Apical Sorting of Bovine Enteropeptidase Does Not Involve Detergent-resistant Association with Sphingolipid-Cholesterol Rafts*

(Received for publication, July 17, 1998, and in revised form, September 17, 1998)

Xinglong Zheng, Deshun Lu, and J. Evan Sadler‡
From the Division of Hematology and Oncology, Department of Medicine and Barnes-Jewish Hospital, Howard Hughes Medical Institute, Department of Biochemistry and Molecular Biophysics, Washington University School of Medicine, St. Louis, Missouri 63110

Enteropeptidase is a heterodimeric type II membrane protein of the brush border of duodenal enterocytes. In this location, enteropeptidase cleaves and activates trypsinogen, thereby initiating the activation of other intestinal digestive enzymes. Recombinant bovine enteropeptidase was sorted directly to the apical surface of polarized Madin-Darby canine kidney cells. Replacement of the cytoplasmic and signal anchor domains with a cleavable signal peptide (mutant proenteropeptidase lacking the amino-terminal signal anchor domain (dSA-BEK)) caused apical secretion. The additional amino-terminal deletion of a mucin-like domain (HL-BEK) resulted in secretion both apically and basolaterally. Further deletion of the noncatalytic heavy chain (L-BEK) resulted in apical secretion. Thus enteropeptidase appears to have at least three distinct sorting signals as follows: the light chain (L-BEK) directs apical sorting, addition of most of the heavy chain (HL-BEK) inhibits apical sorting, and addition of the mucin-like domain (dSA-BEK) restores apical sorting. Inhibition of N-linked glycosylation with tunicamycin or disruption of microtubules with colchicine caused L-BEK to be secreted equally into apical and basolateral compartments, whereas brefeldin A caused basolateral secretion of L-BEK. Full-length BEK was not found in detergent-resistant raft domains of Madin-Darby canine kidney cells or baby hamster kidney cells. These results suggest apical sorting of enteropeptidase depends on N-linked glycosylation of the serine protease domain and an amino-terminal segment that includes an O-glycosylated mucin-like domain and three potential N-glycosylation sites. In contrast to many apically targeted proteins, enteropeptidase does not form detergent-resistant associations with sphingolipid-cholesterol rafts.

Enteropeptidase (enterokinase) is a protease of the duodenal brush border that cleaves and activates trypsinogen. The resultant trypsin then activates other pancreatic digestivezymogens within the lumen of the gut. Deficiency of enteropeptidase causes intestinal malabsorption (1, 2), and intrusion of enteropeptidase into the pancreas may contribute to hemorrhagic pancreatitis (3, 4). Therefore, the localization of enteropeptidase is important to normal digestive physiology.

Enteropeptidase consists of a disulfide-linked heterodimer with a heavy chain of 82–140 kDa and a light chain of 35–62 kDa. Both chains of mammalian enteropeptidases contain 30–50% carbohydrate, and this extensive glycosylation may contribute to the apparent variation in polypeptide masses (reviewed in Ref. 5). Amino acid sequences deduced by cDNA cloning of bovine (6, 7), porcine (8), human (9), mouse (10), and rat enteropeptidase (11) indicate that active two-chain enteropeptidase is derived from a single-chain precursor. The amino-terminal heavy chain contains domains that are homologous to sequences of O-glycosylated epithelial mucins, the low density lipoprotein receptor, complement components C1r and C1s, the macrophage scavenger receptor, and a recently described MAM motif. The carboxy-terminal light chain is homologous to the trypsin-like serine proteases (reviewed in Ref. 5). Studies of recombinant bovine enteropeptidase demonstrate that membrane association is mediated by a signal anchor sequence near the amino terminus (12).

The structures on enteropeptidase that direct it to apical membranes have not been characterized. Basolateral targeting generally is mediated by discrete amino acid sequence motifs in the cytoplasmic domains of transmembrane proteins, whereas the nature of apical targeting signals remains controversial. Apical targeting appears to depend on distributed features of protein ectodomains or transmembrane domains, and there may be several apical targeting mechanisms (13). For some proteins, apical targeting requires N-linked oligosaccharides (14, 15), or juxtamembrane segments with clustered O-linked oligosaccharides (16), or interactions of transmembrane domains (17) or glycosylphosphatidylinositol anchors (18, 19) with the lipid bilayer. Apical sorting determinants may function, in part, by promoting association with sphingolipid-cholesterol rafts that deliver proteins to the apical cell surface (20). However, some apical proteins appear not to associate with rafts and lack any of the currently recognized apical sorting signals (e.g Ref. 21), and proteins may be sorted differently in different cell types (13). Therefore, the mechanism of apical protein targeting remains poorly understood.

We have employed Madin-Darby canine kidney (MDCK) cells, a well characterized system for the study of protein sort-
Apical Sorting of Enteropeptidase

ing (13, 22), to investigate the targeting of enteropeptidase. The results indicate that signals involved in apical delivery reside in the catalytic domain and in an amino-terminal segment that includes the mucin-like domain. Apical delivery of this type II transmembrane protein requires intact post-Golgi transport vesicles and depends on N-linked glycosylation. Unlike many other apically targeted proteins, delivery of enteropeptidase appears not to involve detergent-resistant association with sphingolipid-cholesterol rafts.

**EXPERIMENTAL PROCEDURES**

**Construction of Plasmids—**Plasmid pBEK, containing the full-length cDNA sequence of bovine enteropeptidase, was assembled in vector pBluescript II KS+ (Stratagene) from cDNA clones isolated previously (7). The cDNA insert of pBEK was cloned into the SmaI site of plasmid pNUT (23) to produce expression plasmid pNUTBEK. Plasmids pNUTHL, pNUTL, and pBEKL were described previously (12). In pNUTHL and pBEKL, the sequence encoding amino acids 1–197 was replaced by a signal peptide cassette consisting of the cleavable signal peptide of prothrombin, a His tag, thrombin cleavage site, and the stop codon. The plasmid pNUTL, the same signal peptide cassette replaced the sequence encoding amino acids 1–783. Additional plasmids derived from pBEK were constructed using oligonucleotide site-directed mutagenesis as described previously (12). In plasmid pdsA, the sequence encoding the signal-anchor domain (residues 1–49) was replaced by the prothrombin signal peptide cassette from plasmid pNUTL.

The epitope tag DYKDDDDK to the carboxy terminus of full-length recombinant enteropeptidase, the DNA sequence 5′-gac tag aag gac gag gat aag tag-3′ was inserted before the stop codon of plasmid pBEK, generating the plasmid pBEKflag. Plasmid pDSALflag was derived from pdsA by inserting the same oligonucleotide sequence before the codon for 9 g of plasmid pcDNA3-BEK, pcDNA3-BEKflag, pcDNA3.1-dSA, pcDNA3.1-dSAdLflag, pcDNA3.1-dSAH, and pcDNA3-L, respectively.

**Transfections—**Baby hamster kidney (BHK) cells were grown in six-well plates and transfected with 5 μg of plasmid pNUT-PEK, pNUTL, and pNUTL and 30 μg of Lipofectin (Life Technologies, Inc.) in serum-free Dulbecco’s modified Eagle’s medium (DMEM). After 5 h fetal bovine serum was added to 10%. After an additional 18 h, cultures were split 1:10 for selection in 0.5 mg/ml methotrexate for 10 days. Madin-Darby canine kidney (MDCK-II) cells (ATCC) were transfected with 5 μg of plasmid pNUT-PEK, pNUTL, pdsA, or pDSALflag plasmid and pSAH, and pBEKL, the same signal peptide cassette replaced the sequence encoding amino acids 1–783. Additional plasmids derived from pBEK were constructed using oligonucleotide site-directed mutagenesis as described previously (12). In plasmid pdsA, the sequence encoding the signal-anchor domain (residues 1–49) was replaced by the prothrombin signal peptide cassette from plasmid pNUTL. The epitope tag DYKDDDDK to the carboxy terminus of full-length recombinant enteropeptidase, the DNA sequence 5′-gac tag aag gac gag gat aag tag-3′ was inserted before the stop codon of plasmid pBEK, generating the plasmid pBEKflag. Plasmid pDSALflag was derived from pdsA by inserting the same oligonucleotide sequence before the codon for residues 787, thereby deleting the light chain and appending the DYKDDDDK tag. The DNA inserts of pBEK, pBEKflag, pdsA, or pDSALflag plasmids and pSAH, and pBEKL, the same signal peptide cassette replaced the sequence encoding amino acids 1–783. Additional plasmids derived from pBEK were constructed using oligonucleotide site-directed mutagenesis as described previously (12). In plasmid pdsA, the sequence encoding the signal-anchor domain (residues 1–49) was replaced by the prothrombin signal peptide cassette from plasmid pNUTL.

**Polynuclear Antibodies—**The cDNA sequence encoding the light chain of bovine enteropeptidase (amino acids 784–1035) was cloned into the XhoI site of plasmid pET-28a(+) (Novagen) to yield plasmid pETL. Epicurean coli BL21 (DE3) (Stratagene, La Jolla, CA) transformed with plasmid pETL were grown in LB broth containing ampicillin, and protein expression was induced with 1 mM isopropyl-1-thio-

**Acrylamide gel electrophoresis, DMEM, Dulbecco’s modified Eagle’s medium; MOPS, 4-morpholinopropanesulfonic acid; PMSF, phenylmethylsulfonyl fluoride; PBS, phosphate-buffered saline; BHK, baby hamster kidney; PNGase, peptide N-glycosidase; endo H, endoglycosidase H; MES, 4-morpholineethanesulfonic acid; CHAPS, 3-(3-cholamidopropyl)dimethy lammonio)-1-propanesulfonic acid; BSA, bovine serum albumin.

*2 D. Lu, X. Zheng, and J. E. Saldier, manuscript in preparation.*
ing at 100 °C for 5 min with 30 μl of Laemmli sample buffer (Bio-Rad) containing 1% β-mercaptoethanol. The cell lysate, flow-through, and eluate fractions from streptavidin-agarose were analyzed by SDS-polyacrylamide gel electrophoresis and Western blotting.

**Tryptsin Accessibility of BEK and HL-BEK Expressed in BHK Cells**—Transfected BHK cell lysate was passed through a 22-gauge needle, and the solution was diluted by addition of 900 μl of extraction buffer. Enteropeptidase was immunoprecipitated from soluble and insoluble fractions with anti-Lv (5 μl/ml) for analysis by SDS-PAGE and autoradiography as described above.

**Isolation of Low Density Membrane Domains**—Detergent-insoluble glycoprotein-enriched raft domains were prepared by a modification of the Triton X-100 procedure of Pike and Casey (28). Confluent cells in 100-mm dishes were rinsed twice with ice-cold PBS and scraped into 1 ml of lysis buffer containing 25 mM MES, pH 6.5, 150 mM NaCl, 1% Triton X-100, 10 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride, and 0.3 mM aprotinin. Lysates were incubated for 20 min on ice with intermittent gentle agitation and then mixed with an equal volume of 90% sucrose in MBS (25 mM MES, pH 6.5, 150 mM NaCl). Six ml of 30% sucrose in MBS followed by 4 ml of 5% sucrose in MBS were layered on top of each sample. The gradients were centrifuged at 4 °C for 23 h at 175,000 × g (39,000 rpm, Beckman SW40 rotor). Fractions of 1.2 ml were collected, and the small insoluble pellet was resuspended in 600 μl of lysis buffer by homogenization (25,000 rpm, 1 min) with a wall-mounted Teflon pestle (Sigma). Sucrose gradients were fractionated with 10% trichloroacetic acid on ice for 30 min, and pellets were resuspended in 100 μl of 0.2 N NaOH. Samples (20 μl) of trichloroacetic acid-concentrated fractions were analyzed by SDS-PAGE and immunoblotting with either anti-Lv (1:4,000) or anti-caveolin IgG (1:10,000) (Transduction Laboratories, Lexington, KY).

**Antibody-induced Patching**—BHK cells expressing BEK were cultured in 150-mm dishes to 90% confluency in DMEM, 10% fetal bovine serum, and incubated twice with 10 ml of ice-cold PBS. Cells were incubated without (control) or with anti-Lv (preabsorbed with non-transfected BHK cell lysate coupled on Affi-Gel 10) at a dilution of 1:1000 in serum-free DMEM, 0.1% BSA at 12 °C for 60 min. After washing three times with 10 ml of ice-cold PBS, cells were further incubated with goat anti-Lv for 1 h at room temperature (1:1000) at 12 °C for 60 min. Cells were scraped into 25 ml of extraction buffer, 0.1% Triton X-100, 10 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride, and 0.3 mM aprotinin. Sucrose gradient centrifugation and Western blotting were performed as described above. For immunofluorescence, cells grown on slide chambers were either fixed with acetate:ethanol (1:9) on ice for 10 min and then incubated with anti-Lv (1:1000) and Cy3-conjugated anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA) at room temperature for 45 min. After washing, the filters were cut out and mounted on slides with glycerol mounting medium (Sigma) and examined with a confocal laser-scanning microscope (Zeiss AxioPlan/Bio-Rad MRC1024).

**Apical Sorting of Enteropeptidase**

**Effects of Tunicamycin, Brefeldin A, and Colchicine on Targeting of Enteropeptidase—** MDCK II cells transfected with pcDNAI-L were grown on six-well Costar Transwell filters (pore size 0.4 μm) until a tight monolayer was formed as shown by the transepithelial resistance. The filters were washed with PBS and fixed at 4 °C for 15 min in PBS containing 1.5% paraformaldehyde (25) or ethanol:acetic acid (9:1). Cells were washed sequentially with PBS followed by PBS containing 0.5% Triton X-100, 1% BSA, and 1% goat serum and then incubated in PBS containing 20 μg/ml rabbit anti-Lv serum (1:100) or anti-caveolin IgG (1:500) (Transduction Laboratories, Lexington, KY), 1% BSA, and 1% goat serum at 4 °C overnight. Cells were washed three times with PBS containing 0.5% Triton X-100 and then incubated with Cy3-labeled goat anti-rabbit antibody (15 μg/ml diluted with PBS containing 1.5% non-immune goat serum) (Jackson ImmunoResearch, West Grove, PA) at room temperature for 60 min. After washing, the filters were cut out and mounted on slides with glycerol mounting medium (Sigma) and examined with a confocal laser-scanning microscope (Zeiss AxioPlan/Bio-Rad MRC1024).

**Analysis of Enteropeptidase Polarity on MDCK Cells**—To detect membrane-bound enteropeptidase, stably transfected MDCK cells were grown on six-well Costar Transwell filters (pore size 0.4 μm) until a tight monolayer was formed as shown by the transepithelial resistance. Cells were pulse-labeled with 150–200 μCi/ml Tran^35S^-label at 37 °C for 30 min and chased for 30 min with complete DMEM containing 150 μg/ml unlabeled methionine. Either the apical or basolateral cell surface was incubated with 1.5 mg/ml sulfosuccinimidyl-2-(biotinamidoethyl)-1,3-dithiopropionate (NHS-SS-biotin, Pierce) at 4 °C for 30 min and then incubated with 1.5 mg/ml unlabeled methionine. Either the apical or basolateral cell surface was incubated with 1.5 mg/ml diluted with TBS containing 1.5% non-immune goat serum (Jackson ImmunoResearch, West Grove, PA) at 4 °C for 30 min. After washing, the filters were cut out and mounted on slides with glycerol mounting medium (Sigma) and examined with a confocal laser-scanning microscope (Zeiss AxioPlan/Bio-Rad MRC1024).
expected for a secreted glycoprotein, the N-linked oligosaccharides of L-BEK were resistant to endo H (Fig. 1A, lane 11) but sensitive to PNGase (Fig. 1A, lane 12). Digestion of proenteropeptidase (BEK) with neuraminidase and O-glycanase also decreased its apparent mass, in this case by ~14 kDa (Fig. 1B). Deletion of the amino-terminal 197 residues in HL-BEK (Fig. 1B) prevented the change in mobility associated with O-glycanase digestion, suggesting a major O-glycosylated region lies between residues 48 and 197 in the exoplasmic part of the heavy chain. A possible site for this O-linked glycosylation is the mucin-like domain between residues 166 and 192.

None of these variants of enteropeptidase had detectable enzymatic activity prior to cleavage and activation by trypsin, as reported previously (12), indicating that all are synthesized predominantly as single chainzymogens.

Enteropeptidase Is Localized to the Apical Surface of Polarized Cells—Biotin surface labeling of transfected BHK cell lines showed that virtually all of the 180-kDa form of BEK was exposed on the plasma membrane, whereas the 150-kDa species of both BEK and HL-BEK were mainly intracellular (Fig. 2A). Surface localization of 180-kDa BEK also was demonstrated by digestion with trypsin (Fig. 2B). The 180-kDa form of BEK was cleaved into catalytically active two-chain enteropeptidase, whereas the intracellular 150-kDa forms of BEK and HL-BEK were inaccessible to trypsin. When expressed in polarized MDCK cells, BEK was localized to the apical surface as shown by immunofluorescence staining and confocal laser-scanning microscopy (Fig. 3), and by selective biotinylation of apical and basolateral membrane proteins (Fig. 4). As observed for several other apical membrane proteins in MDCK cells (22, 29), pulse labeling of BEK showed that it was delivered directly to the apical surface within 30 min of chase (data not shown).

Multiple Signals Contribute to Apical Sorting of Enteropeptidase—Targeting signals in enteropeptidase were localized further by deletion mutagenesis (Fig. 4). Constructs were prepared that lacked the transmembrane domain (dSA-BEK), the mucin-like domain (HL-BEK), the entire heavy chain (L-BEK), and an additional 286 residues that included the mucin-like region (L-BEK, dSA-BEK).
or the light chain (dSAL-BEK). Because the available antibodies were specific for the enteropeptidase light chain, a short peptide epitope (DYKDDDDK) was attached to the carboxyl terminus of construct dSAL-BEK to facilitate immunoprecipitation and Western blotting. Adding this epitope to full-length BEK in construct BEKflag did not impair expression or apical targeting (Fig. 4), suggesting that the tag would not perturb biosynthesis of other constructs. Cells were grown as tight monolayers on porous filters in Transwell units. The targeting of membrane-associated BEK and BEKflag was assessed by surface biotinylation. The targeting of secreted proteins dSA-BEK, dSAL-BEK, HL-BEK, and L-BEK was assessed by analysis of culture medium exposed selectively to apical and basolateral surfaces. Replacement of the signal anchor domain with a cleavable signal peptide resulted in the secretion of construct dSA-BEK from the apical cell surface, whereas amino-terminal deletion including the mucin-like domain resulted in both apical and basolateral secretion of construct HL-BEK (Fig. 4). Therefore, an apical targeting determinant appears to reside near the amino terminus of enteropeptidase in a segment (residues 48–197) that contains a mucin-like domain and is O-glycosylated.

**Fig. 3.** Immunofluorescence and confocal microscopy of proenteropeptidase (BEK) in transfected MDCK II cells. Transfected MDCK II cells expressing full-length enteropeptidase (BEK) were grown on Costar clear Transwell filters to form a tight monolayer, fixed in 1.5% paraformaldehyde (A and a) or ethanol:acetic acid (9:1) (B and b), and incubated with either anti-Lv (A) or anti-caveolin IgG (B) and Cy3-conjugated goat anti-rabbit IgG, and examined by confocal laser-scanning microscopy as described under “Experimental Procedures.” A and B show x-y scans parallel to the plane of the filter near the apex of the cells. Enteropeptidase exhibits an apical staining pattern (A), whereas caveolin is localized laterally (B). Some cells in this mixed cell line do not express enteropeptidase, and this accounts for the patchy staining in A. The insets (a and b) show scans along the x-z dimension and demonstrate the apical localization of enteropeptidase (a) and basolateral localization of caveolin (b).

**Fig. 4.** Domains required for apical sorting of enteropeptidase. Stably transfected MDCK II cell lines expressing variants of enteropeptidase were grown on Costar Transwell units. For detection of membrane-associated enteropeptidase, cells were labeled from either the apical (Ap) or basolateral (Bl) side with NHS-SS-biotin. Biotinylated proteins in cell lysates were isolated with streptavidin-agarose for SDS-PAGE and Western blotting with anti-L antibody (BEK) or anti-flag antibody (BEK.Flag). For analysis of secreted forms of enteropeptidase (dSA, dSAdL.Flag, HL, and L), conditioned media were collected from either the apical or basolateral side surface of MDCK cells. Cell lines expressing dSA or dSAdL.Flag were biosynthetically labeled with Tram35S-label, and conditioned medium was analyzed by immunoprecipitation and autoradiography. Conditioned medium from cells expressing BEK, BEK.Flag, HL, or L was analyzed by Western blotting with anti-Lv. The amount of apically sorted enteropeptidase was quantitated from two independent experiments and expressed as a percentage of the total.
Apical Sorting of Enteropeptidase

Fig. 5. Effects of tunicamycin, brefeldin A, and colchicine on sorting of the secreted enteropeptidase light chain. Tight monolayers of MDCK II cells expressing enteropeptidase light chain (L) were grown on Costar Transwell filters and treated with 2 μg/ml tunicamycin (Tun), 10 μg/ml brefeldin A (BFA), or 12 μg/ml colchicine (Colch) in serum-free medium for 18–24 h. Control cells (Con) were incubated similarly. A, conditioned medium was collected from the apical (Ap) or basolateral (Bl) surface and analyzed by SDS-PAGE and Western blotting. gp, glycosylated; dgp, deglycosylated. B, the amount of apically secreted L-BEK was quantitated from three independent experiments and expressed as a percentage of the total. C, the transepithelial resistance was not significantly affected by the various treatments.

(Fig. 1B). Deletion of the entire heavy chain again resulted in apical secretion of construct L-BEK (Fig. 4), suggesting that the heavy chain can inhibit the function of a second apical targeting determinant that resides in the enteropeptidase light chain. This light chain sorting determinant also is not required for correct targeting because deletion of the light chain did not impair apical localization of construct dSAL-BEK (Fig. 4). Thus, enteropitidase appears to have at least three distinct targeting signals as follows: the enteropeptidase light chain (L-BEK) directs apical secretion, addition of the heavy chain (HL-BEK) inhibits apical sorting and causes delivery to both apical and basolateral surfaces, and addition of an amino-terminal segment containing the mucin-like domain (dSA-BEK) restores apical sorting.

Effect of Tunicamycin, Colchicine, and Brefeldin A on L-BEK Secretion in MDCK Cells—Some secretory proteins (14) and transmembrane proteins (15) appear to employ N-glycans as apical sorting signals, and inhibition of N-glycosylation often causes delivery to both apical and basolateral surfaces. The serine protease domain of bovine enteropitidase contains three potential N-linked glycosylation sites (7), and to assess the role of N-glycans in the sorting of L-BEK, transfected MDCK cells were treated with tunicamycin (Fig. 5). Glycosylation was inhibited effectively as shown by the ~15-kDa decrease in the apparent mass of L-BEK, and secretion was reduced significantly by retention within the endoplasmic reticulum. However, in contrast to the apical secretion of glycosylated L-BEK, the under-glycosylated protein was secreted randomly from both basolateral and apical surfaces (Fig. 5). A small residual quantity of fully glycosylated L-BEK was secreted apically, indicating that the sorting pathway remained capable of correctly delivering apical glycoproteins after exposure of cells to tunicamycin. Thus, the apical targeting signal of L-BEK appears to include N-glycans. Similar experiments could not be performed for membrane-bound BEK or secreted variants containing the heavy chain because inhibition of glycosylation with tunicamycin caused complete intracellular retention (data not shown).

Apical targeting of some secreted and membrane proteins in MDCK cells is selectively impaired by agents that depolymerize microtubules (30, 31) or by treatment with brefeldin A (32, 33). The behavior of L-BEK is consistent with these findings. Treatment of MDCK cells with colchicine resulted in the delivery of L-BEK randomly to apical and basolateral surfaces (Fig. 5). Treatment with brefeldin A reversed the polarity of L-BEK secretion so that nearly 75% was recovered from the basolateral compartment (Fig. 5). Transmembrane conductivity measurements confirmed that neither colchicine nor brefeldin A altered the integrity of the polarized cell monolayer (Fig. 5C). Apical secretion of L-BEK therefore depends on an intact microtubular network and on brefeldin A-sensitive vesicular transport, probably at a site between the trans-Golgi network and the apical plasma membrane (33).

Enteropeptidase Is Not Associated with Detergent-resistant Glycosphingolipid-Cholesterol Rafts—Many apically directed membrane proteins can be recovered in specialized membrane domains that are rich in glycosphingolipids and cholesterol and that are relatively insoluble in Triton X-100, termed “rafts” (20). However, full-length enteropitidase expressed in either BHK or MDCK cells was completely solubilized by brief treatment of the cells with Triton X-100 at 4 °C (Fig. 6A). Also, when rafts were prepared by sucrose density gradient centrifugation of Triton X-100 extracts, no enteropitidase (BEK) was recovered in the low density fractions 4 and 5 that contain caveolin, a marker for rafts; instead BEK was found in fractions that contain soluble proteins (Fig. 7).

Some apical transmembrane proteins (34) and glycosylphosphatidylinositol-anchored proteins (27) that are incorporated into rafts during biosynthesis appear to acquire resistance to Triton X-100 extraction after departure from the endoplasmic reticulum. Certain intestinal brush border enzymes begin to associate with detergent-resistant rafts while still in their transient high mannose-glycosylated forms (35). To evaluate the possibility that enteropitidase could associate with rafts transiently en route to the cell surface, pulse-chase analyses were performed with BHK cells expressing BEK. When cells labeled with radioactive methionine and cysteine were chased at 37 °C (Fig. 6B), enteropitidase with endo H-sensitive oligosaccharides was detected in the detergent-soluble fractions from the earliest time point and decreased during the following 2 h. Endo H-resistant enteropitidase appeared after 1 h of chase, consistent with oligosaccharide processing in the Golgi. At no time of chase was enteropitidase found to be resistant to detergent extraction.

Growth of cells at 20 °C blocks the intracellular transport of certain proteins, often resulting in their accumulation within the trans-Golgi of MDCK cells (36) or BHK cells (37). To determine whether this block would cause the intracellular accumulation of a transient raft-associated enteropitidase species,
BHK cells expressing BEK were pulse-labeled with radioactive methionine and cysteine and chased at 20 °C (Fig. 6, B and C). Under these conditions enteropeptidase contained endo H-sensitive oligosaccharides at all times of chase through 5 h, indicating that transport was blocked at or before the mid-Golgi, possibly in the endoplasmic reticulum. No detergent-insoluble enteropeptidase was detected. Pulse-labeled MDCK cells expressing BEK also did not contain detergent-insoluble enteropeptidase when chased at 20 °C for 1–5 h (data not shown). The results of these pulse-chase experiments suggest that intracellular association of enteropeptidase with rafts either does not occur or is extremely transient.

Association of certain membrane proteins with rafts may depend on clustering, and patching with antibodies is reported to allow increased recovery of placental alkaline phosphatase in detergent-resistant membrane domains (38). To determine whether patching could force enteropeptidase to associate with rafts, transfected BHK cells were incubated with anti-Lv and anti-rabbit immunoglobulin after fixation with 4% paraformaldehyde, which does not permeabilize cells. However, cell-surface staining is clearly distinguished by focusing through the cell, and the typical surface immunofluorescence pattern can be seen over the nucleus where the endoplasmic reticulum does not interfere. A similar fine punctate surface pattern was seen for caveolin (Fig. 8C). Preincubation of cells with anti-caveolin and anti-rabbit immunoglobulin gave no signal, confirming the integrity of the cells prior to permeabilization and the low background immunofluorescence of the method (Fig. 8D). Precubation of cells with anti-Lv and anti-rabbit immunoglobulin resulted in redistribution of enteropeptidase into large surface patches (Fig. 8B). Despite patching with anti-Lv, enteropeptidase was not recovered in detergent-resistant low density membrane fractions after treatment of cells with Triton X-100 at 4 °C (Fig. 9).

**DISCUSSION**

Enteropeptidase is a type II integral membrane protein that is found in the apical microvilli of enterocytes in the duodenum and proximal jejunum (5). As shown by immunofluorescence microscopy (Fig. 3) and by selective cell-surface biotinylation (Fig. 4), the signals that specify its apical localization in enterocytes are functional in MDCK cells, a well characterized model system for the study of protein sorting in which most apical proteins are delivered directly to the apical cell surface (22, 29). The correct localization of enteropeptidase and other apical membrane proteins may be critical to their biological function, but the mechanisms of apical protein targeting are not well characterized. Whereas basolateral targeting often depends on compact structural motifs exposed in the cytoplasmic domains of transmembrane proteins, apical targeting instead appears to depend on any of several distributed features of other domains. These may include characteristics of the transmembrane domain (17), the presence of a glycosylphosphatidylinositol membrane anchor (18, 19), extracellular N-glycans (14, 15), or an O-glycosylated juxtamembrane segment (16).

In enteropeptidase at least two such apical sorting determinants appear to coexist, N-glycans and an O-glycosylated segment near the amino-terminal transmembrane domain. Bovine enteropeptidase has 19 potential N-linked glycosylation sites,
three of which are in the carboxyl-terminal light chain (7). Many of these sites are utilized, as indicated by the large shift in apparent mass upon digestion with PNGase (Fig. 1). Unfortunately, inhibition of \(N\)-glycosylation with tunicamycin caused the intracellular degradation of full-length enteropeptidase (BEK) so that the role of \(N\)-glycans in targeting of the native protein could not be evaluated. However, the enteropeptidase light chain (L-BEK) was secreted apically and inhibition of \(N\)-glycosylation randomized its sorting (Fig. 5). Thus, \(N\)-glycans are required for apical sorting of the enteropeptidase light chain, as observed for several other secreted and membrane-bound glycoproteins (13).

A second sorting determinant is present near the amino terminus of the enteropeptidase heavy chain, and like the \(N\)-glycosylated light chain, it can direct the apical targeting of secreted variants of enteropeptidase. Replacement of the endogenous transmembrane domain with a cleavable signal peptide (dSA-BEK) and additional deletion of the light chain (dSAL-BEK) did not prevent apical targeting, but removal of residues 48–197 (HL-BEK) caused secretion from both apical and basolateral surfaces (Fig. 4). This segment is \(O\)-glycosylated (Fig. 1B), probably within a mucin-like domain between residues 166 and 192, and it also contains three potential \(N\)-glycosylation sites. Whether these oligosaccharides contribute to the apical targeting function of this segment has not been determined.

Other \(O\)-glycosylated proteins may be targeted apically independent of \(N\)-glycosylation. For example, the apical sorting of a neurotrophin receptor (p75 NTR), a type I membrane protein, depends on an \(O\)-glycosylated juxtamembrane segment that remains functional in the absence of \(N\)-glycosylation and does not require membrane association for targeting (16). The \(O\)-glycans of p75 NTR have not been proved to play a direct role in sorting, and it remains possible that other structural features constitute the apical sorting determinants. However, these results suggest that \(O\)-glycans as well as \(N\)-glycans may support apical targeting. Cells that are unable to complete complex-type oligosaccharides appear to target apical glycoproteins correctly (39, 40), suggesting that the mannose-rich core regions of \(N\)-glycans may comprise a sorting signal. Because mannose does not commonly occur in \(O\)-glycans, functional targeting motifs within \(N\)-linked and \(O\)-linked oligosaccharides could differ structurally and be recognized by distinct lectin-like components of the sorting pathway, as suggested by

---

**Fig. 8. Antibody-induced patching of enteropeptidase.** Cells expressing full-length enteropeptidase (BEK) were fixed on ice and subsequently incubated with Cy3-conjugated goat anti-rabbit IgG and rabbit antibody to either enteropeptidase (A) or caveolin (C). Under these conditions, both antigens display a diffuse, finely punctate immunofluorescence pattern. Alternatively, cells were first incubated with Cy3-conjugated goat anti-rabbit IgG and antibody to either enteropeptidase (B) or caveolin (D), followed by fixation. Under these conditions, anti-caveolin does not have access to caveolin on the inner leaflet of the plasma membrane and there is only background immunofluorescence (D); anti-enteropeptidase causes the accumulation of enteropeptidase in large surface patches. Photographs were taken focusing on the apex of the cells to emphasize the pattern visible on the plasma membrane.

**Fig. 9. Effect of antibody-induced cross-linking on the association of enteropeptidase with detergent-resistant rafts.** BHK cells expressing enteropeptidase (BEK) were incubated at 12 °C without (−Abs) or with (+Abs) rabbit anti-Lv and goat anti-rabbit IgG antibodies. Cells were extracted on ice with MES buffer, pH 6.5, containing 1% Triton X-100 and subjected to sucrose gradient centrifugation. Fractions were analyzed for enteropeptidase and caveolin (Cav) by SDS-PAGE and Western blotting as described under “Experimental Procedures.”

---
Apical Sorting of Enteropeptidase

Scheiffele and colleagues (14). For example, VIP36 is an N-acetylgalactosamine-binding lectin of exocytic vesicles that might mediate targeting by O-glycans (41), and similar mannose-binding lectins might mediate targeting by N-glycans.

The heavy chain of enteropeptidase appears to contain a third functionally defined signal that can inhibit apical targeting in certain contexts. Addition of most of the heavy chain to the light chain caused the delivery of construct HL-BEK to both apical and basolateral surfaces, suggesting that the heavy chain inhibits apical targeting directed by the light chain (Fig. 4). In contrast, construct dSAL-BEK was secreted apically, indicating that the targeting function of the juxtamembrane domain was not inhibited by inclusion of the remainder of the heavy chain (4). In addition, construct HL-BEK caused a shift of apical targeting directed by the light chain (Fig. 4). Secreted glycoproteins also are not incorporated directly into rafts but nevertheless can be targeted apically, again raising the question of whether some apical targeting pathways may be independent of rafts. However, transient raft association within the cell could be sufficient for delivery to the apical plasma membrane, after which secreted or transmembrane proteins could dissociate and diffuse away from the rafts that brought them there. Apical secretory proteins could bind to rafts indirectly, perhaps through interaction of their N-glycans with lectin-like sorting receptors (14). Similarly, apical transmembrane glycoproteins could interact with rafts directly through transmembrane domain-lipid interactions or indirectly through protein-protein interactions that involve intrinsic raft proteins, and these interactions may not be stable after disruption of the supporting lipid bilayer with detergent (38, 45). If such a model were correct, then the ability to detect raft association could be sensitive to the assay conditions. For example, the glycoprotein clusterin is secreted apically from MDCK cells and could be solubilized completely by extraction of cells with Triton X-100 at pH 7.5 (46); however, extraction at pH 6.2 was reported to allow detection of CHAPS-insoluble clusterin, suggesting a weak association with rafts (45). Depletion of cellular cholesterol also caused mis-sorting of clusterin, and this result indirectly supports a function of cholesterol-rich rafts in its apical targeting (45). At the present time we have not recovered enteropeptidase in low density raft membranes at pH 6.5, even after patching enteropeptidase on the cell surface with antibodies. Therefore, if rafts participate in targeting enteropeptidase, the mode of involvement remains to be defined.

Acknowledgment—We thank Dr. Linda Pike (Washington University) for assistance in the preparation of detergent-insoluble rafts.

REFERENCES

1. Hadorn, B., Tarlows, M. J., Lloyd, J. K., and Wolf, O. H. (1969) Lancet i, 812–813.
2. Haworth, J. C., Gourley, B., Hadorn, B., and Sumida, C. (1971) J. Pediatr. 78, 481–490.
3. Hammond, J. B., and Mann, N. S. (1977) Dig. Dis. 22, 182–188.
4. Grant, D. (1986) Int. J. Pancreatol. 1, 167–183.
5. Lu, D., and Sadler, J. E. (1996) in Handbook of Proteolytic Enzymes (Barrett, A. J., Rawlings, N. D., and Woessner, J. F., Jr., eds.) pp. 50–54, Academic Press Ltd., London.
6. LaValle, E. R., Rahemtulla, A., Racie, L. A., DiBlassio, E. A., Ferenz, C., Grant, D., Light, A., and McCoy, J. M. (1993) J. Biol. Chem. 268, 23311–23317.
7. Kitamoto, Y., Yuan, X., Wu, Q., McCourt, D. W., and Sadler, J. E. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 5788–5792.
8. Matsushima, M., Ichinoe, M., Yahagi, N., Kakei, N., Tsukada, S., Miki, K., Kurokawa, K., Tashiro, K., Shiokawa, K., Shinomiya, K., Uemaya, H., Inoue, H., Takahashi, T., and Takahashi, K. (1994) J. Biol. Chem. 269, 19976–19982.
9. Kitamoto, Y., Nele, R. A., Denis-Keller, H., and Sadler, J. E. (1995) Biochemistry 34, 4562–4568.
10. Yuan, X., Zheng, X. L., Lu, D. S., Rubin, D. C., Pung, C. Y. M., and Sadler, J. E. (1998) Am. J. Physiol. 37, G324–G349.
11. Yahagi, N., Ichinoe, M., Matsushima, M., Matsuura, Y., Miki, K., Kurokawa, K., Fukumachi, H., Tashiro, K., Shiokawa, K., Kageyama, T., Takahashi, T., Inoue, H., and Takahashi, K. (1996) Biochem. Biophys. Res. Commun. 219, 806–812.
12. Lu, D., Yuan, X., Zheng, X., and Sadler, J. E. (1997) J. Biol. Chem. 272, 31293–31300.
13. Keller, P., and Simons, K. (1997) J. Cell Sci. 110, 3001–3009.
14. Scheiffele, P., Peranen, J., and Simons, K. (1995) Nature 378, 96–98.
15. Gut, A., Kappeler, F., Hyka, N., Balda, M. S., Hauri, H. P., and Matter, K. (1998) EMBO J. 17, 1919–1929.
16. Yamazaki, C., Le Gall, A. H., Rüdiger, A. N., Molneauze, L., Bievic, A., and Rodriguez-Boulan, E. (1997) J. Cell Biol. 139, 929–940.
17. Scheiffele, P., Both, M. G., and Simons, K. (1997) EMBO J. 16, 5501–5508.
18. Brown, D. A., Crise, B., and Rose, J. K. (1999) Science 285, 1499–1501.
19. Lisanti, M. P., Canas, I. W., Davatz, M. A., and Rodriguez-Boulan, E. (1989) J. Cell Biol. 109, 2145–2156.
20. Simons, K., and Ikonen, E. (1997) Nature 387, 569–572.
21. Alonzo, M. A., Fan, L., and Aloe, A. (1997) J. Biol. Chem. 272, 30748–30752.
22. Matter, K., and Mellman, I. (1994) J. Cell Biol. 123, 1919–1929.
23. Palmiter, R. D., Behringer, R. R., Quaife, C. J., Maxwell, F., Maxwell, I. H., and Rodolfo, C., and Milgrom, E. (1997) J. Biol. Chem. 272, 5241–5248.
24. Marnolo, M. P., Bull, P., and Gonzalez, A. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 1834–1839.
25. Brown, D. A., and Rose, J. K. (1992) Cell 68, 533–544.
28. Pike, L. J., and Casey, L. (1996) J. Biol. Chem. 271, 26453–26456
29. Wandinger-Ness, A., Bennett, M. K., Antony, C., and Simons, K. (1990) J. Cell Biol. 111, 987–1000
30. Parezyk, K., Haase, W., and Kondor-Koch, C. (1989) J. Biol. Chem. 264, 16837–16846
31. Rindler, M. J., Ivanov, I. E., and Sabatini, D. D. (1987) J. Cell Biol. 104, 231–241
32. Low, S. H., Wong, S. H., Tang, B. L., Tan, P., Subramaniam, V. N., and Hong, W. (1991) J. Biol. Chem. 266, 17729–17732
33. Low, S. H., Tang, B. L., Wong, S. H., and Hong, W. (1992) J. Cell Biol. 118, 51–62
34. Skibbens, J. E., Roth, M. G., and Matlin, K. S. (1989) J. Cell Biol. 108, 821–832
35. Danielsen, E. M. (1995) Biochemistry 34, 1596–1605
36. Matlin, K. S., and Simons, K. (1983) Cell 34, 233–243
37. Griffiths, G., Pfeiffer, S., Simons, K., and Matlin, K. (1985) J. Cell Biol. 101, 949–964
38. Harder, T., Scheiffele, P., Verkade, P., and Simons, K. (1988) J. Cell Biol. 141, 929–942
39. Parezyk, K., and Koch-Brandt, C. (1991) FEBS Lett. 278, 267–270
40. Wagner, M., Morgans, C., and Koch-Brandt, C. (1995) Eur. J. Cell Biol. 67, 84–86
41. Fiedler, K., and Simons, K. (1996) J. Cell Sci. 109, 271–276
42. Hanada, K., Nishijima, M., Akamatsu, Y., and Pagano, R. E. (1995) J. Biol. Chem. 270, 6254–6260
43. Arreaza, G., and Brown, D. A. (1995) J. Biol. Chem. 270, 32641–32647
44. Nichols, B. L., Eldering, J., Avery, S., Hahn, D., Quaroni, A., and Sterchi, E. (1998) J. Biol. Chem. 273, 3076–3081
45. Keller, P., and Simons, K. (1998) J. Cell Biol. 140, 1357–1367
46. Graichen, R., Loseh, A., Appel, D., and Koch-Brandt, C. (1996) J. Biol. Chem. 271, 15854–15857