Basolateral Sorting Signals Differ in Their Ability to Redirect Apical Proteins to the Basolateral Cell Surface*

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Polarized sorting of membrane proteins in epithelial cells is mediated by cytoplasmic basolateral signals or by apical signals in the transmembrane or exoplasmic domains. Basolateral signals were generally found to be dominant over apical determinants. We have generated chimeric proteins with the cytoplasmic domain of either the asialoglycoprotein receptor H1 or the transferrin receptor, two basolateral proteins, fused to the transmembrane and exoplasmic segments of aminopeptidase N, an apical protein, and analyzed them in Madin-Darby canine kidney cells. Whereas both cytoplasmic sequences induced endocytosis of the chimeras, only that of the transferrin receptor mediated basolateral expression in steady state. The H1 fusion protein, although still largely sorted to the basolateral side in biosynthetic surface transport, was subsequently resorted to the apical cell surface. We tested whether the difference in sorting between trimeric wild-type H1 and the dimeric aminopeptidase chimera was caused by the number of sorting signals presented in the oligomers. Consistent with this hypothesis, the H1 signal was fully functional in a tetrameric fusion protein with the transmembrane and exoplasmic domains of influenza neuraminidase. The results suggest that basolateral signals per se need not be dominant over apical determinants for steady-state polarity and emphasize an important contribution of the valence of signals in polarized sorting.

In epithelial cells, plasma membrane proteins are sorted in a polarized manner either to the apical or the basolateral surface. Sorting signals specifying basolateral surface expression have been identified in the cytoplasmic domains of many membrane proteins. Most of them belong to two common classes (for review, see Refs. 1 and 2) characterized either by an essential tyrosine residue in a sequence context NPLY or YXXØ (where Ø is a bulky hydrophobic residue) (3), or by a dileucine motif (4, 5). In several cases, signals of either type also function in endocytosis from the plasma membrane and in direct lysosomal sorting from the trans-Golgi network (TGN)1 to endosomes and lysosomes (for review, see Refs. 2 and 6). Other basolateral sorting signals contain neither an essential tyrosine nor a dileucine motif (e.g. the polyimmunoglobulin receptor (7)).

Little is known about the identity of apical signals. However, they appear to be localized to the noncytosolic segments of membrane proteins because truncation mutants lacking the cytosolic and transmembrane portions were generally found to retain apical polarity (8–11). N-Linked glycans have been shown to act as apical determinants for certain secretory and membrane proteins (12–14). Other proteins, however, did not depend on N-glycosylation for correct delivery (15–17), which suggests several mechanisms for apical sorting. Recently, a role of the transmembrane domain in apical sorting has been demonstrated for several proteins (18–20).

Generally, cytoplasmic basolateral signals were found to be dominant over exoplasmic apical determinants. Mutant forms of basolateral membrane proteins lacking their cytosolic signals were often sorted predominantly to the apical surface (e.g. low density lipoprotein receptor, Fc receptor, lysosomal acid phosphatase (21–23)), reflecting the existence of recessive apical determinants. Insertion of a tyrosine into the small cytosolic domain of influenza hemagglutinin redirected the protein to the basolateral surface (24). Most importantly, fusion of cytosolic basolateral signals to transmembrane and exoplasmic domains of apical proteins resulted in basolateral transport (18, 23, 25–28). Based on these observations, it appeared that binding of cytoplasmic signals to recognition proteins in the coats forming basolateral transport vesicles at the TGN generally overruled the interaction of apical determinants with their sorting machinery.

In this study, we have analyzed the polarized transport of two fusion proteins, HN and TN, consisting of the exoplasmic and transmembrane domains of aminopeptidase N (APN) and the cytosolic portions either of the asialoglycoprotein receptor subunit H1 or of the transferrin receptor (TfR). APN is an apical, resident plasma membrane protein (29). Apical sorting information resides in the exoplasmic portion because soluble forms lacking the membrane anchor were secreted apically as well (8, 9). In contrast, H1 and TfR are basolateral proteins that cycle between the plasma membrane and endosomes. The information for endocytosis and basolateral sorting resides in the cytosolic portion of both proteins. A tyrosine motif (Tyr-5) is essential for both efficient internalization and polarized expression of H1 (30, 31). Internalization of TfR was shown to depend on the motif YXRYP (32), whereas basolateral transport depends mainly on residues 29–35 containing neither a tyrosine nor a dileucine motif (33, 34). Surprisingly, the two fusion proteins H; PBS, phosphate-buffered saline; MHC, major histocompatibility complex.

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# The abbreviations used are: TGN, trans-Golgi network; APN, aminopeptidase N; TfR, transferrin receptor; MDCK, Madin-Darby canine kidney; NA, neuraminidase; endo H, endo-β-D-N-acetylgalcosaminidase H; PBS, phosphate-buffered saline; MHC, major histocompatibility complex.

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proteins, expressed in Madin-Darby canine kidney (MDCK) cells, showed different fates with respect to endocytosis, to initial polarized sorting at the TGN, and to steady-state polarity. The results might be explained by the number of signals presented by different protein oligomers, suggesting an important contribution of the valence of signals in polarized sorting.

**EXPERIMENTAL PROCEDURES**

**DNA Constructs**—The cDNA sequences encoding the cytoplasmic domain of the human asialoglycoprotein receptor subunit H1 (codons 1–40) and of the mouse transferrin receptor (plasmid pMTR-1, from L. Kühn; codons 1–64) were fused to the transmembrane and exoplasmic domains of human APN (codons 10–967) by polymerase chain reaction and “splicing by overlap extension” (35, 36). The resulting hybrid cDNAs were subcloned into the expression plasmid p LJ (37). Similarly, codons 1–40 of H1 were fused to the transmembrane and exoplasmic domains of neuraminidase (NA; codons 7–450), and the resulting construct, HNA, was subcloned into the expression plasmid pCB6.

**Cell Culture, Labeling, and Immunoprecipitation**—Maintenance and transfection of MDCK cell lines (strain II) were as described previously (29). Clonal cell lines resistant to 1 mg/ml G418-sulfate were isolated and screened for expression by surface antibody binding or metabolic labeling and immunoprecipitation using an antisera directed against human APN or against influenza virus NA (an antisera raised against total virus, strain A/WSN/33; from D. Nayak, UCLA). Labeling with [35S]methionine or [35S]sulfate and immunoprecipitation was performed as described previously (29, 38). Cell lines expressing NA and HNA were incubated overnight with 10 mM sodium butyrate before experiments to enhance expression (18). For deglycosylation, the immune complexes were released from protein A-Sepharose by boiling in 50 mM sodium citrate, pH 6, 1% SDS, and incubated with 1 milliunit of endo-β-N-acetylglucosaminidase H (endo H) for 5 h at 37 °C. To identify disulfide-linked oligomers, samples were boiled in SDS-sample buffer with or without 0.5 mM N-mercaptoethanol before gel electrophoresis.

**Polarity Assays**—For separate access to the apical or basolateral cell surface, MDCK cell lines were grown to confluence on polycarbonate filters (0.4-μm pore size) in 24-mm Transwell chambers (Costar). Tightness of the monolayers was assayed as described (39). Polarized surface distribution of wild-type and chimeric APN was determined by surface antibody binding (39) or by surface immunoprecipitation. For the latter procedure, filter-grown cells were incubated for 30 min in methionine-anti-APN serum (diluted 1:500) from either the apical or basolateral surface, rinsed five times, and scraped into lysis buffer. Immune complexes were precipitated with protein A-Sepharose, and the samples were analyzed by SDS-gel electrophoresis and fluorography. Initial surface appearance was assayed similarly by surface immunoprecipitation, except that cells were starved for 30 min at 20 °C to accumulate proteins at the TGN. The TGN was then labeled with [35S]sulfate for 60 min at 20 °C and for an additional 30 min at 37 °C. Antibody was present in the apical or basolateral compartment throughout the labeling time and an additional 60 min at 4 °C. The cells were lysed and immune complexes isolated with protein A-Sepharose. Nonprecipitated material was subjected to a second immunoprecipitation as a control for total expression. Polarity of wild-type and chimeric NA was determined either by metabolic labeling, surface biotinylation, and consecutive precipitation with antibody-protein A and avidin–agarose as described (18) or by surface immunoprecipitation, SDS-gel electrophoresis, blotting, and detection with streptavidin–horseradish peroxidase.

**Endocytosis Assay**—MDCK cell lines were grown to subconfluence in 35-mm plates, labeled with [35S]sulfate for 1 h, and washed at 4 °C with PBS without calcium, which opens the tight junctions and facilitates access of reagents to the basolateral surfaces (40). The cell surface was then biotinylated for 30 min at 4 °C using 1 mg/ml sulfo-NHS-biotin (sulfo-NHS-SS-biotin; Pierce) in PBS. Excess reagent was quenched by washing the cells three times with 50 mM glycine in PBS and twice with PBS. Cells were incubated for 0–30 min at 37 °C in complete minimal essential medium supplemented with 10 mM HEPES, pH 7.2, and washed with PBS at 4 °C. Except for the control cells, biotin exposed on the cell surface was removed by two 20-min incubations with a freshly prepared solution of reduced glutathione (50 mM in 75 mM NaCl, 1 mM EDTA, 1% bovine serum albumin, 75 mM NaOH). Excess glutathione was quenched by a 5-min incubation with 5 mM iodoacetamide in PBS at 4 °C. Cells were washed and subjected to immunoprecipitation. Precipitated material was released from the beads by boiling in 2% SDS, diluted 10-fold with 0.5 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate in PBS, and reprecipitated with avidin agarose. The samples were analyzed by SDS-gel electrophoresis and fluorography. The signal recovered from control cells not stripped with glutathione was defined as 100%. The efficiency of biotinylation and avidin precipitation was determined to be ~30% for HN. Because approximately half of the chimeras are intracellular at steady state based on the endocytosis experiments shown below, the majority of the polypeptides at the cell surface were recovered.

**RESULTS**

**Expression of Wild-type and Chimeric APN in MDCK Cells**—APN, H1, and TIR are type II membrane proteins with a cytoplasmic NH2-terminus. The cytoplasmic domains of H1 and TIR (Fig. 1) were fused to the transmembrane and exoplasmic portions of APN to construct the chimeric proteins HN and TN, respectively. The borders between the cytoplasmic portion and the transmembrane segment are delimited unambiguously in all sequences by a positively charged amino acid preceding a hydrophobic stretch of at least 20 amino acids.

MDCK cells were transfected with the chimeric cDNAs, and expressing clonal cell lines were isolated. In Fig. 2, lanes 1–4, expression of APN, HN, and TN was analyzed by labeling the corresponding cell lines for 1 h with [35S]methionine followed by immunoprecipitation using an APN-specific antisera. Two forms of wild-type APN with apparent molecular masses of ~120 and ~150 kDa were detected, corresponding to the high mannose and complex glycosylated forms of the protein (29). The apparent molecular masses of the two forms of HN and TN were slightly higher in agreement with the added size of their cytoplasmic portions. Parental MDCK cells were devoid of immunoreactive material. All three proteins could also be labeled with [35S]sulfate (Fig. 2, lanes 5–8). Because sulfation occurs in the TGN, radioactivity was incorporated exclusively in the mature forms. Conversion of the high mannose to the complex glycosylated forms, as analyzed in pulse-chase experiments (Fig. 3), proceeded with a half-time of ~30 min for APN and TN and of ~1 h for HN. Both the rates and the extent of conversion indicate that the chimeras passed endoplasmic reticulum quality control quite efficiently and similarly to wild-type APN. It has been shown previously that APN forms noncovalent dimers in the endoplasmic reticulum and that dimerization is required for its transport out of the endoplasmic reticulum (41, 42). Rapid transport to the cell surface thus indicates that the heterologous cytoplasmic tails did not disturb dimerization of the APN portions in the chimeras.

**The Cytoplasmic Domains of H1 and of TIR Mediate Endocytosis**—Endocytosis of H1 is critically dependent on the same

**Fig. 1.** Cytoplasmic sequences of APN, H1, TIR, and NA. Transmembrane domains are shown in italics, endocytosis motifs in boldface, and basolateral signals are underlined.
tyrosine signal necessary for basolateral sorting (31). To test whether in the chimera this bifunctional signal is effective for interaction with clathrin adaptors at the plasma membrane, endocytosis of HN was analyzed. Cells expressing the chimeras, wild-type APN, or H1TS (a variant of H1 which was tagged at the luminal COOH terminus with a tyrosine-sulfation site (38)) were [35S]sulfate labeled and then derivatized at the surface at 4 °C with the impermeant, cleavable reagent sulfo-NHS-SS-biotin. After incubation at 37 °C for different times, surface biotin was stripped at 4 °C by incubation with glutathione. Internalized biotinylated proteins were isolated with protein A-Sepharose and analyzed by SDS-gel electrophoresis. The high mannose and complex glycosylated forms are indicated by hm and c, respectively.

To test whether HN is sorted differentially, cells were specifically endocytosed, although at different rates (Fig. 4). TN was internalized at a rate of 0.7%/min; TN and H1TS were labeled with [35S]methionine and with [35S]sulfate, surface biotinylated at 4 °C, and shifted to 37 °C for different times to allow internalization. Non-endocytosed surface biotin was removed with glutathione at 4 °C. To test whether selective degradation at the basolateral side might cause predominantly apical polarity of the HN chimera, its half-life was determined in a pulse-chase experiment. The [35S]sulfate-labeled constructs were equally stable over a time period of 8 h, showing extrapolated half-lives of −12 h for APN and −15 h for HN and TN (Fig. 6). This suggested that the opposite polarities of HN and TN were the result of different sorting mechanisms and that the cytosolic signal of H1 was not sufficient to determine the polarity of the chimera.

The Cytoplasmic Domain of H1 Is Not Sufficient for Basolateral Polarity of an APN Chimera—To assess the polarity of the chimeric proteins, the cells were grown to confluence on filters and then incubated at 4 °C first with APN-specific antibodies added either to the apical or basolateral side and then with 125I-protein A added to both surfaces. Cell-associated radioactivity was measured by scintillation counting. The means of at least three independent experiments are shown.

To test whether selective degradation at the apical side might cause predominantly apical polarity of the TN chimera, its half-life was determined in a pulse-chase experiment. The [35S]methionine-labeled constructs were equally stable over a time period of 8 h, showing extrapolated half-lives of −12 h for APN and −15 h for HN and TN (Fig. 6). This suggested that the opposite polarities of HN and TN were the result of different sorting mechanisms and that the cytosolic signal of H1 was not sufficient to determine the polarity of the chimera.

The Cytoplasmic Domain of H1 Mediates Initial Basolateral Sorting of the APN Chimera—To test whether HN is sorted
directly to the apical surface or transcytosed via the basolateral surface, we determined the polarity of newly synthesized molecules at their first arrival at the cell surface. To improve sensitivity, the cells were labeled with [35S]sulfate for 60 min at 20 °C, which blocks surface transport, and for an additional 30 min at 37 °C. To capture the protein as it appeared at the cell surface, antibody was present in the apical or basolateral compartment throughout the labeling time and an additional 60 min at 4 °C. 30 min at 37 °C is insufficient for a significant amount of protein to be transported first to one cell surface (t1/2 > 10 min (45)) and then to be transcytosed to the other (e.g. t1/2 ~30 min for the polymeric immunoglobulin receptor (46)). After lysis, immune complexes were isolated. APN and TN showed the same initial polarity as at steady state (Fig. 7). In contrast, HN first appeared predominantly on the basolateral surface, although with lesser efficiency than TN. This indicates that the signal in the cytoplasmic domain of H1 is largely functional to direct HN from the TGN to the basolateral side but is unable to maintain this initial polarity.

The Cytoplasmic Domain of H1 Is Dominant in a Tetrameric Chimera with Neuraminidase—A possible explanation for the reduced efficiency of the basolateral and endocytic determinants of H1 in the chimera compared with those of the TIR is the oligomeric state of the proteins. H1 forms noncovalent homotrimers through an exoplasmic α-helical coiled-coil stalk segment (47, 48). The TIR and APN, on the other hand, are dimers (41, 42, 49). In the dimeric context of the APN chimeras, two copies of the cytoplasmic signals are presented. At least three copies of the basolateral signal of H1 might be required for efficient basolateral sorting and maintenance. To test this hypothesis, we constructed the chimera HNA, consisting of the cytoplasmic domain of H1 fused to the transmembrane and exoplasmic domains of NA, which was also redirected to the basolateral side (18). In addition, most studies in which basolateral sorting signals were either mutated inside normally basolateral proteins or matched against apical determinants in chimeric proteins suggested that basolateral signals were generally dominant over apical determinants.

In contrast, the cytoplasmic portion of H1 did not invert the steady-state polarity of APN when fused to its transmembrane and exoplasmic domains. Although it was sufficient to direct at least a majority of the newly synthesized fusion proteins to the basolateral surface initially, it failed to maintain this polarity, and the protein was redistributed to the apical side in subsequent sorting steps. This is reminiscent of the sorting pathway of apical proteins in hepatocytes (51): apical proteins are first delivered to the basolateral surface and then transported to the apical surface by transcytosis. In the intestinal epithelial cell line Caco-2, an intermediate situation has been observed with some apical proteins transported directly to their destination and others at least in part via the basolateral membrane (52). Because basolateral proteins always take a direct route in
Panel A, MDCK cells expressing HNA were pulse labeled with [35S]methionine for 30 min and chased for up to 120 min as indicated. Immunoprecipitated protein was incubated with (+) or without (−) endo H and analyzed by SDS-gel electrophoresis under reducing or nonreducing conditions. Panel B, filter-grown cell lines were labeled with [35S]methionine for 5 h, biotinylated at 4 °C from the basolateral (B) or apical (A) side, lysed, and precipitated first with streptavidin-agarose and then with an antiseraum against NA and protein A-Sepharose. Samples were analyzed by SDS-gel electrophoresis and fluorography and quantified using a PhosphorImager. Parental MDCK cells (−) were analyzed in lanes 5 and 6. The molecular masses of marker proteins are shown in kDa.

Caco-2 cells, basolateral sorting in the TGN appears to be less exclusive than in endosomes upon endocytosis. In MDCK cells, both apical and basolateral proteins are generally sorted directly (53). However, it has been demonstrated previously that there are two stations for polarized sorting also in MDCK cells: in exocytosis at the level of the TGN and upon endocytosis in endosomes (34, 50). Using the low density lipoprotein receptor as a model protein, it was shown that the same signals directing polarized sorting in the TGN also control recycling and transcytosis after internalization from either cell surface domain, suggesting related machineries (50). Analysis of TIR mutants revealed differences in the recognition of basolateral determinants within the biosynthetic and endosomal pathways (34). HN is a further example illustrating different properties of the two machineries for polarized sorting. The basolateral signal in HN is still recognized in the TGN, but not in endosomes. H1 and TIR are sorted by different types of signals. Whereas basolateral expression of H1 depends on a typical tyrosine motif, YQDL (31), the main basolateral determinant of TIR involves a stretch of 13 amino acids (residues 29–41) which does not contain any known motifs (34). This might thus suggest that different mechanisms exist for basolateral targeting (at least in endosomes), only some of which are dominant over apical determinants. However, other tyrosine motifs, similar to that in H1, have also been shown to be dominant (23, 24, 27, 28). In addition, there is evidence that both H1 and TIR are transported from the TGN to the cell surface indirectly via endosomal compartments (45, 54). Candidate coats for recognition of basolateral proteins in the TGN are clathrin-associated AP-1 adaptor complexes, for which there is evidence that they recognize at least some tyrosine motifs (55–58). Recently, clathrin/AP-1 coats were detected on sorting endosomes of MDCK cells, i.e. endosomal structures accessible to TIR and polymeric immunoglobulin receptor from the apical and basolateral surface (59). Because in addition clathrin/AP-1 coats and basolateral sorting were similarly sensitive to brefeldin A treatment, these endosomal clathrin coats were proposed to be involved in basolateral surface transport of TIR (59).

As an alternative to different basolateral sorting mechanisms, the basolateral signals in HN and TN may have different affinities for interaction with common coat components of basolateral transport vesicles. A potentially important factor in defining the interaction affinity of oligomeric proteins is the valence of signals presented. H1 forms noncovalent oligomers through an exoplasmic α-helical coiled-coil stalk segment that readily forms heteromers in vitro (48). H1 heteromers have also been observed in in vivo cross-linking experiments (47). The TIR, on the other hand, is a dimer (49), as are APN (41, 42) and its derivatives. The behavior of HN suggests that at least three copies of the basolateral signal of H1 are required in a protein complex for basolateral expression and efficient endocytosis. The concept was tested with a tetrameric fusion construct of the cytoplasmic domain of H1 to NA. This protein was expressed basolaterally, consistent with the notion that the H1 signal needs to be presented at higher valence to be functional.

The endocytosis signal of H1, which involves the same tyrosine motif required for basolateral sorting, is sufficient to induce internalization of HN. However, the internalization rate of the dimeric chimera is clearly lower than that of trimeric H1, consistent with a role of signal valence in defining the affinity for clathrin/AP-2-coated pits at the plasma membrane. A correlation between the number of functional endocytosis motifs in a protein oligomer and its internalization rate has also been shown by introducing a second tyrosine signal into the cytoplasmic domain of the TIR (60) and by coexpression at different ratios of wild-type and Tyr-to-Ala mutant chains of the macrophage asialoglycoprotein-binding protein (61). Arneson and Miller (62) analyzed the oligomeric requirements of major histocompatibility complex (MHC) class II invariant chain, which associates as a trimer with three MHC class II αβ dimers and contains the main determinants for transport of the complex from the TGN to an endosomal MHC class II compartment. At least two functional tails of invariant chain were required in a complex for efficient endosomal sorting, whereas one tail was sufficient to induce endocytosis from the plasma membrane.

Very recently, another exception to the rule that basolateral signals are dominant over apical ones was published: the short cytoplasmic domain of hemagglutinin, a normally apical protein, mutated to contain tyrosine motifs was functional to redistribute the mutant proteins to the basolateral side but was not sufficient to do so when fused to lactase-phlorizin hydrolase, an apical enzyme of the small intestine (63). This finding suggested that not all mechanisms of apical sorting are necessarily recessive. Alternatively, this result could also be explained in terms of different valences of signals presented in the oligomers because hemagglutinin oligomerizes to trimers (64), whereas lactase-phlorizin hydrolase only forms dimers (42). Whether also in this case biosynthetic sorting to the basolateral membrane is still functional is not known. Our observations together with these results suggest that basolateral signals per se need not be dominant over apical determinants and emphasize an important contribution of the valence of signals in polarized sorting.

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