Enteropeptidase, a type II transmembrane protein of the enterocyte brush border, is sorted directly to the apical membrane of Madin-Darby canine kidney II cells. Apical targeting appears to be mediated by an N-terminal segment that contains a 27-amino acid residue O-glycosylated mucin-like domain consisting of two short mucin-like repeats, A and B. Targeting signals within these repeats were characterized by using green fluorescent protein (GFP) as a reporter. Constructs with a cleavable signal peptide and both repeats A and B were secreted apically. Similar constructs lacking mucin repeats were secreted randomly. Either repeat A or B was sufficient to direct apical targeting of GFP. O-linked oligosaccharides alone were not sufficient for targeting because fusion to a different O-glycosylated motif did not alter the random secretion of GFP, and several constructs with mutations in either repeat A or B were O-glycosylated and secreted randomly. In addition, repeat B appears to contain an apical targeting signal that functions in the absence of glycosylation. Density gradient centrifugation indicated that, unlike several other apically targeted membrane and soluble proteins, apical sorting of mucin-GFP chimeric proteins does not appear to utilize lipid rafts.

Enteropeptidase, a serine protease localized to the brush border of duodenal enterocytes (1), is targeted directly to the apical surface of Madin-Darby canine kidney II (MDCK) cells (2). Apical sorting of enteropeptidase may involve at least two distinct signals. One is located in the C-terminal serine protease domain and depends on N-glycosylation; another is proposed to be located in an N-terminal segment that includes an O-glycosylated mucin-like domain and three potential N-glycosylation sites (2). The apical targeting signals within the N-terminal region of enteropeptidase have not been characterized structurally.

Several distinct classes of apical sorting signals have been identified, suggesting the existence of several apical targeting mechanisms. For example, apical sorting can be mediated by transmembrane domains (3–5), by glycosylphosphatidylinositol anchors (6, 7), by PDZ-interacting domains (8, 9), or by N-linked oligosaccharides (10, 11). In some proteins, such as sucrase-isomaltase (12) and dipetidyl peptidase IV (13, 14), O-linked oligosaccharides also appear capable of mediating apical sorting. The juxtamembrane segment of the neurotrophin receptor p75 contains clustered O-linked oligosaccharides and is required for apical targeting (15, 16). The O-linked glycan-dependent apical sorting of sucrase-isomaltase also is accompanied by association with glycosphingolipid and cholesterol-rich membrane microdomains or lipid rafts (13, 14).

To characterize apical sorting determinants within the N-terminal segment of enteropeptidase, we employed a modified green fluorescent protein (GFP) as a reporter (18) in transfected MDCK cell lines expressing various chimeric enteropeptidase-GFP proteins. The results demonstrate that either of two short O-glycosylated mucin-like repeats of enteropeptidase can confer apical targeting on a heterologous protein. However, O-linked oligosaccharides are not sufficient for apical targeting, and some apical targeting activity is retained by a peptide that is not glycosylated. The apical targeting function also does not appear to involve stable interaction with lipid rafts.

**EXPERIMENTAL PROCEDURES**

**Construction of Deletion Mutants**—Plasmids pSMAB(His)6-GFP and pSHIS(g)GFP were derived from pHL-BEK, which encodes the human prothrombin signal peptide and His6 tag from pHIL-BEK (19) linked to the codon for the Ala166 of plasmid pBEK (2) (8). A fragment (SMAB) encoding the human prothrombin signal peptide (S) plus His6 tag linked to two mucin-like repeats (SMAB) (amino acids 166–192) of bovine enteropeptidase (20) was made by PCR (GeneAmp reagents, PerkinElmer Life Sciences) with N-terminal primer (5’-gcagcttgcagtagtgtagcatg-3’ (BamHI site underlined)) and C-terminal primer (5’-cgagcttgcagtagtgtag-3’ (BamHI site underlined)). Fragment SHIS-encoded the prothrombin signal peptide and His6 tag without mucin-like repeats was generated by PCR with the same N-terminal primer and a different C-terminal primer (5’-gcagcttgcagtagtgtag-3’ (BamHI site underlined). The PCR fragments SMAB(His)6 and SHIS(g)GFP were digested with XhoI and BamHI and ligated into the same sites of vector pEGFPN1 (GenBank™ accession number U55792, CLONTECH Laboratories, Palo Alto, CA) to generate pSMAB(His)6-GFP and pSHIS(g)GFP, respectively. A fragment encoding the prothrombin signal peptide (S) was generated from template pSHIS(g)GFP with primers 5’-gcagcttgcagtagtgtagcag-3’ (XhoI site underlined) and 5’-ca-
overnight. After washing, the membrane was incubated with peroxi-
dents. After 24 h, medium was collected from the apical and basolateral 
(oPTI-MEM, Invitrogen) added to both apical and basolateral compart-
ents. After 5 h, fetal bovine serum was added to
modified Eagle's medium. After 5 h, fetal bovine serum was added to
viable colonies were identified by fluorescence microscopy and Western blotting
with anti-GFP monoclonal antibody (MMS-116P, Berkeley Antibody Co.,
results. 

**Transferase—MDCK II cells** (American Type Culture Collection)
were cultured in Dulbecco's modified Eagle's medium supplemented
with 10% fetal bovine serum (Invitrogen) as described (2). Cells growing
in 6-well tissue culture plates were washed with phosphate-buffered
saline and incubated with 5 μl of phosphatidic acids premixed (1.6 w/v)
with 30 μl of PerFect lipid (pfx-2, Invitrogen) in serum-free Dulbecco’s
modified Eagle’s medium. After 5 h, fetal bovine serum was added to
10%. After an additional 18 h, cultures were split 1:50 and cultured in

**Glycosidases** were obtained from Oxford
Arthrobacter ureafaciens

Enzymatic removal of O-linked oligosaccharides reduced the apparent mass of each protein to 34 and 36 kDa (Fig. 2B, lanes 6 and 8), and these values are similar to the calculated masses of 34.5 and 36.6 kDa, respectively, for the polypeptides alone.

The specificity of these glycosidases suggests that the oligosac-
charide structures consist almost exclusively of monosialylated or disialylated structures, which are related to Sia2–3Galβ1–3
Sia2–6GalNAC-O-Ser/Thr (22). Although mucin repeat A contains a potential N-glycosylation site (Asn-Phe-Ser), further digestion with N-glycanase or endoglycosidase H did not affect the mass of $\text{M}_{\text{Ab}}$-GFP and His$_{8}$-$\text{M}_{\text{Ab}}$-GFP, indicating that this site is not utilized (data not shown). As expected, GFP (27.5 kDa) was not glycosylated (Fig. 2B, lanes 1 and 2). However, His$_{8}$-gGFP was O-glycosylated, presumably on one or more of the Ser/Thr residues flanking the His$_{8}$ tag sequence. The removal of O-linked oligosaccharides reduced the apparent mass from 33–37 to 50 kDa (Fig. 2B, lanes 3 and 4), which is similar to the calculated mass of 29.6 kDa for the polypeptide alone. Despite this O-glycosylation, His$_{8}$-gGFP was secreted randomly (Table 1).

The number of mucin repeats in enteropetidease varies considerably, with one repeat in human, two in bovine and porcine, three in rat, and four in mouse enteropetidease (21). Such heterogeneity suggests that targeting signals could reside in single mucin repeats. Therefore, the behavior of chimeric prote-
ns containing mucin repeat A or B was examined. When stably expressed in MDCK cells, constructs containing either mucin repeat were secreted apically (Fig. 3A and Table 1), indicating that both repeats possess functional targeting sig-
al. Glycosidase digestions demonstrated that each mucin repeat was O-glycosylated (Fig. 3B) and not N-glycosylated (data not shown).

**RESULTS**

**Targeting Activity of the Enteropetidease Mucin Domain—**
Previous studies suggested that apical sorting of enteropetide-
ase in MDCK cells depends on a signal near the N terminus, between amino acid residues 50–197 (2). This juxta membrane
region contains four potential N-glycosylation sites and a mucin domain consisting of two tandem O-glycosylated Ser/Thr-
rich repeats (Fig. 1A) (20, 21). Potential targeting signals within these mucin repeats were characterized by using green fluorescent protein (GFP) as a reporter (Fig. 1B) (18). Control constructs (GFP and His$_{8}$-gGFP) were secreted randomly (Fig.

**DISCUSSION**

Glycosidase digestions—Glycosidases were obtained from Oxford
Glycosciences (Bedford, MA). Samples of concentrated conditioned medium (40 μl) were digested with Arthrobacter ureafaciens neuraminidase (0.5 million/microliter) at 37 °C for 1 h in 100 mM sodium acetate, pH 5.0, followed by digestion with O-glycanase (0.05 million/microliter) in 100 mM sodium citrate phosphate, pH 6.0, and 100 μg/ml bovine serum albumin at 37 °C for 16 h. Digested samples were analyzed by SDS-PAGE and Western blotting with monoclonal anti-GFP IgG as described above.

**Analysis of Raft Association—**Detergent-insoluble glycosphingolipid-

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**GFP** (27.5 kDa) was not glycosylated (Fig. 2B, lanes 1 and 2). However, His$_{8}$-gGFP was O-glycosylated, presumably on one or more of the Ser/Thr residues flanking the His$_{8}$ tag sequence. The removal of O-linked oligosaccharides reduced the apparent mass from 33–37 to 50 kDa (Fig. 2B, lanes 3 and 4), which is similar to the calculated mass of 29.6 kDa for the polypeptide alone. Despite this O-glycosylation, His$_{8}$-gGFP was secreted randomly (Table 1).

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**Analysis of Raft Association—**Detergent-insoluble glycosphingolipid-
edified raft domains were prepared by cell lysis in ice-cold Triton
X-100 (for 10 min without agitation) and then sucrose density gradient centrifugation as described previously (2). Proteins in the fractions were precipitated with 10% trichloroacetic acid on ice for 1 h, and pellets were resuspended in 200 μl of 0.2 N NaOH. Samples (20 μl) of trichloroacetic acid-concentrated fractions were analyzed by SDS-
PAGE and immunoblotting with either monoclonal anti-GFP IgG (1: 10,000) or monoclonal anti-caveolin IgG (1:10,000; C37120, Transduc-
sion Laboratories, Lexington, KY) as described above.
Glycosylation and Targeting—The enteropeptidase mucin repeats contain 11 Ser/Thr residues that could be O-glycosylated (Fig. 1A) (20). Replacement of all Ser/Thr residues by alanine abolished the targeting activity of mucin repeat A, whether present in one or three copies (Fig. 4 and Table I). Mucin repeat B had reduced but significant apical targeting activity after all Ser/Thr residues were mutated, suggesting that features of the amino acid sequence may contribute to targeting (Fig. 4 and Table I). Glycosidase digestions confirmed that none of these constructs had N-linked or O-linked oligosaccharides (data not shown).

The role of O-linked glycosylation was investigated further by restoring selected Ser/Thr residues, singly or in clusters. For mucin repeat A, all constructs that contained any Ser/Thr residues were O-glycosylated and constructs MAS172A/T173A/S175A-GFP and M AT173A-GFP lost apical targeting activity (Fig. 5 and Table I). These data suggest that O-glycosylation is not sufficient for apical targeting and that a central cluster of Ser/Thr residues may be important for apical targeting of M A-GFP. As observed for mucin repeat A, all mucin repeat B constructs that contained Ser/Thr residues were O-glycosylated, including MB-T184A/T185A/S186A-GFP, which has only one Thr residue (Fig. 6). Mutation of Thr-185 alone randomized the targeting of MB-T185A-GFP, whereas all other mutants tested retained apical targeting similar to that of MB-GFP (Fig. 6 and Table I). Therefore, O-glycans appear not to be required, but the modification of selected Ser/Thr residues can inhibit or potentiate the apical targeting of M B-GFP.

MAB-GFP Does Not Associate with Lipid Rafts—Sphingolipid and cholesterol-rich membrane microdomains, or lipid rafts, have been proposed to participate in the delivery of many apically sorted proteins (23), but enteropeptidase appears to be an exception. Bovine enteropeptidase, a type II transmembrane protein, was targeted to the apical membrane of transfected MDCK cells, but association with lipid rafts could not be demonstrated (2). Similar results were obtained for secreted MAB-GFP (Fig. 7), which contains a subset of the apical targeting signals found in full-length enteropeptidase. Triton X-100 lysates were prepared from MDCK cells expressing GFP and MAB-GFP, and the extracts were fractionated by sucrose gradient centrifugation (2). Caveolin, a marker for lipid rafts, was recovered in low-density fractions 4–5 near the top of the gradient (Fig. 7). In contrast, GFP (Fig. 7A) and MAB-GFP (Fig. 7B) were recovered in denser fractions at the bottom of the
Therefore, MAB-GFP is secreted apically but does not appear to associate intracellularly with lipid rafts.

**DISCUSSION**

The results presented here show that small mucin-like peptides of 12–15 amino acid residues, derived from bovine enteropeptidase, can direct the apical secretion of GFP. O-glycosylation contributes to the efficiency of apical targeting, but no specific O-glycan appears to be required. For mucin repeat B, some apical targeting activity persists after all glycosylation sites are eliminated by mutagenesis (Table I). Several O-glycosylated peptides have no targeting activity in this system, indicating that glycosylation alone is not sufficient. Some apical membrane and secreted proteins associate with lipid rafts, but this is not the case for transmembrane or soluble variants of enteropeptidase (2) Apically secreted mucin-GFP chimeric proteins also did not associate with rafts (Fig. 7). Therefore, enteropeptidase mucin-like domains contain relatively compact apical targeting signals that depend on both O-glycosylation and amino acid sequence context but appear to function independent of lipid rafts. This appears to be the first example of an O-glycosylated peptide that can confer apical targeting on a randomly sorted heterologous protein.

Previous studies have implicated O-glycans in the apical targeting of several proteins, although the evidence is indirect.

**TABLE I**

| Inserted amino acid sequence | Construct name          | Apical secretion |
|------------------------------|-------------------------|------------------|
| (none)                      | GFP                     | 53 ± 2           |
| FTASVMGSSHHHHHSSR           | (His)_{6}GFP            | 57 ± 1           |
| ASLENPSIPTATTS              | M_{AP}-GFP              | 90 ± 3           |
| ASLENPSIPTATTS              | M_{AP}-GFP              | 81 ± 6           |
| ASLENPSIPTATTS              | M_{AMP}-GFP             | 49 ± 3           |
| ASLENPSIPTATTS              | M_{AM}-GFP              | 52 ± 3           |
| ASLENPSIPTATTS              | M_{S167A}-GFP           | 75 ± 10          |
| ASLENPSIPTATTS              | M_{T173A}-GFP           | 55 ± 2           |
| ASLENPSIPTATTS              | M_{S172A/T173A/S175A}-GFP | 62 ± 5         |
| ASLENPSIPTATTS              | M_{T175A/T179A/S180A}-GFP | 96 ± 1.3      |
| ASLENPSIPTATTS              | M_{AP}-GFP              | 81 ± 10          |
| ASLENPSIPTATTS              | M_{AM}-GFP              | 68 ± 3           |
| ASLENPSIPTATTS              | M_{T185A}-GFP           | 52 ± 4           |
| ASLENPSIPTATTS              | M_{T184A/T185A/S186A}-GFP | 76 ± 12       |
| ASLENPSIPTATTS              | M_{T181A}-GFP           | 81 ± 13          |

**FIG. 3.** Targeting activity of individual mucin repeats. The indicated proteins were analyzed for the polarity of protein secretion (left) and sensitivity to digestion with neuraminidase and O-glycanase (right), as described under Fig. 2.

**FIG. 4.** Effect of mutating all O-glycosylation sites. Codons for all Ser/Thr residues were changed to Ala in mucin repeat A (M_{AMall}) or mucin repeat B (M_{BMall}), and the corresponding sequences were inserted between a prothrombin signal peptide and GFP. One construct contained three copies of the mutated M_{AMall} repeat (M_{AMallx3}). The polarity of protein secretion was assessed as described under Fig. 2.

**FIG. 5.** Mutation of selected Ser/Thr residues in mucin repeat A. The amino acid sequence inserted before GFP is given in Table I for the indicated constructs. The polarity of protein secretion (Panel A) and sensitivity to digestion with neuraminidase and O-glycanase (Panel B) were assessed as described under Fig. 2.
were assessed as described in the Fig. 2 legend. O-
sensor (16) are membrane proteins with their stalk domains abolishes apical targeting (12, 16). These
targeted to the apical surface of MDCK cells, and deletion of regions adjacent to their transmembrane domains. Both are
- glycosylated stalk regions near their transmem-
O-glycosylation mediates apical targeting,

**Fig. 6. Mutation of selected Ser/Thr residues in mucin repeat**

**Panel A** The amino acid sequence inserted before GFP is given in Table I for the indicated constructs. The polarity of protein secretion (Panel A) and sensitivity to digestion with neuraminidase and O-glycanase (Panel B) were assessed as described in the Fig. 2 legend.

**Panel B** The amino acid sequence inserted before GFP is given in Table I for the indicated constructs. The polarity of protein secretion (Panel A) and sensitivity to digestion with neuraminidase and O-glycanase (Panel B) were assessed as described in the Fig. 2 legend.

**Fig. 7. Relationship of chimeric M_{AB}-GFP to lipid rafts.** MDCK cells expressing GFP (Panel A) or M_{AB}-GFP (Panel B) were lysed with ice-cold 1% Triton X-100, and the lysate was subjected to sucrose density gradient centrifugation. Fractions were collected from the top (fraction 1) to the bottom (fraction 10) of each gradient and analyzed by Western blotting for GFP or caveolin as described under “Experimental Procedures.”

and mixed. Sucrase-isomaltase (12) and the neurotrophin receptor (16) are membrane proteins with O-glycosylated stalk regions adjacent to their transmembrane domains. Both are targeted to the apical surface of MDCK cells, and deletion of their stalk domains abolishes apical targeting (12, 16). These data suggest that O-glycosylation mediates apical targeting, but other studies suggest that sorting signals reside elsewhere. Replacement of the N-terminal transmembrane domain with a cleaved signal peptide causes the random secretion of sucrase-isomaltase despite the presence of the O-glycosylated stalk, suggesting that membrane association contributes to targeting and the stalk region is not sufficient (12). The replacement of Gln-117 by Arg, at a location near the N terminus of the isomaltase domain, causes random delivery to the apical and basolateral membranes of MDCK cells and suggests that features of sucrase-isomaltase distinct from its stalk region are necessary for targeting (24). Furthermore, O-glycosylation does not correlate with the apical targeting of aminopeptidase N and lactase-phlorizin hydrolase, which are apical membrane proteins with O-glycosylated stalk regions near their transmembrane domains. Deletion of these stalk regions does not affect their apical targeting in MDCK cells (25, 26).

These various studies indicate that O-glycosylation can contribute to apical targeting but is not sufficient and sometimes is not necessary. Enteropeptidase mucin-like domains exhibit similar properties, in that their apical targeting activity is diminished by mutagenesis of only certain O-glycosylated residues (e.g. Thr-173 or Thr-185, Table I). Such residues might bear oligosaccharides that are particularly potent targeting signals, possibly disialylated species. Clustered O-linked oligosaccharides might be required for targeting, and disruption of a central member of the cluster could be sufficient to impair targeting. Although the accumulating data do not exclude a direct targeting function for O-glycans, other models appear to be equally plausible. For example, O-glycans could play an indirect role, supporting a primary targeting signal that resides elsewhere in the protein (27). Alternatively, features of protein and oligosaccharide structure could collaborate to form a complete targeting signal that contains both carbohydrate and peptide determinants, in which case these structures could be spatially close together. Any of these models would be compatible with the observation that short O-glycosylated peptides can target GFP for apical secretion.

The association of membrane proteins with lipid rafts often correlates with their delivery to apical cell surfaces but, as noted for the proposed relationship between O-glycosylation and targeting, many exceptions are known. Sucrase-isomaltase, aminopeptidase N, aminopeptidase A, and dipeptidyl peptidase IV associate mainly with lipid rafts (12, 24, 28), whereas lactase-phlorizin hydrolase (29), maltase-glucosylnase (28), and (full-length) enteropeptidase (2) do not, yet all are apical membrane enzymes of the intestinal brush border. Similar variability has been reported for apically secreted proteins, natural or engineered. Thyroglobulin (30) and soluble prohormone convertase 2 (31) have been recovered bound to lipid rafts, whereas clusterin (32) and the ectodomains of placental alkaline phosphatase and the neurotrophin receptor have not (33). Similarly, the mucin-like domains of enteropeptidase direct apical secretion of GFP in the absence of a demonstrable association with lipid rafts (Fig. 7). These results indicate that stable binding to lipid rafts is not a requirement for apical protein sorting. Weaker associations that are not preserved during the isolation of rafts might contribute to targeting, and different experimental approaches would be required to identify them.

Apical targeting can occur with or without stable raft association, glycosylation, or membrane anchoring. However, the apical targeting of specific proteins has been found to depend on one or more of these particular features. This heterogeneity may reflect the existence of multiple apical sorting pathways that employ distinct signals. Alternatively, the apparent diversity of signals could reflect an indirect role of various protein

**Fig. 6. Mutation of selected Ser/Thr residues in mucin repeat**

**Panel A** The amino acid sequence inserted before GFP is given in Table I for the indicated constructs. The polarity of protein secretion (Panel A) and sensitivity to digestion with neuraminidase and O-glycanase (Panel B) were assessed as described in the Fig. 2 legend.

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structures, such as oligosaccharides, in the stabilization of a single class of sorting determinant that could be protein-based (27). These classes of mechanism need not be mutually exclusive. Studies of the relatively simple targeting signals present in enteropeptidase mucin-like domains may facilitate the evaluation of these models.

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