N-Linked Oligosaccharides on the Meprin A Metalloprotease Are Important for Secretion and Enzymatic Activity, but Not for Apical Targeting*

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The α and β subunits of meprins, mammalian zinc metalloendopeptidases, are extensively glycosylated; ~25% of the total molecular mass of the subunits is carbohydrate. The aim of this study was to investigate the roles of the N-linked oligosaccharides on the secreted form of mouse meprin A. Recombinant meprin α and mutants in which one of the 10 potential Asn glycosylation sites was mutated to Gln were all secreted and sorted exclusively into the apical medium of polarized Madin-Darby canine kidney cells, indicating that no specific N-linked oligosaccharide acts as a determinant for apical targeting of meprin α. Several of the mutant proteins had decreased enzymatic activity using a bradykinin analog as substrate, and deglycosylation of the wild-type protein resulted in loss of 75–100% activity. Some of the mutants were also more sensitive to heat inactivation. In studies with agents that inhibit glycosylation processes in vivo, tunicamycin markedly decreased secretion of meprin, whereas castanospermine and swainsonine had little effect on secretion, sorting, or enzymatic properties of meprin. When all the potential glycosylation sites on a truncated form of meprin α (α(1–445)) were mutated, the protein was not secreted into the medium, but was retained within the cells even after 10 h. These results indicate that there is no one specific glycosylation site or type of oligosaccharide (high mannose- or complex-type) that determines apical sorting, but that core N-linked carbohydrates are required for optimal enzymatic activity and for secretion of meprin α.

Meprins belong to the astacin family of metalloendopeptidases and to the metzin family (1, 2). The prototype of the family is the crayfish astacin (EC 3.4.24.21), a 20-kDa monomer secreted from the hepatopancreas. The meprins are oligomers composed of α and/or β subunits that are evolutionarily related, but differ in function (1, 3). Meprin β subunits are integral membrane proteins; mature α subunits are secreted from cells unless associated with β subunits (4, 5). Meprin A (EC 3.4.24.18) is defined as those isoforms that contain the α subunit; this includes membrane-bound α/β hetero-oligomeric forms and a secreted α homo-oligomeric form (1, 6). Meprins are tissue-specific proteases that are implicated in developmental processes as well as in normal and pathological processes in adult tissues (1, 7, 8). They are secreted from or localized in mammalian brush-border membranes of the intestine and kidney epithelial cells (1, 9, 10). Secreted meprin A is found in mouse urine and in the lumen of the colon (6, 8). Meprins are capable of degrading proteins such as collagen and gelatin; hormones such as parathyroid hormone, luteinizing hormone-releasing hormone, and melanocyte-stimulating hormone; and small peptides such as bradykinin, angiotensins, and gastrin (3, 11). The meprins may therefore be involved in activation or inactivation of important extracellular proteins and peptides and are highly regulated at transcriptional and post-transcriptional levels themselves.

The deduced amino acid sequences of meprin α subunits from mouse, rat, and human cDNAs have been determined (12). The subunit encodes an amino-terminal signal sequence (see Fig. 1, S), a protease (astacin-like) domain, a MAM (meprin, A5 protein, protein-tyrosine phosphatase) domain, a MATH (meprin and tumor necrosis factor receptor-associated factor homology) domain, an AM (after MATH) domain, an I (inserted) domain, an epidermal growth factor (EGF)-like domain, a putative transmembrane-spanning domain (T), and a cytoplasmic domain (C) (13). The mouse meprin α subunit is extensively glycosylated, containing ~25% carbohydrate (14). It contains 10 potential N-linked glycosylation sites (15). The functions of the oligosaccharides of meprins, however, have not been investigated.

Appropriate glycosylation of a number of proteins is important for proper expression and function. For example, nonglycosylated rabies virus glycoprotein is not expressed at the cell surface, nor is it effective as a vaccine (16); N-glycosylation of GD3 synthase is necessary for folding, catalytic activity, and trafficking to the Golgi complex (17); and glycosylation of the human high affinity immunoglobulin E receptor is necessary for efficient folding in the endoplasmic reticulum (18).

The features of meprins that direct the enzymes to apical membranes have not been characterized; however, the final destination of these proteases is critical for normal tissue physiology and for pathological processes. Basolateral targeting of transmembrane proteins generally is mediated by discrete amino acid sequence motifs in the cytoplasmic domain (19). For apical targeting, some proteins, such as erythropoietin, require specific N-linked oligosaccharides (20); other proteins require juxtamembrane segments with clustered O-linked oligosaccharides or interactions of transmembrane domains or glycosyl-phatidylinositol anchors with the lipid bilayer (21–23).
Because meprin α does not contain O-linked oligosaccharides, and the transmembrane and cytoplasmic domains are removed in the endoplasmic reticulum (ER) during biosynthesis, those features cannot be factors; however, N-linked oligosaccharides are possible targeting moieties.

Madin-Darby canine kidney (MDCK) cells, a well-characterized cell line for study of biogenesis of epithelial cell polarity (e.g. Refs. 20–23), were used in this study to determine whether N-linked oligosaccharides are required for the apical targeting of meprin α or whether they affect secretion, folding, and activity of the meprin α subunit. Mutational analysis, in which potential glycosylation sites were eliminated (see Fig. 1), and inhibitors of biosynthesis and processing of N-linked oligosaccharides were employed for this study.

EXPERIMENTAL PROCEDURES

Plasmid Construction and Mutagenesis—The pcDNAI/Amp plasmid (Invitrogen) containing full-length wild-type mouse meprin α subunit cDNA, the α(1–528) COOH-terminal truncation mutant, and the MATH deletion (ΔMATH) were described previously (see Fig. 1) (13). These wild-type and mutant cDNAs in pcDNAI/Amp were subcloned into vector pcDNA3.I(+) (Invitrogen) at an EcoRI site to generate expression plasmids. The COOH-terminal truncation mutant α(1–445) was generated by the polymerase chain reaction using a mutagenic antisense primer (5′-CCGCTCGAGTCATGCAGGGCAGGGGGTTTCT-3′) that changed the codon of the wild-type transcript at Gly 446 to a stop codon. Pfu DNA polymerase (Promega) was used for polymerase chain reactions to minimize base misincorporations. Site-directed mutagenesis was done using the Transformer site-directed mutagenesis kit (CLONTECH). For each mutant, the Asn residue in the Asn-terminus (N-X-Ser/Thr) sequence was changed to Gln using mutagenic primers. The selection primer was designed to change a Thr sequence was changed to Gln using mutagenic primers. The selection primer was designed to change a XhoI site for site-directed mutagenesis. All constructs were verified as correct by DNA sequence analyses.

Tissue Culture and Transfection—MDCK-II cells (ATCC 34 CCL) and human embryonic kidney 293 cells (ATCC 1573 CRL) were maintained in Dulbecco’s modified Eagle’s medium (DMEM) (Life Technologies, Inc.) supplemented with 10% fetal calf serum, 50 units/ml penicillin, and 50 μg/ml streptomycin (complete DMEM) in a 37°C incubator with 5% CO₂.

The pcDNA3.I(+) expression plasmids containing wild-type or mutant meprin α cDNA (Fig. 1) were transfected into MDCK cells by the calcium phosphate precipitation method; 10 μg of plasmids were added to cells grown on 100-mm tissue culture plates (Falcon). Twenty-four h after transfection, the cells were treated with trypsin and diluted 1:20 in 0.6 mg/ml Geneticin (G418, Life Technologies, Inc.) to obtain stable transfectants.

For transient expression of recombinant wild-type and mutant meprin α, the expression plasmids were transfected into human embryonic kidney 293 cells by the calcium phosphate precipitation method using 10 μg of expression plasmid and 1 μg of helper plasmid pVA1 per 100-mm tissue culture plate (24). Cells were grown to ~90% confluency by overnight incubation in complete DMEM. The medium was replaced with serum-free Opti-MEM I (Life Technologies, Inc.) and maintained in the incubator for an additional 48 h.

Preparation of Media and Cell Lysates—The tissue culture media of transfected 293 cells were collected after 48 h of transfection and subjected to centrifugation at 16,000 × g for 20 min. The supernatant fractions were concentrated 10-fold to 500 μl per plate using Centricon-30 and Microcon-30 concentrators (Amicon, Inc.). The cells were washed twice with phosphate-buffered saline, removed from the plates with a rubber scraper, and subjected to centrifugation at 300 × g for 5 min. The sedimented cells were suspended in phosphate-buffered saline, containing 0.1% Triton X-100, sonicated for 1 min at 4°C, and subjected to centrifugation at 100,000 × g for 1 h; the supernatant fraction is referred to as the cell lysate.

Analysis of Polarity of Meprin α Secretion—Stably transfected MDCK cell lines were maintained in complete DMEM containing 0.6 mg/ml Geneticin. To detect polarity of secretion of recombinant wild-type and mutant meprin α, the cells were grown on Millicell-PCF 0.4-μm culture inserts (Millipore Corp.) on six-well plates until a monolayer was formed (3–6 days). Then media were replaced with fresh serum-free Opti-MEM I. After an additional 48 h of incubation, media and cells were collected and prepared as described above.

SDS-PAGE and Immunoblotting—The collected media and cell lysates were subjected to electrophoresis using 7.5% SDS-polyacrylamide gels (25). Immunoblotting was performed as described previously (26). The proteins were probed with anti-mouse meprin α polyclonal antibody produced in rabbits. Meprin subunits were detected using the enhanced chemiluminescence method (Pierce).

Pulse-Chase Experiments—The transfected cells were preincubated for 1 h at 37°C in DMEM lacking methionine and supplemented with 10% dialyzed fetal bovine serum. The cells were pulse-labeled for 30–60 min with [35S]methionine/cysteine (100 μCi/ml/dish; ICN Pharmaceuticals) and then incubated in fresh serum-free Opti-MEM I. At the indicated times, the media were separated from the cells; washed twice with phosphate-buffered saline; lysed in phenol; and concentrated 20-fold using 1% Triton X-100, 0.5% sodium deoxycholate, 0.02% sodium azide, and proteinase inhibitors (100 μg/ml each antipain, chymotrypsin, leupeptin, pepstatin, and phenylmethylsulfonyl fluoride); and subsequently sonicated for 1 min. The media were subjected to centrifugation at 6500 × g for 10 min.

Immunoprecipitation of Labeled Meprins—The cells and media were
mixed with 40 μl of Pansorbin (Calbiochem) for 1 h at 4°C to prevent nonspecific binding of protein A beads and then centrifuged at 6500 × g for 30 min. The supernatant fractions were incubated with 15 μl of anti-mouse meprin α antibody at 37°C for 10 min and then stored at 4°C for 16 h. The immunocomplexes were mixed with 40 μl of protein A-Sepharose beads (50% gel suspension; Sigma) for 3 h at 4°C with gentle agitation. The beads were washed three times with 0.1% Triton X-100, 200 mM EDTA, and 10 mM Tris-HCl (pH 7.5); washed another three times with the same buffer containing 1 mM NaCl and 0.1% sodium laurel sarcosinate; and then washed twice with 5 mM Trit-HCl (pH 7.0). The sedimented beads were boiled for 5 min at 100°C with 50 μl of 0.1% SDS containing 0.5 mM EDTA, 5 μl sucrose, and 5 mM Tris-HCl (pH 8.0) with 2-mercaptoethanol.

Endoglycosidase Digestion—For total deglycosylation, the immunoprecipitated or secreted proteins were denatured by boiling for 5 min at 100°C with 5 mM Tris-HCl (pH 8.0) containing 0.2% SDS. The samples were adjusted to a final concentration of 50 mM sodium acetate buffer (pH 6.0) containing 0.75% Triton X-100 and 100 μg/ml protease inhibitors (apain, chymostatin, leupeptin, pepstatin, and phenylmethylsulfonyl fluoride). The mixtures were incubated with 10 milliliters of endoglycosidase H (Endo-H) or 1.0 unit of endoglycosidase F/VN-glycosidase F (Endo-F) (Roche Molecular Biochemicals) and incubated at 37°C for 18 h. Reactions were stopped by boiling the samples in SDS-PAGE sample buffer. To investigate the effects of deglycosylation on enzymatic activity, samples containing meprin were incubated with Endo-F as described above, except that no boiling or detergents were included in the protocol.

Trypsin Treatment—Trypsin (Sigma) was added to the collected media at a final concentration of 1 μg/ml in 20 mM Tris-HCl (pH 7.5) to activate meprin subunits. After incubation at 25°C for 30 min, soybean trypsin inhibitor (type II-S, Sigma) was added at a 2-fold excess over trypsin. Samples were incubated at 25°C for 15 min and then kept at 4°C until analysis.

Activity Determinations—A fluorogenic bradykinin analog (BK-4), 2-amino-2-phenylbenzoyl-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg-Lys(dinitrophenyl)-Gly-OH (obtained from Dr. Graham Knight, Strengeways Research Laboratory, Cambridge, United Kingdom), was used as a substrate (26). For specific activity calculations, the amount of meprin protein in each sample was determined by immunoblotting and densitometry. All point mutations of activity, at least two independent sample preparations were used, and values are reported as averages of triplicate assays.

Effects of Tunicamycin, Castanospermine, Swainsonine, Brefeldin A (BFA), and Colchicine—MDCK cells stably expressing wild-type mouse meprin α were grown on 100-mm tissue culture plates until a monolayer was formed. Cells were preincubated with or without 2 μg/ml tunicamycin (Roche Molecular Biochemicals) for 18 h and then subjected to pulse-chase experiments. Tunicamycin (2 μg/ml) was maintained in all media during the pulse-chase experiments.

To examine the effects of other compounds, MDCK cells stably expressing wild-type mouse meprin α were grown on Millicell-PCF culture inserts on six-well plates until a tight monolayer was formed as determined by microscopic examination. Then cells were preincubated with or without 10 μg/ml BFA (Sigma) or 12 μg/ml colchicine (Calbiochem) for 2 h or with or without 100 μg/ml castanospermine (Roche Molecular Biochemicals) or 5 μg/ml swainsonine (Calbiochem) for 18 h in complete DMEM. After preincubation, the media were replaced with Opti-MEM 1 with or without each compound at the same concentration and incubated for 24 h. The cells and media were collected, prepared as described above, and subjected to immunoblot analyses.

RESULTS

Wild-type Meprin A Is Secreted into the Apical Medium—MDCK cells stably transfected with mouse meprin α subunit cDNA were cultured on polycarbonate filters, and apical and basolateral media were analyzed for meprin protein after 24 h (Fig. 2). Wild-type meprin α was exclusively secreted into the apical medium with a molecular mass of ~95 kDa (Fig. 2). When MDCK cells were perturbed by treatment with compounds such as BFA, which impairs vesicular transport, or colchicine, which depolymerizes microtubules, apical sorting of meprin was markedly affected (Fig. 2). In the presence of BFA, 52% of the secreted meprin was found in the apical medium; and in the presence of colchicine, 66% was targeted to the apical medium. There was no indication that these compounds caused cell death or general toxicity from the appearance of cells, and the cystosine enzyme lactate dehydrogenase was not detectable in the apical or basolateral medium (data not shown). These results indicate that meprin A is normally secreted into the apical medium by a mechanism that requires an intact microtubule network and vesicular transport system.

Point Mutations of Asn in Potential N-Linked Oligosaccharide Sites: Effects on Enzymatic Activity and Thermal Stability, but Not on Apical Sorting—The mouse meprin α subunit contains 10 potential N-linked oligosaccharide chain sites: Asn4, in the prosequence; Asn152, Asn174, and Asn175 in the protease domain; Asn205 and Asn238 in the MAM domain; Asn252 in the AM domain; and Asn346, Asn535, and Asn514 in the AM domain (Fig. 1). A series of mouse meprin α mutants were constructed in which GlcNAc was substituted for Asn at the 10 potential N-glycosylation sites. Each of the mutants was stably transfected into MDCK cells, and apical and basolateral media were analyzed by immunoblotting and densitometry. All point mutants were predominantly secreted into the apical medium like the wild-type meprin (Fig. 3). In a few instances, a small amount of meprin α was detectable in the basolateral medium; however, it was never found to be >10% of the total secreted meprin. These results indicate that individual N-linked oligosaccharide chains are not critical determinants of apical targeting.

The enzymatic activities of the secreted mutant proteins against BK-4 varied over a range of 0.9 ± 0.1 to 5.9 ± 0.8 μmol/min/mg, with the wild-type activity at 3.2 ± 0.4 (Table 1). Only one mutant had a higher specific activity and Kcat/Km than the wild-type enzyme, N452Q in the MATH domain. Most of the other mutants had lower activities (28–75% of the wild-type enzyme), except for N553Q, a mutation in the AM domain,
The antibodies or point mutants of MDCK cells were stably transfected with cDNA of the wild-type protein noted in susceptibility of the mutants to degradation by higher concentrations of trypsin (up to 100 μg/ml) as determined by densitometry (data not shown). These results indicate that although the individual N-linked oligosaccharides are not essential for activity, Asn$^{11}$, Asn$^{553}$, Asn$^{546}$, Asn$^{614}$ (in the prosequence, protease, MAM, and AM domains) of the meprin α subunit contribute to optimal enzymatic activity. Partial deglycosylation of secreted wild-type meprin A under non-denaturing conditions also resulted in loss of enzymatic activity against BK$^+$ (Fig. 4). In the first four lanes of Fig. 4, meprin samples were activated by trypsin before denaturation and Endo-F treatment. In the last four lanes, the samples were not treated with trypsin before endoglycosidase treatment or gel electrophoresis; they were, however, incubated with trypsin after Endo-F treatment to measure enzymatic activity. Thus, the proenzyme and active enzyme were both exposed to Endo-F. Endo-F completely removed N-linked oligosaccharides after denaturation of meprin subunits, but only partially deglycosylated the subunits under non-denaturing conditions. The proenzyme lost 75% activity after Endo-F treatment, whereas the activated enzyme treated with Endo-F had no detectable activity. Thus, partial deglycosylation had marked effects on enzymatic activity.

Differences in vulnerability to heat inactivation were observed between the wild-type protein and some meprin α potential N-glycosylation site mutants (Fig. 5). Wild-type meprin A retained 55% activity against BK$^+$ after incubation at 55 °C for 20 min. Similar results were obtained for six of the Asn mutants: N41Q, N330Q, N426Q, N452Q, N546Q, and N614Q. However, mutants N234Q, N270Q, and N614Q were 90% inactive after 20 min of incubation at 55 °C. The N152Q mutant was the most unstable mutant and lost 94% activity in 5 min at 55 °C. These results indicate that the mutations at Asn$^{152}$, Asn$^{546}$, and Asn$^{614}$ in the protease domain and at Asn$^{614}$ in the AM domain decrease thermal stability of the protein.

**Effects of N-Glycosylation Inhibitors on Biosynthesis, Sorting, and Secretion of Meprin A—**To examine the role of N-linked oligosaccharides of meprin α in biosynthesis, pulse-chase experiments with or without tunicamycin were performed (Fig. 6). Tunicamycin inhibits the first step of the N-glycosylation pathway, i.e., the transfer of GlcNAc-1-P from UDP-GlcNAc to dolichol-P to form dolichyl-P$^+$-GlcNAc (27). The wild-type meprin α subunit was synthesized as an ~100-kDa protein in MDCK cells, processed to a 95-kDa protein, and then gradually secreted into the medium. When treated with tunicamycin, the meprin α proteins associated with cells had a molecular mass of ~75 kDa; however, the expression level was

| Specific activity | $k_{cat}/K_m$ | $\mu\text{mol} \text{min}^{-1} \text{mg}^{-1}$ | $s^{-1}$ |
|-------------------|--------------|---------------------------------|---------|
| Wild-type         | 3.2 ± 0.4    | 6.5 × 10^3                      |         |
| N41Q             | 1.4 ± 0.2    | 2.8 × 10^4                      |         |
| N152Q            | 0.9 ± 0.1    | 1.7 × 10^4                      |         |
| N234Q            | 2.4 ± 0.3    | 4.9 × 10^4                      |         |
| N270Q            | 2.0 ± 0.2    | 4.1 × 10^4                      |         |
| N330Q            | 2.1 ± 0.2    | 4.3 × 10^4                      |         |
| N426Q            | 1.4 ± 0.3    | 2.8 × 10^4                      |         |
| N452Q            | 5.9 ± 0.8    | 1.2 × 10^5                      |         |
| N546Q            | 2.2 ± 0.3    | 4.5 × 10^4                      |         |
| N614Q            | 3.3 ± 0.4    | 6.7 × 10^4                      |         |
| ND               | 1.6 ± 0.2    | 3.3 × 10^4                      |         |

$^a p < 0.05$ relative to the wild-type protein (by Student's $t$ test).

where the activity was the same as that of the wild-type enzyme. The decreased activities were not due to degradation of the subunit during trypsin activation, and no differences were noted in susceptibility of the mutants to degradation by higher concentrations of trypsin digestion as described under “Experimental Procedures.” Samples of media from both sides were subjected to SDS-PAGE, followed by immunoblotting using anti-mouse meprin α polyclonal antibodies.
markedly decreased, and the protein was not detected in the medium. Tunicamycin markedly decreased the biosynthesis and secretion of meprin α into the medium.

Castanospermine, an inhibitor of glucosidases I and II in the Golgi complex, had little if any effect on sorting and secretion of meprin α in MDCK cells (Fig. 7). In the presence of castanospermine, the wild-type meprin α subunit was exclusively secreted into the apical medium. Upon treatment with swainsonine, 88% of the protein was secreted into the apical medium. The wild-type meprin α subunit was resistant to Endo-H, but sensitive to Endo-F, indicating that it was secreted as a protein containing complex-type oligosaccharide chains (Fig. 7B). The Endo-F treatment decreased the molecular mass of the wild-type protein from 95 to 75 kDa, as previously observed. When treated with O-glycosidase and neuraminidase, no bands with N-Glycosylation of Meprin α 25581

FIG. 5. Thermal stability of the wild-type protein and transcripts mutated at potential N-linked glycosylation sites. The secreted wild-type and mutant proteins were incubated at 25 °C for 30 min with 10 μg/ml trypsin for activation, followed by inactivation of trypsin with a 2-fold excess of soybean trypsin inhibitor. The activated meprin proteins were then incubated at 55 °C for 5–30 min, and samples were removed to measure activity. Activity was determined using 24 μM fluorogenic BK-P. The dashed line indicates wild-type meprin α and Asn mutants N41Q, N330Q, N426Q, N452Q, N546Q, and N553Q. Control, CST, SW.

FIG. 6. Effect of tunicamycin on biosynthesis and secretion of wild-type meprin α. MDCK cells stably transfected with wild-type mouse meprin α cDNA were grown on 100-mm tissue culture plates until formation of a monolayer. The cells were incubated with (+) or without (−) 2 μg/ml tunicamycin for 18 h and then subjected to pulse-chase radiolabeling experiments. The cells were pulse-labeled with [35S]methionine/cysteine for 30 min and incubated in nonradiolabeled media for 2, 5, or 10 h in the continued absence or presence of tunicamycin. The cell lysates or media were immunoprecipitated with anti-mouse meprin α polyclonal antibodies. The immune complexes were analyzed by SDS-PAGE, followed by fluorography.

FIG. 7. Effect of castanospermine or swainsonine on the apical targeting of wild-type meprin α in MDCK cells. A, MDCK cells were stably transfected with cDNA for mouse wild-type meprin α and grown on Millipore-PCF culture inserts. The cells were incubated with or without 100 μg/ml castanospermine (CST) or 5 μg/ml swainsonine (SW) for 18 h in complete DMEM. The media were then replaced with Opti-MEM I with or without each compound at the same concentration and incubated for 24 h. The apical (Ap) and basolateral (Bl) media were prepared as described under “Experimental Procedures.” Samples were subjected to SDS-PAGE (7.5% gels with 2-mercaptoethanol), followed by immunoblotting using anti-mouse meprin α polyclonal antibodies. B, the apical media were incubated at 37 °C for 18 h with or without Endo-H (H) or Endo-F (F).

N-Linked Oligosaccharides Are Required for Secretion into the Medium—To further examine whether oligosaccharides are required for secretion and sorting of meprin subunits, a multiple-site mutant (α(1–445)/N*) in which all of the N-linked glycosylation sites were changed from Asn to Gln was constructed (see Fig. 1). It has been previously demonstrated that the α(1–528) and α(1–452) truncation mutants and ΔMAH deletion mutants (see Fig. 1) are secreted when they are expressed in human embryonic kidney 293 cells (13). To determine whether these mutants are sorted as the wild type to the apical membrane, they were stably transfected into MDCK cells, and the polarity of their secretion was examined. The α(1–528) and α(1–445) truncation mutants and ΔMAH deletion mutants were all secreted (>90%) into the apical medium (Fig. 3). When the truncation and multiple Asn mutants were transiently transfected into human embryonic kidney 293 cells, the α(1–445) mutant was primarily found in the medium; however, there was no detectable secretion of the multiple Asn mutant (data not shown).

To determine the fate of the multiple-site mutant lacking N-linked oligosaccharides, pulse-chase experiments were performed (Fig. 8). The radiolabeled α(1–445) mutant was observed in the cell and medium; the cell-associated protein had
a molecular mass of ~65 kDa and was secreted into the medium within 2 h (Fig. 8A). The multiple-site mutant lacking N-linked oligosaccharides was initially synthesized as a protein of ~50 kDa. However, the protein was predominantly retained in the cell (Fig. 8B). During a 10-h period of incubation with unlabeled amino acids, no secreted protein was detected in the medium. Deglycosylation of the multiple mutant with Endo-F did not change the molecular size, whereas α-(1–445) was shifted from 65 to 50 kDa (Fig. 8C). This confirmed that the multiple-site mutant had no N-linked oligosaccharides. These results indicate that N-linked oligosaccharides are required for secretion of the meprin α subunit into the medium.

![Image](https://example.com/image.png)

**Fig. 8.** Pulse-chase experiments with a truncation mutant with and without multiple potential N-glycosylation sites mutated. A and B, human embryonic kidney 293 cells transfected with α-(1–445), a truncation mutant of mouse meprin α, or the truncated transcript with multiple potential N-glycosylation sites mutated (α-(1–445)/N*), respectively, were pulse-labeled with [35S]methionine/cysteine for 30 min. The cell lysates or media were immunoprecipitated with anti-meprin α polyclonal antibody. The immune complexes were analyzed by SDS-PAGE, followed by fluorography. C, transfected cells were pulse-labeled with [35S]methionine/cysteine for 1 h. The cell lysates were immunoprecipitated with anti-meprin α polyclonal antibody. The immune complexes were incubated at 37°C for 18 h with or without Endo-F. The mixtures were analyzed by SDS-PAGE, followed by fluorography.

**DISCUSSION**

The results obtained with the multiple Asn mutant and the tunicamycin experiments herein demonstrate that some core glycosylation is required for secretion of meprin α subunits. This implies that carbohydrate residues are essential for the correct folding of the nascent protein. N-Glycosylation is required for secretion of several proteins (e.g., Ref. 28); however, there are proteins secreted by carbohydrate-independent mechanisms such as the intestinal brush-border enzyme aminopeptidase N (29). Thus, rather than the glycosylation itself being essential for secretion, it is more likely the effect of glycosylation on the structure of a specific protein that is determinative for movement through the secretory pathway. It is of interest that the meprin multiple Asn mutant is retained in the cell rather than being degraded in the ER. Previous work had demonstrated that meprin transcripts lacking the MAM domain were degraded in the ER by a proteasomal route, indicating that those transcripts were recognized as unfolded proteins and were targeted for transport into the cytosol (13). The lack of oligosaccharides, as in the α-(1–445)/N* mutant, did not result in proteasomal degradation, but rather ER retention, implicating carbohydrates as essential factors in retrograde transport into the cytosol as well as transport of meprin to the cell surface.

The results clearly indicate that no one specific N-linked oligosaccharide chain is required for secretion or apical sorting of the homo-oligomeric form of mouse meprin A. All 10 of the potential Asn glycosylation sites were mutated for this study because it is not known how many of the sites are glycosylated. However, previous work using lectin binding of meprin peptides established that at least three of the potential glycosylation sites are linked to high mannose or complex oligosaccharides in mouse kidney (15). One glycosylated site is Asn330 in the MAM domain; this site is linked to bisected biantennary complex oligosaccharides. The other identified glycosylated sites are in the AM domain (Asn546, Asn553, and Asn614); at least two of these sites contain high mannose and/or complex biantennary oligosaccharides. Mutation of those four Asn residues and consequently the elimination of their oligosaccharide chains, however, had no effect on sorting. The potential glycosylation sites in the MAM and AM domains are not highly conserved sites. Only Asn553 is conserved in both rat and mouse meprin α, and the N553Q mutation did not affect sorting, enzymatic activity, or resistance of the subunit to thermal inactivation. In addition, mutants that are truncated before the AM and MATH domains are secreted apically by MDCK cells (as shown in Fig. 3), further confirming that glycosylation and other factors in these domains do not play an essential role in targeting the protein to the apical membrane.

Apical sorting of meprin A does require intact tubules and vesicles, as demonstrated by treatment of cells with BFA and colchicine. The secreted form of meprin in the presence of BFA migrates slightly faster than under control conditions, indicating some alteration in glycosylation. The complex-type glycosylation that occurs in the Golgi apparatus, observed for meprin secreted from kidney and intestine and for recombinant meprin secreted from 293 or MDCK cells, is not essential for targeting or correct folding of the protein. This conclusion is based on the results of the experiments in which meprin was synthesized and secreted in the presence of castanospermine and swainsonine. These inhibitors prevent the removal of glucose and mannose residues from the core N-linked glycosylated sites, thereby preventing complex glycosylation. These inhibitors will also decrease association of newly synthesized glycoproteins with lectin-like chaperones such as calnexin and calreticulin (30). However, calnexin has been shown to interact
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with unfolded proteins lacking N-glycans and thus may be an important chaperone in the folding of various forms of the meprin molecule (31).

The picture that emerges from this and previous (13, 24, 26) work is that core glycosylation, the MAM domain, and COOH-terminal proteolytic processing (which removes the I, epidermal growth factor, transmembrane, and cytoplasmic domains and releases meprin α from the membrane) are all required for movement of the meprin α protein from the ER into the secretory pathway. In the absence of any one of these factors, the meprin protein is retained in the ER or transported back to the cytosol for proteasomal degradation. The data also indicate that intersubunit disulfide bridging, oligomerization, complex glycosylation, specific-site glycosylation, and enzymatically active or activatable forms of meprin are not essential for secretion.

The three protease domain Asn mutants and the N614Q mutant in the AM domain were more vulnerable to heat inactivation than the wild-type protein or other single-site mutants. For the protease domain mutants, it is likely that instability is due to the Asn-to-Gln mutation rather than effects on glycosylation, as there is no indication that astacin, meprins, or other members of the astacin family contain oligosaccharides in the catalytic domain. In addition, none of the three Asn residues are invariant in the astacin family proteins. The meprin protease domain model, based on the crystallographic structure of astacin, allows one to predict the consequences of the three mutations made within the protease domain (Fig. 9). The mutated Asn residue closest to the active site is Asn152, −8 Å from the active-site Glu residue, in a β-sheet strand. N152Q was the most unstable mutant, indicating that the additional methyl group of this mutant is sufficient to perturb the structure of the protein. Asn234 is −9 Å from the active-site Tyr residue, beneath the proposed S1′ subsite. It is possible that N152Q and N234Q alter structure by disrupting zinc coordination at the active site. Asn270 is predicted to be on the opposite face of the active site, and mutation of this residue also affects the rate of thermal inactivation. This observation is consistent with those obtained previously showing that structural perturbations removed from the active site decrease the stability and enzymatic activity of meprin (34). The enhanced thermal inactivation of the AM domain N614Q mutant is of interest in that this residue is very near the COOH terminus of the secreted mature meprin α subunit, Arg615 for meprin A from mouse kidney (14). Alterations in Asn614 glycosylation or substitution of a Gln residue at this site may affect COOH-terminal proteolytic processing and, consequently, the mature enzyme structure.

Many of the mutants with single Asn mutations had altered enzymatic activity against bradykinin. For the protease domain mutants, the most marked decrease in activity was observed for the N152Q mutant. This mutation is near the proposed S1 subsite that would bind the Phe residue of BK. The addition of a methyl group may disrupt the S1 site enough to affect binding of Phe, thus lowering the catalytic activity. The other protease domain mutations would not be predicted to directly interfere with substrate binding, but may indirectly affect the active-site structure. The N41Q mutation in the prosequence domain also decreased enzymatic activity, indicating that alterations of the prosequence affect folding of the enzyme or activation of the enzyme. The Asn mutations in the MAM and AM domains that decreased meprin activity illustrate that changes in the noncatalytic domains affect the catalytic domain. The N452Q mutation was the only one that increased the specific activity and the catalytic efficiency. This may be due to conformational changes that result in increased binding or turnover of substrate; however, we cannot rule out that the mutation changes the antibody recognition and consequently our estimate of the amount of protein used in experiments. In general, the mutational data and data on the deglycosylation of the mature enzyme by Endo-F indicate that oligosaccharides play an important role in the catalytic properties of meprin. This may result from conformational alterations of the active site or effects on the subunit or domain-domain relationships that alter the interaction of enzyme and substrate. The interdependence of the domains for correct folding, activity, and structure has been demonstrated previously with domain deletions and truncation mutants; these data indicate that oligosaccharides play an important part in determining how the noncatalytic portions of the enzyme affect the function of the enzyme.

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FIG. 9. Asn residues in potential glycosylation sites of the meprin α protease domain. An α-carbon trace of the meprin α protease domain is shown, where N (Asn78) and C (His187) represent the amino and carboxyl termini of the protease domain, respectively (32). Asn residues in potential glycosylation sites are shown as wire frames in pink; active-site residues (His167, Glu168, His171, His177, and Tyr226) are shown in green. Dots represent van der Waals radii. The catalytic zinc and water are shown in space fill and are colored brown and red, respectively. The diagram was produced using the program RasMol (33).
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