domain-domain interactions, a misfolded protease domain can affect the association of the protein in the MAM and TRAF domains (12). One possible mechanism by which the absence of the Asn-152 and Asn-270 glycans affects intersubunit disulfide bond formation in the MAM domain involves shuffling of the disulfide bonds during oxidative folding of the protein. In this process, nonnative disulfide pairings form first and are subsequently rearranged by an oxidoreductase (37). If cysteine residues are missing, as in the case of meprin cysteine mutants, the shuffling process is stalled in nonnative pairings and intersubunit disulfide bonds do not form (10, 13, 31). Misfolding and/or the inability to form the two intradomain disulfide bonds in the protease domain could trap the protein in a conformation that does not allow crucial cysteines in the protease and/or MAM domains to interact with the oxidoreductase or to find their native bonding partners. Based on a homology model of the protease domain, the proximity of the Asn-152 and Asn-270 glycans to the two intradomain disulfide bonds would allow them to influence disulfide bond formation, a phenomenon that was observed in a glycopeptide from the nicotinic acetylcholine receptor (38–40). The inability to form the noncovalent interface is likely a result of improper intersubunit disulfide bond formation, as well as overall misfolding of the protein.

The protease domain glycans are also implicated in the stabilization of the oligomer against chemical and thermal denaturation. The conformational flexibility of the glycan moieties stabilizes glycoproteins by compensating for the entropy lost once the dynamics of the protein backbone are decreased (41). For instance, the Asn-234 glycan in the meprin α protease domain lies within a loop region in the C-terminal portion of this domain, based on homology modeling, where it likely decreases the flexibility of this region (38). Removing the Asn-234 and Asn-270 glycans enzymatically did not affect the struc-
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The glycans of the MAM and TRAF domains were originally hypothesized to be the most likely candidates to influence the oligomeric state of meprin A, given that these domains mediate oligomerization in other proteins and contain other elements that direct meprin A oligomerization (10, 12, 13, 31). However, the glycans in these domains were not necessary by themselves to form the large molecular mass oligomers. Furthermore, a double MAM mutant (N330Q/N426Q) had no effect on disulfide bonding, oligomerization, or activity. The glycans of the TRAF domain, although not contributing to the structure of the protein, may prove to be important in mediating interactions with other proteins, such as MBP, the only endogenous inhibitor of meprin that has been identified to date (24). The macroheterogeneity observed at Asn-546 and Asn-553 in the TRAF domain could be an important factor in regulating the interaction of meprin A with other cell surface or extracellular proteins by creating populations of meprin molecules with different binding properties. Although the Asn-452 glycan was also removed by PNGaseF along with Asn-234 and Asn-270, it may not contribute as much to oligomer stability as the other two glycosylation sites because the N452Q mutant was shown to have wild-type heat stability (25). However, because of its relative accessibility at the surface of the protein, it may also mediate interactions with other glycan-binding proteins. The absence of a glycan at Asn-614 near the putative C terminus may be necessary to allow cleavage of the protein in this region, or the tertiary structure of the protein may simply prevent the oligosaccharide transferase complex from adding a glycan to this sequon (43). The orientation of the domains of meprin and the location of the glycans remain speculative, because crystallization of the protein has proven difficult due in part to the heterogeneity of the glycans. Common strategies to circumvent this heterogeneity include removing some of the glycans (enzymatically or by mutagenesis) and using glucosidase inhibitors or glucosidase-deficient cells that leave the glycans in high mannose form (44). This study has demonstrated that the first strategy would likely be more successful if the MAM and/or TRAF domain glycans were eliminated by mutagenesis, because the protease domain glycans are critical for correct folding, oligomerization, and stability. Using a deglycosidase to remove Asn-234 and Asn-270 from the mutant protein after purification would eliminate two more of the glycans. In addition, selective reduction of the intersubunit disulfide bond to yield a noncovalent dimer would likely make the protein much more amenable to crystallization.

The glycans of meprin A have been shown to be critical factors in determining meprin A function by promoting important protein–protein interactions between meprin subunits (homooligomerization), but these glycans may also mediate interactions between meprin and other cell-surface and secreted proteins (hetero-oligomerization). For instance, determining which glycans are recognized by MBP will be important in understanding how this endogenous inhibitor of meprins functions to decrease meprin activity. Furthermore, the exact composition of the glycans of meprin may also play a role in protein–protein interactions, serving as specific binding epitopes. Because the type of glycans can be altered in diseased states such as cancer, a change in the glycan profile may modulate the interaction of meprins with other important cell-surface and secreted molecules.

Acknowledgments—We thank Dr. Channe Gowda for use of carbohydrate analysis instrumentation and helpful advice. We appreciate the help of Dr. Eric Snyder in the analysis of meprin glycosylation sites by electrospray mass spectrometry. We also thank Dr. Ira Ropson for use of the CD and fluorescence instruments, as well as for helpful discussions.

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