Transcytosis of Aminopeptidase N in Caco-2 Cells Is Mediated by a Non-cytoplasmic Signal*

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In Caco-2 cells, aminopeptidase N is transported to the apical membrane from the trans Golgi network by both the direct and the indirect pathway (Matter, K., Brauchbar, M., Bucher, K., and Hauri, H.-P. (1990) Cell 60, 429-437). The aim of this study was to determine the importance of the transmembrane or cytoplasmic domain of aminopeptidase N for transport of aminopeptidase N by the indirect pathway by analysis of mutated forms of aminopeptidase N recombinantly expressed in Caco-2 cells. A tail-less and two secretory forms of aminopeptidase N, all deprived of the cytoplasmic tail, were transported to the basolateral plasma membrane in proportions equivalent to the wild type enzyme. This shows that no cytoplasmic basolateral sorting signal is involved in directing aminopeptidase N to the basolateral plasma membrane. Both the wild type and the tail-less aminopeptidase N were transcytosed from the basolateral to the apical plasma membrane, whereas no transcytosis of two secretory forms could be detected, showing that the transmembrane domain is important for efficient transcytosis to take place. A significant difference in transcytosis kinetics of the human and the porcine wild type aminopeptidase N was observed. This indicates that transcytosis of aminopeptidase N from the basolateral to the apical membrane does not occur by default transport but involves an active sorting mechanism.

The plasma membrane of epithelial cells is divided into an apical and a basolateral domain by tight junctions. Polarized epithelial cells are able to selectively direct membrane-bound proteins to either of these membrane domains. It has been shown that there are at least two pathways to the apical plasma membrane. A direct pathway from the trans Golgi network to the basolateral membrane followed by transcytosis to the apical domain (for a review, see Rodriguez-Boulan and Powell (1992)). Proteins transported to the basolateral membrane seem to be signal mediated by ill-defined part(s) of the extracellular domain (Mostov, 1994). Transcytosis is promoted by ligand binding (Hirt et al., 1991a; 1991b) and phosphorylation of Ser-664 (Hirt et al., 1993; Casanova et al., 1990). The transport to the apical plasma membrane seems to be signal mediated by ill-defined part(s) of the extracellular domain (Mostov et al., 1987).

Another protein, dipeptidyl peptidase IV, can also use the indirect pathway to achieve an apical expression in both MDCK cells (Casanova et al., 1991b) and Caco-2 cells (Matter et al., 1990). It has been reported that dipeptidyl peptidase IV in MDCK cells carries a basolateral sorting signal on the cytoplasmic domain and an apical sorting signal on the extracellular domain (Weisz et al., 1992). In both MDCK cells (Weisz et al., 1992) and Caco-2 cells (Matter et al., 1990), only part of the newly synthesized dipeptidyl peptidase IV molecules use the indirect pathway, whereas the rest uses the direct pathway to the apical membrane.

We have previously shown that aminopeptidase N (APN) carries an apical sorting signal on the extracellular domain directing the protein to the apical side in MDCK cells (Vogel et al., 1992a, 1992b), and thus, in this respect, APN resembles both dipeptidyl peptidase IV and the polymeric immunoglobulin receptor. In MDCK cells, it has been shown that the majority of newly synthesized APN molecules are transported via the direct pathway (Wessels et al., 1990). In Caco-2 cells, APN is transported to the apical side via both the direct and the indirect pathways (Matter et al., 1990; Le Bivic et al., 1990). We have set out to investigate the sorting signals directing the indirect transport of APN in Caco-2 cells with special attention to possible sorting signals in the cytoplasmic tail.

In the present study, we found that the transport of APN to the basolateral membrane is independent of sorting signals in the cytoplasmic domain. However, the transmembrane domain is necessary for transcytosis of APN from the basolateral to the apical plasma membrane. In addition, we show that the Caco-2 cell line, which is of human origin, is able to transcytose human APN more efficiently than porcine APN.

MATERIALS AND METHODS

Cell Culture and DNA Constructions—Caco-2 cells (a gift from M. Spiess, Biocenter, Basel) were grown in minimal essential medium supplemented with 2 mM L-glutamine, 10% fetal bovine serum, 1% non-essential amino acids, 100 units/ml penicillin, and 100 μg/ml streptomycin, phosphate-buffered saline; s-NHS-biotin, sulfo-N-hydroxysuccinimidobiotin; s-NHS-SS-biotin, sulfosuccinimidyl 2-(biotinamido)ethyl-1,3-dithiopropionate; PAGE, polyacrylamide gel electrophoresis; APN, aminopeptidase N.
von Damm, Confluent monolayers of cells were washed once in minimal essential medium lacking methionine but containing 10% dialyzed fetal bovine serum and spun 3000 × g for 5 min. The supernatant was then incubated with streptavidin-agarose (50 μl of a 50% slurry) at 4°C overnight. The streptavidin beads were washed four times with 25 mM Tris-HCl, pH 7.8, 500 mM NaCl, and 0.5% Triton X-100 and then three times in 10 mM Tris-HCl, pH 7.8, and 150 mM NaCl. The streptavidin precipitate was analyzed on 10% SDS-PAGE gels. The gels were dried, exposed to x-ray films, or analyzed on a phosphorimager.

Enzymatic Assay—The monoclonal antibody G43 (a kind gift from Dr. B. Delmas, Institut National de la Recherche Agronomique, Jouy-en-Josas France) was raised against porcine APN and does not cross-react with human APN (Delmas et al., 1994). The monoclonal antibody 43E6 (a kind gift from Dr. R. A. Ashmun, St. Jude Children’s Research Hospital, Memphis, TN) reacts with human APN, and the supernatant from this cell line was incubated 3 h with NHS-SS-biotin (50 μl of a 50% slurry). The cells were then washed four times with PBS/CM prewarmed to 37°C and biotinylated from the basolateral side with 0.5 mg/ml s-NHS-SS-biotin dissolved in prewarmed PBS/CM for 20 min at 37°C. The cells were then chased again for 0 or 4 h as described above. If indicated the cells were reduced in freshly prepared 50 mM 2-mercaptoethanesulfonic acid (MESNA) in 50 mM Tris-HCl, pH 8.7, 100 mM NaCl, 2.5 mM CaCl2 for 3 × 20 min. The cells were washed in PBS/CM containing 20 mM iodoacetamide and subsequently dissolved in lysis buffer (10 μg/ml of pTEJ-4 containing 10% SDS-PAGE gels and quantitated as described above. The assay was performed in duplicate.

RESULTS

Secretory Forms of APN Are Secreted from Both Sides of Caco-2 Cells—We have previously investigated the recombiant expression and secretion of two secretory forms of human APN (anchor-minus and anchor/stalk-minus) in MDCK cells (Vogel et al., 1992a, 1992b). To investigate the way in which these forms of APN are secreted from Caco-2 cells, eukaryote expression vectors carrying the cDNA for anchor-minus and anchor/stalk-minus APN (Fig. 1) were transfected into Caco-2 cells, and stable clones were isolated. The media of the clones were assayed for aminopeptidase enzymatic activity. 24-h media from non-transfected Caco-2 cells contained low (0–3 mil-liunits/ml) concentrations of aminopeptidase activity, whereas 24-h media from transfected clones chosen for this study contained at least 0.6 mil-liunits/ml. 24-h media from transfected clones grown on polycarbonate filters were collected from the apical and basolateral sides and assayed for aminopeptidase activity. For each construct, two clones were investigated with essentially the same result. The anchor-minus APN is secreted 36 ± 5% to the apical side and 64 ± 5% to the basolateral side, and anchor/stalk-minus is secreted 48 ± 6% to the apical side and 52 ± 6% to the basolateral side (Table I). The distribution of the secretory forms of APN between the apical and basolateral domains is thus indeed different from the endogenous wild type membrane-bound human APN, which is mainly present on the apical membrane (Matter et al., 1990; Le Bivic et al., 1990). We were unable to detect apical to basolateral or basolateral to apical transcytosis when the secretory forms of APN were applied at either side of filter-grown non-transfected Caco-2 cells.

It has previously been shown that in Caco-2 cells about 40% of the endogenous human APN is transported by the indirect pathway (Matter et al., 1990), leaving about 60% to the direct pathway using cell culture conditions (5-13 days post-confluent) similar to ours (3-6 days post-confluent). Using 15-20 days post-confluent cells, 60–70% of the endogenous APN was transported by the indirect pathway (Le Bivic et al., 1990). The present study thus shows that Caco-2 cells transport the secre-
The N-terminal sequences of the wild type porcine APN, porcine APN mutated in tyrosine 6 (Tyr-6), porcine APN with deleted tail (tail-less), wild type human APN, anchor-minus human APN, and anchor/stalk-minus human APN. The transmembrane-spanning parts are underlined by a single line, and the hemagglutinin signal peptide is underlined by a double line. The hemagglutinin signal peptide cleavage site is marked by an arrow.

The Cytoplasmic Domain is of No Importance for the Steady State Cell Surface Distribution of APN—To investigate the role of the cytoplasmic tail, two mutations in the APN cDNA were made. To distinguish the recombinant protein from the endogenous human APN in Caco-2 cells, we used the porcine APN cDNA and analyzed the protein with a monoclonal antibody (G43) specific to porcine APN (Delmas et al., 1994). A cDNA coding for a form of the porcine APN where the 9-amino acid N-terminal cytoplasmic sequence is exchanged for a Met-Ala-Arg sequence (tail-less) was constructed (Fig. 1). In the other construct, the only tyrosine in the cytoplasmic tail of the porcine APN was mutated to alanine (Tyr-6).

The tail-less, Tyr-6, and wild type porcine APN cDNAs were transfected into Caco-2 cells, and stable clones were isolated. The clones were screened using $^{35}$S-methionine labeling followed by immunoprecipitation, and clones expressing tail-less, Tyr-6, and wild type APN were identified. Pulse-chase experiments showed that the tail-less and the Tyr-6 APN were converted from the high mannoseto the complex glycosylated form with kinetics very similar to that of the wild type porcine APN (data not shown).

To determine the polarized distribution of the recombinant proteins, filter-grown transfected Caco-2 cells were incubated at 4 °C with saturating concentrations of antiserum G43 either on the apical or the basolateral side, followed by incubation with $^{125}$I-labeled Fab fragments. For each construct, two clones were investigated with essentially the same result. Total cellular extracts were counted on a γ counter. Quantitation of two to five experiments showed (Table I) that about 70% of the cell surface expressed wild type, tail-less, and Tyr-6 porcine APN located on the apical side. These results show that the cytoplasmic tail has no influence on the steady state distribution of the porcine APN between the apical and the basolateral membrane.

Human Wild Type APN Is More Efficiently Polarized Than Porcine Wild Type APN—It has earlier been demonstrated that at steady state about 98% of the endogenous human APN resides on the apical membrane (Le Bivic et al., 1990). This is noticeably better polarization than the polarization of the recombinantly expressed wild type porcine APN reported in

### Table I

| Assay type          | Antibody binding assay | Biotinylation assay | Enzymatic activity secreted |
|---------------------|------------------------|--------------------|-----------------------------|
| Endogenous          | 95 ± 12 (n = 3)        | 94 ± 7 (n = 3)     |                             |
| human APN           |                        |                    |                             |
| Recombinant         |                        |                    |                             |
| human APN           | 36 ± 5 (n = 11)        | 48 ± 6 (n = 10)    |                             |
| Anchor-minus        |                        |                    |                             |
| human APN           | 68 ± 6 (n = 4)         | 73 ± 15 (n = 3)    |                             |
| Anchor/stalk-minus  |                        |                    |                             |
| human APN           | 69 ± 6 (n = 3)         | 77 ± 13 (n = 6)    |                             |
| Porcine APN         |                        |                    |                             |
| Tail-less           | 68 ± 6 (n = 4)         | 73 ± 15 (n = 3)    |                             |
| porcine APN         |                        |                    |                             |
| Tyr-6 porcine APN   | 72 ± 10 (n = 4)        |                    |                             |
this study. To investigate this difference in polarity, cells were transfected with an expression vector carrying the cDNA for wild type human APN, and a stable clone expressing aminopeptidase activity in amounts comparable to the clones expressing wild type porcine APN was isolated. After 3 days of confluence, the aminopeptidase N activity that could be ascribed to the endogenous human aminopeptidase N was maximally 20% of the total aminopeptidase activity in all the clones used in this paper. The antibodies available to us against human APN did not function well in the antibody-binding assay to determine the steady state distribution on the two membrane domains. Instead, a clone expressing recombinant wild type human APN was labeled with [35S]methionine for a short pulse, chased overnight, and biotinylated from either the apical or the basolateral side. Thereafter, the cells were extracted and immunoprecipitated with a monoclonal antibody against human APN (43E6), streptavidin precipitated, analyzed on 10% SDS-PAGE gels, and quantitated. The analysis showed that Caco-2 cells transfected with an expression vector containing the wild type human APN cDNA had 94 ± 7% of the surface human APN on the apical side (Table I). Non-transfected Caco-2 cells had 95 ± 12% of the surface human APN on the apical side. In a similar assay, transfected Caco-2 had 77 ± 13% of the surface wild type porcine APN on the apical side and 73 ± 15% of the surface tail-less APN on the apical domain. These results indicate that the difference in sorting efficiency is not dependent on expression levels since Caco-2 cells polarize the human APN with the same efficiency regardless of whether the cells also express recombinant human APN or not.

This difference between the human and the porcine APN was further investigated by studying the appearance of newly synthesized proteins on the two cell surface domains. Cells were pulse labeled with [35S]methionine for 20 min, and after various chase times the plasma membrane proteins present in either the apical or basolateral domain were biotinylated. Biotinylated APN was purified by immunoprecipitation using protein A-agarose, released from the beads by boiling, and reprecipitated with streptavidin-agarose. The resulting purified antigens were then analyzed on SDS-PAGE gels and quantitated. Caco-2 cells expressing recombinant wild type human APN were investigated, and the enzyme was found to be transported initially to both the apical and the basolateral membrane with a clear transient pool at the basolateral membrane (Fig. 2, panel A). The basolateral fraction disappeared after 3 h of chase, which resembles what has been reported for the endogenous human APN (Matter et al., 1990; Le Bivic et al., 1990). The porcine APN was also transported to the two membranes simultaneously (Fig. 2, panel B), and a transient pool of APN was observed at the basolateral side, albeit its disappearance from the basolateral side is slower and less effective than that of the human APN. The basolateral pool is still clearly visible after an overnight chase (data not shown). Caco-2 cells are thus able to polarize the human APN more efficiently than the porcine APN.

Both Wild Type and Tail-less Porcine APN Are Transcytosed—The appearance of newly synthesized tail-less APN on cell surface domains was also investigated as described above. The tail-less APN also appeared on both membranes, with a transient basolateral pool resembling that of the wild type porcine APN. No significant difference could be seen between the wild type porcine APN and the tail-less form.

To assess directly whether the newly synthesized wild type and tail-less porcine APN that appear transiently on the basolateral membrane are routed to the apical cell surface, we used the following protocol. Wild type or tail-less porcine APN expressing cells, grown on filters, were pulse labeled with...
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FIG. 3. Transcytosis of wild type and tail-less porcine APN in Caco-2 cells. Cells were pulsed for 20 min and chased for 60 min. The cells were thereafter biotinylated from the basolateral side with s-NHS-SS-biotin, a cleavable analogue of biotin, at 37 °C and further chased for 0 or 4 h. After this second chase, cells were reduced with MESNA from the apical (A) or basolateral (B) side or reduction was omitted (none). The biotinylated aminopeptidase N was purified by the double immunoprecipitation and streptavidin precipitation protocol described in the legend to Fig. 2, analyzed by SDS-PAGE gels, and quantitated. The means of at least two independent experiments are shown.

\[ ^{35} \text{S} \text{methionine for 20 min, chased for 60 min, and then biotinylated on the basolateral surface with s-NHS-SS-biotin at 37 °C for 20 min followed by a second chase for 0 or 4 h. At the indicated times, the cells were reduced twice with MESNA from the apical side or from the basolateral side. These experiments show that both wild type and tail-less APN, biotinylated from the basolateral side, can be partly reduced from the apical side (Fig. 3) after a 4-h chase. A control experiment showed that without a second chase, the biotin could be reduced from the basolateral side but not from the apical side. Thus, the wild type and tail-less porcine APN molecules are transcytosed from the basolateral to the apical side to approximately the same degree. \]

DISCUSSION

It is well known that both porcine and human APN form homodimers (Danielsen, 1994, Jascur et al., 1991). Thus, it is possible that the recombinant and endogenous APN expressed together in Caco-2 cells in this study form heterodimers. However, the anchor-minus and anchor/stalk-minus APN secreted from Caco-2 cells are unlikely to represent molecules that pass through the cell in a heterodimeric state (between soluble and membrane-bound APN) since we have previously experienced, during purification of the enzyme, that the dimer is very stable (Sjöström and Norén, 1982). When analyzing the recombinant-expressed porcine aminopeptidase N, it is possible that maximally ¼ of the molecules (estimated from the expression level of aminopeptidase enzymatic activity of non-transfected and transfected cells) are in a heterodimer state and thus, if present, constitute a minor fraction.

Transport from the trans-Golgi network to the Plasma Membrane—It has been demonstrated for several proteins that the signal for basolateral sorting resides in the cytoplasmic domain (for a review, see Matter and Mellman (1994)). To our knowledge, no basolateral sorting signal has been identified so far on the ectodomain of a membrane protein. The secretory and tail-less forms of APN do not carry a cytoplasmic basolateral sorting signal since they do not have a cytoplasmic tail. They are, however, still routed to the basolateral domain in proportions equivalent to the wild type enzymes. This strongly indicates that no cytoplasmic basolateral sorting signal is involved in directing APN to the basolateral membrane in Caco-2 cells.

Thus, the basolateral transport is either guided by a new class of basolateral sorting signals located on the ectodomain or the transport to the basolateral domain in this cell type occurs by default.

Transcytosis from the Basolateral to the Apical Plasma Membrane—In this study, we found that the basolateral pool of wild type human APN disappears faster than that of the wild type porcine aminopeptidase N and that the wild type human APN obtains and maintains a more polarized distribution after 2 h of chase than that of wild type porcine APN. This indicates that the human APN is transcytosed more efficiently than the porcine APN. The registered rate of transcytosis of the porcine forms could be due to dimer formation between the recombinant porcine form and the endogenous human APN. The porcine forms might thus be unable to transcytose on their own, making the difference in kinetics even bigger.

It is not known whether the transcytotic transport from the basolateral to the apical domain is a signal-mediated process, but the finding in this study that the porcine and the human APN are transcytosed with different kinetics provides evidence that this is not a default pathway, and hence active sorting is involved. The signal involved is not located in the cytoplasmic part since the human and the porcine APN transcytose with significantly different kinetics despite a 100% identity in the cytoplasmic tail. However, the transmembrane domain is necessary for the ability to transcytose efficiently, as seen by the lack of transcytosis of the soluble forms. Whether a transcytosis signal is located in the transmembrane domain or it is merely the membrane attachment that is important to ensure efficient endocytosis is not yet known.

In this study, we found that the Caco-2 cell line that is derived from a human colon adenocarcinoma is able to sort human APN more efficiently than porcine APN. When the porcine APN cDNA was expressed in MDCK cells, 92 ± 5% of the total surface-expressed APN was located on the apical domain, suggesting that the difference in polarization in Caco-2 cells is not due to accidentally introduced mutation(s) in the cloned porcine cDNA. It could be hypothesized that polarization is an old and well conserved trait, but the present study indicates that the mechanisms leading to

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polarization have undergone some modification during evolution. The differences between the efficiency in sorting of the human and porcine APN may provide an opportunity to map the sorting signal operating on the transcytotic pathway in Caco-2 cells. There is 79% identity between the human and the porcine APN amino acid sequences (Delmas et al., 1992). A range of chimeras between the human and porcine APN has already been analyzed in MDCK cells to map a virus binding side on APN (Delmas et al., 1994), and they were found to be well expressed on the cell surface indicating a correct three-dimensional structure of the chimeric molecules that allows cell surface expression.

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REFERENCES

Aroeti, B., and Mostov, K. E. (1994) EMBO J. 13, 2297–2304
Aroeti, B., Kosen, P. A., Kuntz, I. D., Cohen, F. E., and Mostov, K. E. (1993) J. Cell Biol. 123, 1149–1160
Casanova, J. E., Breitfeld, P. P., Ross, S. A., and Mostov, K. E. (1990) Science 248, 742–745
Casanova, J. E., Apodaca, G., and Mostov, K. E. (1991a) Cell 66, 65–75
Casanova, J. E., Mishumi, Y., Ikehara, Y., Hubbard, A. L., and Mostov, K. E. (1991b) J. Biol. Chem. 266, 24428–24432
Danielsen, E. M. (1994) Biochemistry 33, 1599–1605
Delmas, B., Geff, J., L’Haridon, R., Vogel, L. V., Sjöström, H., Norén, O., and Laude, H. (1992) Nature 357, 417–420
Delmas, B., Geff, J., Kut, E., Sjöström, H., Norén, O., and Laude, H. (1994) J. Virol. 68, 5216–5224
Graham, F. L., and Van der Eb, A. J. (1973) Virology 52, 456–467
Hirt, R. P., Hughes, G. J., Frutiger, S., Michetti, P., Perreux, C., Poulain-Godefroy, O., J.粪妊娠, N., Neutra, M. R., and Kraehenbuhl, J. P. (1993) Cell 74, 245–255
Jascur, T., Matter, K., and Hauri, H.-P (1991) Biochemistry 30, 1908–1915
Johansen, T. E., Schaller, M. S., Tolstoy, S., and Schwartz, T. W. (1990) FEBS Lett. 267, 289–294
LeBlanc, A., Quaroni, A., Nichols, B., and Rodriguez-Boulan, E. (1990). Cell Biol. 111, 1351–1361
Matter, K., and Meliman, I. (1994) Curr. Opin. Cell Biol. 6, 545–554
Matter, K., Brauchbar, M., Bucher, K., and Hauri, H.-P. (1990) Cell 60, 429–437
Mostov, K. E., Breitfeld, P., and Harris, J. M. (1987) J. Cell Biol. 105, 2031–2036
Okamoto, C. T., Shia, S.-P., Bird, C., Mostov, K. E., and Roth, M. G. (1992) J. Biol. Chem. 267, 9925–9932
Rodriguez-Boulan, E., and Powell, S. K. (1992) Annu. Rev. Cell Biol. 6, 395–427
Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
Sjöström, H., and Norén, O. (1982) Eur. J. Biochem. 122, 245–250
Sjöström, H., Norén, O., J. epeesin, L., Staun, M., Svensson, B., and Christiansen, L. (1978) Eur. J. Biochem. 88, 503–511
Song, W., Bomse, M., Casanova, J., Vaerman, J.-P., and Mostov, K. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 163–166
Southern, P. J., and Berg, P. (1982) Mdl. Appl. Genet. 1, 327–341
Vogel, L. K., Sjöström, H., and Norén, O. (1992a) FEBS Lett. 306, 14–17
Vogel, L. K., Spiess, M., Sjöström, H., and Norén, O. (1992b) J. Biol. Chem. 267, 2794–2797
Weisz, O. A., Machamer, C. E., and Hubbard, A. L. (1992) J. Biol. Chem. 267, 22282–22288
Wessels, H. P., Hansen, G. H., Fuhrer, C., Look, A. T., Sjöström, H., Norén, O., and Spiess, M. (1990) J. Cell Biol. 111, 2923–2930
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