Aminopeptidase N Is Directly Sorted to the Apical Domain in MDCK Cells

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Abstract. In different epithelial cell types, integral membrane proteins appear to follow different sorting pathways to the apical surface. In hepatocytes, several apical proteins were shown to be transported there indirectly via the basolateral membrane, whereas in MDCK cells a direct sorting pathway from the trans-Golgi-network to the apical membrane has been demonstrated. However, different proteins had been studied in these cells. To compare the sorting of a single protein in both systems, we have expressed aminopeptidase N, which already had been shown to be sorted indirectly in hepatocytes, in transfected MDCK cells. As expected, it was predominantly localized to the apical domain of the plasma membrane. By monitoring the appearance of newly synthesized aminopeptidase N at the apical and basolateral surface, it was found to be directly sorted to the apical domain in MDCK cells, indicating that the sorting pathways are indeed cell type-specific.

Polarized epithelial cells have two distinct plasma membrane domains separated by tight junctions: the basolateral membrane facing the interior (i.e., the serum) and the apical membrane facing the exterior of the organism (i.e., the lumen of the organ). The two domains differ in their protein and lipid composition (reviewed by Simons and Fuller, 1985; Simons and van Meer, 1988), reflecting their specialized biological functions. The question of how the constituents are sorted to the two surface domains has been intensively studied (see recent reviews of Bartles and Hubbard, 1988; Caplan and Matlin, 1989; Rodriguez-Boulan and Nelson, 1989; Wandinger-Ness and Simons, 1990).

In rat hepatocytes, the sorting pathways of two basolateral membrane proteins, the asialoglycoprotein receptor and a 48-kD protein called CE9, and of three apical membrane proteins, aminopeptidase N (APN), dipeptidylpeptidase IV, and a 105-kD protein called HA4, have been examined. All of them were found to be delivered first to the basolateral membrane. From there, the ones destined to the apical domain were transported further to their final location (Bartles et al., 1987).

The sorting pathways in intestinal cells are not completely clear. The results from one study point to a direct transport of APN to the apical membrane (Danielsen and Cowell, 1985). The results from another study indicate that all of the newly synthesized enzyme transits the basolateral membrane before it reaches the apical domain (Massey et al., 1987).

In MDCK cells (Richardson et al., 1981), the biosynthetic pathways of two basolateral markers, Na+/K+ -ATPase and the envelope glycoprotein of vesicular stomatitis virus, and of two apical markers, influenza virus hemagglutinin and an endogenous 114-kD glycoprotein (gp14), were studied. Sorting of apical and basolateral proteins was shown to take place intracellularly in the trans-Golgi network from where separate transport vesicles depart to either surface domain (Matlin and Simons, 1984; Misek et al., 1984; Fuller et al., 1985; Pfeiffer et al., 1985; Rindler et al., 1985; Caplan et al., 1986; Griffiths and Simons, 1986; Bennett et al., 1988; Lisanti et al., 1989a; Le Bivic et al., 1990).

A likely interpretation of these findings is that different sorting pathways for apical membrane proteins are operative in different epithelial cell types. Alternatively since different proteins have been studied so far in MDCK cells and in hepatocytes, the two observed sorting pathways might be protein specific. According to this hypothesis, one would expect to find certain proteins to follow the direct pathway and others to follow the indirect pathway in the same cell. This is in fact what has been observed in the intestinal epithelial cell line Caco-2. Sucrase-isomaltase was found to be sorted directly to the apical domain, whereas two other apical markers, APN and dipeptidylpeptidase IV, were transported partially via the basolateral membrane (Matter et al., 1990).

To find out whether the two apical pathways reflect differ
ences in the sorting machineries of the cells studied or rather features of the proteins themselves, we have examined the sorting of APN in MDCK cells. This apical protein is naturally expressed in hepatic, intestinal, as well as renal epithelia, and its transport has already been characterized in hepatocytes (Bartles et al., 1987) and Caco-2 cells (Eilers et al., 1989; Matter et al., 1990). MDCK cells express endogenous canine APN, however, only in small amounts. To facilitate the analysis, we have transfected the human APN (hAPN) cDNA, which recently had been cloned from Caco-2 cells (Olsen et al., 1988) and from myeloid cells (Look et al., 1989), into MDCK cells. Our results show that a dominating fraction of hAPN is directly transported to the apical domain in MDCK cells and confirm cell type specific differences in protein sorting.

Materials and Methods

Cell Culture and Transfection

Cell culture reagents were purchased from Gibco Laboratories (Grand Island, NY). MDCK cells (strain II, Richardson et al., 1981), HepG2 cells, and Caco-2 cells (a gift from H. P. Hauri, Biocenter, Basel) were grown in minimal essential medium supplemented with 2 mM L-glutamine, 10% FCS (Inotech, Switzerland), 100 U/ml penicillin, and 100 µg/ml streptomycin at 37°C with 7.5% CO₂. For expression of hAPN, the retroviral shuttle vector pZIPneoSV(X)-1 containing the cDNA of hAPN (Look et al., 1989) was transfected into MDCK cells. To a 100-mm plate of cells (50% confluent) in 2.5 ml complete medium, 30 µg of plasmid DNA was added followed by 30 µg Polybrene (Pluka, Switzerland). The cells were incubated for 6 h at 37°C with occasional rocking of the plate. The medium was then replaced gently with 5 ml 25% dimethyl sulfoxide in complete medium at room temperature and incubated for precisely 3 min. The cells were gently washed with 10 ml serum-free medium which was replaced by 10 ml complete medium. The next day the medium was renewed; 2 d later the cells were split 1:20 and grown in selective medium containing 1 mg/ml G418-sulfate, which was replaced every 4 d. After 10 d separate colonies were isolated using cloning cylinders and screened for expression by immunoblot analysis. Of 10 clones, 6 were found to express hAPN. The polarity of hAPN expression was analyzed in two of them and was found to be the same. The clone chosen for further analysis was named MN. For separate expression of hAPN, the retroviral expression vector pZIPneoSV(X)-1 carrying the cDNA of hAPN (see Materials and Methods) was transfected with antiserum diluted 1:500 in DPBS/0.5% BSA at 4°C. After rinsing the cells three times, they were incubated for 30 min with [125I]protein A (~10⁵ cpm/ml) in DPBS/0.5% BSA. Cells were washed three times with DPBS/0.5% BSA, twice with DPBS, lysed in hot sample buffer, and analyzed by SDS-gel electrophoresis.

EM and Immunocytochemical Analysis

For ultrastructural analysis, filter-grown MDCK and MN cells were fixed in 2.5% glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.2, for 30 min at 4°C. After removal of the filters, the cells were postfixed in 1% osmium tetroxide in phosphate buffer for 15 min at 4°C. They were then treated with 1% uranyl acetate in water for 1 h before dehydration in graded series of ethanol and embedding in Epon. Ultrathin sections were cut on an Ultrotome III (LKB Instruments, Inc., Gaithersburg, MD) using a Jumdi diamond knife, stained in lead citrate (Reynolds, 1963) for 10-20 s, and finally examined in a Philips electron microscope 201c operated at 60 kV. The length of the apical and basolateral membranes were measured by a planimeter (Numonics Corp., Lansdale PA).

For immunocytochemical analysis, gold probes were prepared by the method of Slot and Geuze (1985). Sheep anti-rabbit IgG (SBL, Stockholm, Sweden) was conjugated to 5-nm gold particles essentially as described by Wang and Larsson (1985) with the modification that the IgG-gold complexes were purified by ultracentrifugation instead of gel chromatography. Filter-grown cells were fixed in 3% glutaraldehyde and 2% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.2, for 2 min at 4°C, rinsed in phosphate buffer, and incubated three times for 5 min in TBS and 20 min in TBS containing 3% BSA. The filters were then incubated either on the apical or the basolateral side with the preadsorbed antiserum (diluted 1:250) in TBS/0.5% BSA, twice with DPBS, lysed in hot sample buffer, and analyzed by SDS-gel electrophoresis.

Expression of hAPN in MDCK Cells

To study the sorting pathway of hAPN, MDCK cells were transfected with the retroviral expression vector pZIP-neoSV(X)-1 carrying the cDNA of hAPN (see Materials and Methods). Stable cell lines were isolated by selection in
Figure 1. Expression of human aminopeptidase N in MDCK cells. Approximately equal amounts of protein of total cell extracts of MN cells (transfected MDCK cells expressing hAPN; lane 1), mock-transfected MDCK cells (lane 2), Caco-2 cells (lane 3), and HepG2 cells (lane 4) were subjected to immunoblot analysis. A rabbit antiserum raised against human microvillar membrane and preadsorbed to canine kidney proteins was used (as described in Materials and Methods). The positions of marker proteins and their molecular masses in kD are indicated; hAPN is marked by an arrowhead. G418-containing medium and screened for expression of hAPN by immunoblot analysis. An antiserum was used that had been raised against human intestinal microvillar membranes and preadsorbed to dog kidney membranes. Fig. 1 shows the immunoreactive material present in total cellular extracts of the transfected cell line MN (Fig. 1, lane 1), which was chosen for further analysis, in comparison to mock-transfected MDCK cells (Fig. 1, lane 2), Caco-2 cells (Fig. 1, lane 3), and human hepatoma HepG2 cells (Fig. 1, lane 4). MDCK cells are devoid of any immunoreactive material: endogenous canine APN (Louvard, 1980) is not recognized by the preadsorbed antiserum. MN cells express an antigenic protein with the apparent molecular mass of hAPN of ~150 kD, which is also present in Caco-2 cells, as expected. Several additional proteins are also recognized by our poly-

Figure 2. Processing and surface delivery of newly synthesized hAPN in MN cells. (A) Filter-grown MN cells were pulse labeled for 30 min with [35S]methionine and chased for up to 150 min. Appearance of hAPN on the cell surface was assayed by neuraminidase treatment (N) at 12°C from both sides. hAPN from total cell lysate was immunoprecipitated and analyzed by gel electrophoresis and fluorography. The high-mannose glycosylated precursor form of hAPN is indicated by an arrow, the complex glycosylated mature form by an arrowhead. (B) The experiment shown in A was quantitated by densitometric scanning of the fluorograph. The complex glycosylated mature form of hAPN is plotted as the fraction of total hAPN.

Figure 3. Polarized expression of hAPN in MDCK cells. Filter-grown MN cells were treated with neuraminidase at 12°C either on the apical (lane 2), or the basolateral (lane 3), or on both sides (lane 4). Total cell extracts were analyzed by immunoblotting and autoradiography.
hAPN Is Expressed Predominantly on the Apical Surface of MDCK Cells

To determine the polarity of surface expression of hAPN, MN cells were grown to confluence on polycarbonate filters in Transwell units (see Materials and Methods), which allow separate access to both surface domains. In a first assay, intact monolayers were treated with neuraminidase at 12°C on the apical and/or the basolateral surface (Fig. 3). In the cells treated on both sides (Fig. 3, lane 4), all hAPN had an increased electrophoretic mobility on immunoblots (Fig. 3, arrowhead), indicating that practically all hAPN molecules were accessible on the cell surface. Upon neuraminidase treatment of the apical side, the majority of hAPN is shifted (Fig. 3, lane 2), whereas only a small population was affected when neuraminidase was added from the basolateral side (Fig. 3, lane 3). As expected, hAPN is thus localized predominantly on the apical surface of MN cells.

For a quantitative analysis, filter-grown MN and MDCK cells were incubated at 4°C with the preadsorbed antiserum either on the apical or the basolateral side, followed by incubation with [125I]iodinated protein A. Total cellular extracts were fractionated by gel electrophoresis, and radioactive protein A was visualized by autoradiography (Fig. 4 A). While only low background levels of protein A were associated with untransfected MDCK cells (Fig. 4 A, lanes 3 and 4), significant amounts were bound to the apical and to a lesser extent the basolateral surface of MN cells. Quantitation of three independent experiments indicated that 79 ± 4% of surface hAPN is localized in the apical domain (Fig. 4 B).

To complement the biochemical analysis, MDCK and MN cells were characterized by EM. There was no obvious difference in the ultrastructure of the parental and the transfected cells (Fig. 5) with respect to the ratio of apical to basolateral surface (~1:1.5), the density of microvilli (2.0 per μm of crosssection), and the height of the microvilli (≈0.4 μm). hAPN distribution of MN cells was analyzed by preembedding immunogold staining (as described in Materials and Methods). The population of cells was very homogeneously labeled. High density of hAPN labeling was detected in the apical plasma membrane domain of MN cells (Fig. 6 A). The lateral and basal surface was considerably less labeled (Fig. 6, C and D). The labeling was reduced towards the upper portions of the lateral domain, which might reflect a reduced accessibility of this domain to the gold probe. Interestingly, strong labeling was observed on a number of finger-like projections of the basal membrane protruding into the filter (not shown). Almost complete absence of gold particles on the surface of parental MDCK cells demonstrated the specificity of labeling (Fig. 6 B).

hAPN Is Directly Sorted to the Apical Membrane of MDCK Cells

To find out whether hAPN is transported directly to the apical surface or via the basolateral membrane, we monitored the appearance of the newly synthesized protein on either surface domain of MN cells in pulse-chase experiments by surface immunoprecipitation. A prerequisite for these experiments is that antibodies bound to hAPN in one plasma membrane domain do not switch to other antigens (intracellular or from the other surface domain) after lysis of the cells. To test our antibodies, MN cells were grown in four wells, two of which were biosynthetically labeled with specific antiserum in Caco-2 and HepG2 cell extracts. Similar amounts of hAPN are detectable in MN and Caco-2 cells. Yet, based on enzyme assays, MN cells express 15 times more APN activity than the parental MDCK cells (977 mU/mg protein and 64 mU/mg, respectively).

Processing of newly synthesized hAPN and delivery to the cell surface was analyzed by pulse-chase experiments (Fig. 2). MN cells were labeled for 30 min with [35S]methionine and further incubated in medium containing excess nonradioactive methionine for up to 150 min. Cell extracts were analyzed by immunoprecipitation, gel electrophoresis, and autoradiography. hAPN was synthesized as a high-mannose glycosylated precursor of ~130 kD (Fig. 2 A, arrow). hAPN is thus synthesized with a reduced accessibility of this domain to the gold probe. Interestingly, strong labeling was observed on a number of finger-like projections of the basal membrane protruding into the filter (not shown). Almost complete absence of gold particles on the surface of parental MDCK cells demonstrated the specificity of labeling (Fig. 6 B).
One labeled and one unlabeled well were incubated with antiserum, lysed, mixed with the cell lysates of an unlabeled and a labeled well, respectively, and incubated overnight. Immunoprecipitates were then analyzed by gel electrophoresis and fluorography. Radioactive hAPN was precipitated only when the antibodies were initially bound to labeled cells (Fig. 7, lane 1), but not when they first bound to unlabeled MN cells (Fig. 7, lane 2).

To study the time course of appearance of hAPN on the apical and basolateral surface, filter-grown MN cells were labeled with [35S]methionine for 15 min and incubated with excess nonradioactive methionine for up to 75 min. At 4°C either the apical or the basolateral surface was then incubated with antiserum. After lysis of the cells, the bound antigen was precipitated with protein A-Sepharose and analyzed by gel electrophoresis and fluorography, as shown in Fig. 8. As expected, after 30 min of chase, labeled hAPN began to appear at the cell surface. Already at this earliest time point, the protein was found predominantly on the apical side (Fig. 8 A, lane 5). Throughout the time course and up to 24 h chase (as shown in Fig. 8 C), the ratio of apical to basolateral hAPN was very close to the ratio of 4:1 determined for the equilibrium distribution of hAPN. No transient increase of the basolateral hAPN pool, suggesting an indirect route via the basolateral membrane, could be detected. Apical hAPN, therefore, appears to be transported directly to its final location.

Discussion

In all polarized cell systems studied, basolateral membrane proteins were found to be transported directly from the trans-Golgi to the correct surface domain. For apical membrane proteins, different pathways have been observed in different epithelial cell types, as is summarized in Table I. In rat hepatocytes, three endogenous proteins were shown to be sorted indirectly via the basolateral surface to the apical plasma membrane. In MDCK cells, however, influenza virus hemagglutinin and the endogenous protein gpl4 were found to be delivered directly to the apical domain, suggesting a cell type specificity of the mechanism of apical sorting. Alternatively, since different proteins have been analyzed in these studies, the sorting pathways might be protein specific. This is supported by the recent finding that in Caco-2 cells both direct and indirect pathways are used: sucrase-isomaltase follows the direct pathway, APN and dipeptidyl-peptidase IV partially the indirect one (Matter et al., 1990). To address this question, it was important to study the sorting pathways of a single protein in different epithelial cells.

Expression and sorting of APN have already been characterized in hepatocytes and intestinal cells. APN is also endogenously synthesized by MDCK cells, although at a low level. To facilitate the analysis, we transfected the cDNA of hAPN into MDCK cells. The level of expression and the kinetics of processing and surface delivery of hAPN in MDCK cells were very similar to those in Caco-2 cells. Roughly 80% was localized on the apical, and 20% on the basolateral surface. Similar polarity has been determined for other apical markers in MDCK cells (Simons and Fuller, 1985). In pulse-chase experiments, the vast majority of hAPN was transported directly to the apical domain; no transient appearance on the basolateral surface was detected. During the entire time course, the ratio of apical to basolateral hAPN remained constant and identical to that of the equilibrium distribution of 4:1. Basolateral hAPN thus appears to constitute a stable missorted population. However, our experiments do not exclude the possibility of an exchange between the apical and the basolateral hAPN population.

In summary, APN is sorted to the apical domain directly in MDCK cells, entirely via the basolateral membrane in hepatocytes (Bartles et al., 1987), and partially via the
Figure 6. Localization of hAPN by immunogold staining. MN cells (A, C, and D) and untransfected MDCK cells (B) were characterized by preembedding immunogold labeling (5-nm gold particles) of hAPN. Intense labeling was observed in the apical plasma membrane (A). Much less labeling was found in the lateral (C) and basal plasma membrane (D). In untransfected MDCK cells no labeling could be found, neither in the apical plasma membrane (B) nor in the basolateral membrane. bm, basal membrane, lm, lateral membrane. Bars, 0.1 μm.

basolateral domain in Caco-2 cells (Matter et al., 1990). The possibility cannot be entirely excluded that hepatic APN is structurally different from intestinal APN. According to genomic Southern analysis, there is only a single APN gene (Olsen et al., 1989); however, the transcript and the protein might be differently processed (e.g., alternative splicing and posttranslational modifications). More likely, there are cell type–specific differences in the site of sorting of apical membrane proteins which occurs intracellularly in the trans-Golgi network and/or in the basolateral plasma membrane. Three factors might determine the pathway of an apical protein in a given cell. The first factor is the intensity of the direct vesicular flow from the Golgi to the apical membrane. Hepatocytes appear not to have an apical secretory pathway altogether (Coleman, 1987; Bartles and Hubbard, 1988). In Caco-2 cells it seems to be less developed than in MDCK cells: secretory proteins that can be assumed to lack sorting signals are exocytosed roughly to the same amount on both sides in MDCK cell monolayers (Gottlieb et al., 1986), but predominantly on the basolateral side in Caco-2 cells (Rindler and Traber, 1988; Hughson et al., 1989). The second factor is the quality of the sorting signal within apical proteins. The findings by Matter et al. (1990) for Caco-2 cells suggest that sucrase-isomaltase contains a “strong” sorting signal
Surface-bound antibodies do not switch antigen upon cell lysis. Antiserum was allowed to bind to the surface of biosynthetically labeled and unlabeled MN cells. After being washed, the cells were lysed and mixed with lysate of unlabeled and labeled cells, respectively. After an overnight incubation at 4°C, the antibodies were precipitated with protein A-Sepharose and analyzed by gel electrophoresis and fluorography. (lane 1) Antiserum adsorbed to labeled cells; (lane 2) antiserum adsorbed to unlabeled cells. The positions of hAPN (150 kD) and of the buffer front (F) are indicated.

efficiently recognized already in the trans-Golgi network, whereas APN and dipeptidylpeptidase IV have relatively "weak" sorting signals allowing a large population to escape to the basolateral domain by the default pathway. The third factor is the intracellular distribution of the hypothetical apical sorting receptor(s). Except for glycophospholipid-anchored membrane proteins in which the lipid anchor acts as the apical determinant (Lisanti et al., 1989b; Lisanti and Rodriguez-Boulan, 1990), however, sorting signals for vectorial transport in epithelial cells have yet to be identified.

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References

Figure 7. Surface-bound antibodies do not switch antigen upon cell lysis. Antiserum was allowed to bind to the surface of biosynthetically labeled and unlabeled MN cells. After being washed, the cells were lysed and mixed with lysate of unlabeled and labeled cells, respectively. After an overnight incubation at 4°C, the antibodies were precipitated with protein A-Sepharose and analyzed by gel electrophoresis and fluorography. (lane 1) Antiserum adsorbed to labeled cells; (lane 2) antiserum adsorbed to unlabeled cells. The positions of hAPN (150 kD) and of the buffer front (F) are indicated.

Figure 8. Polarized delivery of newly synthesized hAPN to the surface of MN cells. (A) Filter-grown MN cells were pulse labeled with [35S]methionine for 15 min and chased for up to 75 min as indicated. hAPN was immunoprecipitated either from the apical (a) or the basolateral surface (b) and analyzed by gel electrophoresis and fluorography. A representative experiment is shown. (B) The time course of the delivery of newly synthesized hAPN to the apical (filled squares) and basolateral (open squares) surface of MN cells was quantitated by densitometric scanning of the fluorograph shown in A. (C) The ratio of apical to basolateral pulse-labeled hAPN is shown for up to 24 h of chase. The data of up to 75 min of chase are from the experiment shown in A. The values for 2-24 h of chase were determined in an independent pulse-chase experiment with a 1-h pulse (average of three determinations each with SD). For comparison the equilibrium distribution of hAPN is indicated by a line (with SD).

Table 1. Sorting Pathways of Apical Integral Membrane Proteins
<table>
<thead>
<tr>
<th>Protein</th>
<th>Sorting pathway in MDCK cells</th>
<th>in Caco-2 cells</th>
<th>in hepatocytes</th>
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<tbody>
<tr>
<td>Influenza HA gp114</td>
<td>direct*</td>
<td>direct*</td>
<td>indirect*</td>
</tr>
<tr>
<td>Aminopeptidase N</td>
<td>direct†</td>
<td>direct/indirect†</td>
<td>indirect*</td>
</tr>
<tr>
<td>Dipeptidylpeptidase IV</td>
<td>direct/indirect§</td>
<td>indirect*</td>
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<tr>
<td>Sucrase-isomaltase</td>
<td>direct†</td>
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<tr>
<td>HA4</td>
<td>indirect*</td>
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In addition, APN has been reported to be directly sorted in enterocytes from pig small intestinal mucosa explants (Danielsen and Cowell, 1985) and to be indirectly sorted in enterocytes from rabbit jejunum (Massey et al., 1987). Direct sorting of the endogenous apical marker Ag517 has been demonstrated in the intestinal adenocarcinoma cell line SK-CO-15 (Le Bivic et al., 1989). * See recent reviews: Bartles and Hubbard (1988); Caplan and Matlin (1989); Rodriguez-Boulan and Nelson (1989); Wandinger-Ness and Simons (1990). † Le Bivic et al. (1990). § Matter et al. (1990). ‡ This work.