Analysis of the Signals for Polarized Transport of Influenza Virus (A/WSN/33) Neuraminidase and Human Transferrin Receptor, Type II Transmembrane Proteins

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In polarized MDCK cells influenza virus (A/WSN/33) neuraminidase (NA) and human transferrin receptor (TR), type II glycoproteins, when expressed from cloned cDNAs, were transported and accumulated preferentially on the apical and basolateral surfaces, respectively. We have investigated the signals for polarized sorting by constructing chimeras between NA and TR and by making deletion mutants. NATR Δ 90, which contains the cytoplasmic tail and transmembrane domain of NA and the ectodomain of TR, was found to be localized predominantly on the apical membrane, whereas TRNA Δ 35, containing the cytoplasmic and transmembrane domains of TR and the ectodomain of NA, was expressed preferentially on the basolateral membrane. TR Δ 57, a TR deletion mutant lacking 57 amino acids in the TR cytoplasmic tail, did not exhibit any polarized expression and was present on both apical and basolateral surfaces, whereas a deletion mutant (NA Δ 28-35) lacking amino acid residues from 28 to 35 in the transmembrane domain of NA resulted in secretion of the NA ectodomain predominantly from the apical side. These results taken together indicate that the cytoplasmic tail of TR was sufficient for basolateral transport, but influenza virus NA possesses two sorting signals, one in the cytoplasmic or transmembrane domain and the other within the ectodomain, both of which are independently able to transport the protein to the apical plasma membrane.

Plasma membranes of polarized epithelial cells have two domains, apical and basolateral, separated by tight junctions (29). Each domain has a distinct protein and lipid composition (25, 29, 37). In Madin-Darby canine kidney (MDCK) cells, proteins are sorted at the level of the trans-Golgi network where apical and basolateral proteins are incorporated into distinct transport vesicles for direct delivery to the respective plasma membrane domains (19, 28). In contrast, in hepatocytes proteins are sorted via the endocytic-transcytotic route, i.e., both apical and basolateral proteins are transported first to the basolateral surface and subsequently the apical proteins are delivered to the apical surface via transcytosis (1). Intestinal epithelial cell line Caco-2 uses both pathways for polarized sorting (15, 20).

At present, there is no consensus hypothesis explaining the mechanism of polarized sorting of plasma membrane proteins in epithelial cells. One model suggests that apical transport is signal mediated, whereas basolateral transport occurs by default (37). The other model postulates that basolateral sorting is signal mediated and the apical transport occurs by default (25). Little is known about the molecular signals which direct proteins to either the apical or basolateral surface. In MDCK cells, one class of apical signal has been established. This signal was found to be glycosyl-phosphatidylinositol (GPI), which anchors the carboxy terminus of certain proteins to the lipid bilayer. GPI-anchored proteins are sorted exclusively to the apical plasma membrane (3, 17, 18). However, even GPI-anchored proteins appear to be preferentially targeted to the basolateral surface of Fischer rat thyroid epithelial cells (38). Recently, basolateral proteins, e.g., polymeric immunoglobulin receptor (4), FcRII B2 receptor, low-density lipoprotein recep-

tor, lysosomal membrane glycoprotein lgp120 (7, 21), influenza virus hemagglutinin (HA) mutant HA-Tyr543 carrying a tyrosine mutation in its cytoplasmic tail (2), human nerve growth factor receptor mutant carrying a 58-amino-acid (58-aa) deletion in the cytoplasmic domain (16), and lysosomal acid phosphatase (27), have been reported to possess a basolateral transport signal(s) in their cytoplasmic domains.

The infection of polarized MDCK cells by enveloped viruses provides a tool to study the events involved in membrane biogenesis and protein sorting. Enveloped viruses are found to bud asymmetrically from either the apical or the basolateral surface of the infected polarized MDCK cells (30, 32, 35). Influenza viruses and paramyxoviruses bud from the apical domain, whereas vesicular stomatitis virus (VSV) and several retroviruses bud from the basolateral surface (30, 32, 35). The envelope glycoproteins of these viruses are also preferentially sequestered in the same domain from which the viruses bud. Influenza virus has three envelope proteins: HA, a type I membrane glycoprotein; neuraminidase (NA), a type II membrane glycoprotein; and M2, a type III membrane protein. In MDCK cells, HA, NA, and M2 proteins, when expressed individually from cloned cDNAs, are transported and accumulated on the apical surface of the plasma membrane (10, 11, 33), demonstrating that these proteins possess the structural features which are recognized by the sorting machineries involved in both intracellular transport and polarized surface expression.

Previous studies using chimeric type I membrane glycoproteins between influenza virus HA and VSV G as well as deletion mutants of HA suggested that the ectodomain of HA possesses the signal(s) for apical transport (22–24, 34). However, little is known about the sorting signal(s) of type II viral or cellular proteins for polarized transport. In the present study, structural requirements for polarized sorting of type II membrane glycoproteins were analyzed by constructing dele-

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tion mutants in the cytoplasmic tail and by making chimeras between influenza virus NA (8, 11) and human transferrin receptor (TR) (5, 12, 36). Our results with chimeric and deletion mutants demonstrate that the cytoplasmic tail of TR, a type II basolateral protein, has the information for polarized transport, whereas influenza virus NA, a type II apical protein, possesses the signal(s) for polarized sorting on both the cytoplasmic or transmembrane domain and the ectodomain.

MATERIALS AND METHODS

Cell culture. MDCK cells were obtained from the American Type Culture Collection and maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Costar Transwell polycarbonate filter units (Cambridge, Mass.) with a pore size of 0.4 μ m were used. Cells (2×10^6) were plated and grown for 4 to 5 days prior to the experiments to form tight monolayers on the filters.

Construction of chimeric and deletion mutants of NA and TR. Construction of the chimeric NATR Δ 90 and TRNA Δ 35 has been described before (13). The generation of deletion mutants TR Δ 57 and NA Δ 28-35 was accomplished by in vitro mutagenesis (Amersham, Arlington Heights, Ill.). TR Δ 57 was obtained by loop-out mutagenesis from a plasmid pGTR (12) by using a 58-mer DNA primer. In TR Δ 57 cDNA, the nucleotide sequences encoding the 57-aa cytoplasmic domain were removed, while the four amino acids (lysine, proline, lysine, and arginine) just preceding the transmembrane domain were kept. Construction of NA Δ 28-35 was carried out by site-specific mutagenesis and will be reported elsewhere (9). All plasmids were sequenced through the chimeric junctions or deletion points to confirm that the expected codons for amino acids were in phase.

Stable expression in MDCK cells. cDNAs encoding the NA, TR, chimeric NATR Δ 90 and TRNA Δ 35, and deletion mutants TR Δ 57 and NA Δ 28-35, were cloned under the control of the metallothionein promoter of the expression vector pMEP4 containing the hygromycin resistance marker (Invitrogen Corporation, San Diego, Calif.). MDCK cells were transfected by the DNA-calcium phosphate procedure as described elsewhere (31). Clones expressing the hygromycin resistance marker were selected in the presence of hygromycin B (300 μ g/ml; Sigma Chemical Co., St. Louis, Mo.) in the culture medium. Cells expressing the proteins of interest were identified by immunofluorescence and radio immunoprecipitation assays. At least two clones expressing the desired protein from each cDNA construct were randomly selected for further analysis. The polarity of all cell lines used in the experiments was confirmed by determining the transepithelial resistance. Transepithelial resistance was measured with a voltmeter (World Precision Instruments, New Haven, Conn.). All cell monolayers exhibited resistances between 80 and 120 Ω /cm² prior to their use in experiments.

Radiolabeling and immunoprecipitation. For assaying the intracellular synthesis of the desired protein, hygromycin-resistant MDCK cells were induced with 10 μ M CdCl₂ for 16 h and pulse labeled for 2 h with 100 μ Ci each of [³⁵S]cysteine and [³⁵S]methionine (ICN, Irvine, Calif.) per ml. The cells were lysed in 0.5 ml of radioimmunoprecipitation assay (RIPA) buffer without sodium dodecyl sulfate (SDS) (50 mM Tris-HCl [pH 7.6], 150 mM NaCl, 1% Triton X-100, 0.5% deoxycholate, 1 mM phenylmethylsulfonyl fluoride), and cellular debris were removed by centrifugation. For immunoprecipitation, the lysates (0.5 ml) were incubated with either monoclonal anti-TR (Amersham) or (for NA) polyclonal anti-influenza virus (A/WSN/33) antibodies for 2 h on ice,

protein A-Sepharose (5 mg) was added, and the mixture was shaken for 2 to 3 h at 4°C. The beads containing the immune complexes were collected and washed two times with RIPA buffer containing 5 mg of bovine serum albumin per ml and 0.5 M NaCl and finally washed again with the RIPA buffer (13). The immunoprecipitated protein samples were dissolved by boiling the Sepharose beads in 25 μ l of sample buffer (62.5 mM Tris-HCl [pH 6.8], 10% glycerol, 2% SDS, 5% β -mercaptoethanol) and electrophoresed on SDS-10% polyacrylamide gels (14).

Plasma membrane domain selective biotinylation. Cells grown on Costar Transwell units were metabolically labeled for 4 h with 400 μ Ci each of [³⁵S]cysteine and [³⁵S]methionine in modified Eagle's medium lacking cysteine and methionine. Monolayers were washed three times with ice-cold phosphate-buffered saline containing 0.01% Ca²⁺ and Mg²⁺ (PBS⁺), and either the apical or basolateral surface of parallel culture units was biotinylated twice for 20 min with 1 mg of sulfo-*N*-hydroxysuccinimide-biotin (sulfo-NHS-biotin; Pierce Chemical Co., Rockford, Ill.) per ml in PBS⁺. The reaction was quenched by washing the cells with PBS⁺ containing 50 mM lysine. Filters were then cut out of the holder, and the cells were lysed in RIPA buffer without SDS for 30 min under constant agitation at 4°C. Postnuclear supernatant was precleared with Pansorbin (Calbiochem, La Jolla, Calif.) and was immunoprecipitated with monoclonal anti-TR or polyclonal anti-A/WSN/33 virus antibodies. Immunoprecipitates were washed three times with RIPA buffer. The immunoprecipitate was eluted by boiling twice in 100 μ l of elution buffer [1% SDS, 0.2 M Tris-HCl (pH 8.5), 5 mM ethylene glycol-bis(β -aminoethyl ether)-*N,N,N',N'*-tetraacetic acid] for 2 min and then washed with RIPA buffer. Both the eluates and the wash were pooled and incubated with 50 μ l of slurry streptavidin-agarose (Pierce Chemical Co.) beads for 30 min at 4°C. The beads were then washed three times with RIPA buffer and boiled in sample buffer. The supernatant was analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (14). Autoradiograms were quantified by densitometric analysis using an LKB 2222-020 Ultrascan XL laser densitometer (Pharmacia-LKB, Uppsala, Sweden).

Pulse-chase experiments. Cells grown on filters were pulse labeled with 500 μ Ci each of [³⁵S]cysteine and [³⁵S]methionine for 20 min. After being washed with PBS⁺, cells were chased for different times in modified Eagle's medium containing two times the normal cysteine and methionine concentration. Apical and basolateral surfaces were then biotinylated and immunoprecipitated. Biotinylated proteins were isolated and analyzed by SDS-PAGE and quantified as described above.

RESULTS

Construction and expression of the wild type (WT), chimeric mutants, and deletion mutants. Construction of stable MDCK cells expressing NA, TR, NATR Δ 90, TRNA Δ 35, TR Δ 57, and NA Δ 28-35 cDNAs is detailed in Materials and Methods. In NATR Δ 90 and TRNA Δ 35, the cytoplasmic tail and transmembrane domain were switched precisely and joined to the heterologous ectodomain of TR and NA, respectively (13). Figure 1 shows that NATR Δ 90 possesses the cytoplasmic tail (6 aa) and transmembrane domain (29 aa) of NA attached to the TR ectodomain (670 aa). TRNA Δ 35, on the other hand, possesses the cytoplasmic tail (61 aa) and transmembrane domain (28 aa) of TR attached to the NA ectodomain (418 aa) (13). In TR Δ 57 cDNA, the nucleotide sequences encoding the first 57 aa of the cytoplasmic domain TR were removed and the last 4 aa preceding the anchor were kept. NA Δ 28-35 cDNA is expected to code for a protein which does not have 8 aa in the

FIG. 1. (A) Schematic representation of the primary structure of NA, TR, chimeric (NATR Δ90 and TRNA Δ35), and deletion (TR Δ57 and NA Δ28-35) proteins. Dotted boxes and single lines represent the influenza virus NA sequence, and double boxes and double lines represent the human TR sequence. The second line in NA Δ28-35 depicts the secreted form of NA ectodomain. (B) Amino acid sequences of the proteins diagrammed in panel A. Vertical arrows indicate junction sequences. Dots indicate the deletion in NA. The extraneous amino acid acquired from the linker during construction is underlined.

anchor domain (9, 26). Studies to be reported elsewhere have shown that this protein when expressed in nonpolarized cells is cleaved and secreted into the culture medium (9). Although the precise cleavage site was not determined, the molecular weight estimate from the PAGE analysis of the unglycosylated protein after tunicamycin treatment supported the notion that the ectodomain lacking the cytoplasmic and transmembrane domain was secreted into the medium (9, 26). Furthermore, NA Δ28-35, when translated in vitro in the presence of dog pancreatic membrane, is translocated into the endoplasmic reticulum (ER) lumen and is cleaved, indicating that cleavage of NA Δ28-35 occurs during translocation into the ER, possibly by the signal peptidase (9).

These constructs were transfected in MDCK cells, and stable hygromycin-resistant clones expressing NA and TR proteins, as assayed by immunoprecipitation, were obtained. For immunoprecipitation, cells were pulsed with [³⁵S]cysteine and [³⁵S]methionine for 2 h and the WT and mutant proteins were immunoprecipitated with appropriate antibodies and analyzed by SDS-PAGE and fluorography (Fig. 2). The apparent molecular weights of the protein products were in good agreement with the expected molecular weights of the deletion mutants and chimeras.

Polarized expression of the WT NA and TR in transfected MDCK cells. cDNAs encoding the influenza virus NA and human TR proteins were expressed in MDCK cells by using

FIG. 2. Expression of WT, chimeric, and deletion mutants of influenza NA and human TR. Stably transfected MDCK cells were grown on 60-mm-diameter dishes (1×10^6 cells) for 24 h, induced with $10 \mu\text{M}$ CdCl₂ for 16 h, and labeled with $100 \mu\text{Ci}$ each of [³⁵S]cysteine and [³⁵S]methionine for 2 h. Cells were lysed with RIPA buffer, and samples were processed for immunoprecipitation as described in Materials and Methods. Lanes: 1, NA; 2, TR; 3, NATR Δ90; 4, TRNA Δ35; 5, TR Δ57; 6, NA Δ28-35. Marker protein sizes are shown in kilodaltons. The arrowheads indicate the positions of WT NA (lower) and TR (upper) proteins.

the metallothionein promoter vector pMEP4. Two independent MDCK clones were analyzed, yielding identical results. To determine the apical versus the basolateral distribution of the WT NA and TR proteins at steady state, we performed domain-selective biotinylation of cells cultured on permeable filters. Stable MDCK transfectants expressing NA or TR were grown on filter supports for 4 to 5 days to form tight monolayers as determined by transepithelial resistance. The cells were then induced with $10 \mu\text{M}$ CdCl₂ for 16 to 20 h and labeled for 4 h as described in Materials and Methods. The apical or basolateral surface of parallel filters was derivatized by using the non-membrane-permeating biotin analog sulfo-*N*-hydroxysuccinimidyl-biotin (sulfo-NHS-biotin). Cells were lysed, and surface-specific proteins were immunoprecipitated and analyzed as described in Materials and Methods. Results show that about 90% of NA and TR were expressed on apical and basolateral surfaces, respectively (Fig. 3). These data are consistent with those reported previously, although the domain-specific surface biotinylation technique for studying the expression of apical or basolateral surface proteins was not used in previous experiments (5, 11, 36).

Polarized expression of chimeric NA and TR proteins. In order to elucidate the structural features required for apical versus basolateral transport, we made chimeric constructions in which cytoplasmic and anchor domains of the NA and TR proteins were swapped. NATR Δ90 has the TR ectodomain containing the NA cytoplasmic and transmembrane domains, and TRNA Δ35 contains the NA ectodomain joined to the cytoplasmic and transmembrane domain of TR (13). Stable MDCK transfectants expressing NATR Δ90 or TRNA Δ35 were grown on filters and pulse labeled with [³⁵S]cysteine and [³⁵S]methionine for 4 h. The apical or the basolateral surface proteins were biotinylated. Biotinylated proteins were isolated by immunoprecipitation and streptavidin treatment and analyzed by SDS-PAGE and fluorography as described in Materials and Methods. The results (Fig. 4) show that, unlike the WT TR, NATR Δ90 was present predominantly (90%) on the apical surface of the polarized MDCK cells. On the other hand, TRNA Δ35, unlike the WT NA, was found to be localized predominantly (95%) on the basolateral surface of the polarized MDCK cells (Fig. 4). These data indicate that the cytoplasmic and transmembrane domains of NA and TR were important in polarized transport of NATR Δ90 and TRNA Δ35 proteins to the apical and basolateral surfaces, respectively.

FIG. 3. Polarized surface distribution of WT NA and TR. Monolayers of polarized MDCK cells expressing NA and TR were grown on Transwell units and labeled for 4 h with 400 μ Ci each of [35 S]cysteine and [35 S]methionine. The cell surface proteins were isolated by domain-selective biotinylation, immunoprecipitated with specific antibodies, selected with streptavidin-agarose, and analyzed by SDS-PAGE and fluorography, as described in Materials and Methods. The fluorograms were quantified, and results are expressed as the percent apical (lanes A) and basolateral (lanes B) surface expression of each protein (39).

Polarized expression of deletion mutants. Although the results above indicate that the cytoplasmic and transmembrane domains of influenza virus NA and human TR proteins play an important role in polarized transport, the precise role of neither the cytoplasmic tail or transmembrane domain nor the ectodomain could be determined from the chimeric constructs, since both the cytoplasmic tail and transmembrane domain were attached to the heterologous ectodomain with unknown transport behavior. Therefore, we constructed two deletion mutants to determine whether the ectodomain of NA or TR also possesses any signals for polarized sorting. In one construct, TR Δ 57, nucleotides encoding the first 57 aa of 61 cytoplasmic tail residues were deleted and only the methionine and the 4 aa close to the transmembrane domain were kept (Fig. 1). We tried a similar deletion for NA, removing all of the 5 aa except the methionine from the cytoplasmic tail, but this mutant was transport defective in MDCK cells (data not shown) and therefore could not be used in polarized sorting experiments. To circumvent this problem, we used an NA Δ 28-35 deletion mutant in which amino acids from 28 to 35 in the NA transmembrane domain were deleted. This deletion causes the cleavage of the mutant NA after translocation into the ER, and the ectodomain of NA is secreted into the culture medium (9). Stable MDCK transfectants expressing either TR Δ 57 or NA Δ 28-35 were grown on filters. For TR Δ 57, apical or basolateral surfaces were biotinylated as described in Materials and Methods. For NA Δ 28-35, culture media from the apical and basolateral sides of the filter were harvested and immunoprecipitated separately and analyzed by SDS-PAGE and fluorography (Fig. 5).

Surface biotinylation results show that TR Δ 57 protein was present on both apical and basolateral surfaces (Fig. 5). Thus,

FIG. 4. Polarized surface distribution of chimeric NATR Δ 90 and TRNA Δ 35. MDCK cells expressing NATR Δ 90 and TRNA Δ 35 were grown on filters for 5 days and labeled for 4 h with 400 μ Ci each of [35 S]cysteine and [35 S]methionine. The surface proteins were biotinylated, immunoprecipitated, and analyzed as described in the legend for Fig. 3. The fluorograms were quantified, and results are expressed as the percent apical (lanes A) and basolateral (lanes B) surface expression of each protein.

TR lacking the cytoplasmic tail was randomly sorted, indicating that the cytoplasmic tail of TR possesses the sorting signal for polarized expression and demonstrating that the cytoplasmic tail of TR is both necessary and sufficient for basolateral transport of TR. However, in the case of NA Δ 28-35, the NA ectodomain is secreted predominantly from the apical side, indicating that the NA ectodomain, unlike the TR ectodomain, also possesses the signal(s) for polarized transport of the protein to the apical surface.

Pulse-chase analyses of the WT and chimeric proteins. The experiments described above examined the steady-state distribution of the proteins on the cell surface or secretion into the culture medium of polarized epithelial cells. However, such steady-state cell surface distribution could be due to direct delivery of the newly made proteins from the trans-Golgi network to one or both surfaces of the cells. Alternatively, transportation of proteins to one of the cell surfaces could be followed by endocytosis and transcytosis to the opposite surface. To distinguish between these two possibilities, we determined the kinetics of the appearance of the newly synthesized proteins on the apical or basolateral surface of the polarized cells by pulse-chase experiment. Accordingly, polarized MDCK cells were metabolically labeled with [35 S]cysteine and [35 S]methionine for 20 min and the appearance of labeled proteins on the apical and basolateral surfaces at different times during chase was determined as described in Materials and Methods.

The results presented in Fig. 6 show that NA and TR appeared directly at the apical and basolateral surfaces, respectively, as expected. NA appeared on the apical surface after 1 h and continued to increase for up to 4 h (the maximum chase time). Similarly, TR appeared directly on the basolateral surface before 1 h and reached a steady-state level by 2 h. NATR Δ 90 containing the ectodomain of TR exhibited behav-

mutant of TR, TR $\Delta 57$, lacking 57 of 61 aa of the cytoplasmic tail, was missorted and was present on both apical and basolateral surfaces. This could be due to the fact that multiple signals present in the cytoplasmic tail or transmembrane domain and ectodomain of TR act cooperatively to direct TR to the basolateral surface and interruption of any of these signals will cause missorting of the protein. Alternatively, TR possesses a signal for basolateral transport within the cytoplasmic tail and therefore the removal of 57 aa from the cytoplasmic tail containing the signal causes missorting of the protein to both apical and basolateral surfaces. The fact that TRNA $\Delta 35$ in which the cytoplasmic and transmembrane domains of TR are attached to the NA ectodomain is transported to the basolateral surface would argue for the presence of a basolateral sorting signal in the cytoplasmic or transmembrane domain. Furthermore, since TR $\Delta 57$, missing only the cytoplasmic tail but possessing the transmembrane domain, is missorted, this would indicate that the cytoplasmic tail of TR and not the transmembrane domain possesses the basolateral sorting signal.

Influenza virus NA, when expressed from cDNA, is transported to the apical surface of the plasma membrane in polarized MDCK cells. The mutant NA $\Delta 28-35$ has a deletion of 8 aa in the transmembrane domain. This protein was cleaved during translocation into the ER, and the NA ectodomain was secreted into the culture medium of the nonpolarized cells (9, 26). The results in Fig. 5 show that the ectodomain was secreted predominantly from the apical surface of the polarized MDCK cells. These results would indicate either that the ectodomain of NA was secreted apically by default without the active involvement of a sorting signal or that a signal for apical sorting was present in the ectodomain of NA. We prefer the latter alternative for the following reason. The ectodomains of transmembrane apical proteins, e.g., influenza virus HA, human nerve growth factor receptor, placental alkaline phosphatase, and FcR2 B1, are transported apically even after the removal of their cytoplasmic tail or secreted apically upon cleavage by signal peptidase (4, 7, 34, 38). Furthermore, basolateral proteins, e.g., VSV G (38) or TR, are missorted to both apical and basolateral surfaces after removal of their cytoplasmic tail. These findings as well as the results reported here would support the active involvement of sorting signals for both apical and basolateral transport. On the other hand, apical transport by default has been proposed for basolateral proteins like FcR2 B2, LDL receptor, and Igpl20 which are transported predominantly to the apical surface upon removal of their tail (7). However, even for these proteins, the existence of a cryptic (or weak) apical signal within the ectodomain cannot be ruled out.

A/WSN/33 NA also appears to possess an apical sorting signal in the transmembrane domain or cytoplasmic tail, since the chimeric construct, NATR $\Delta 90$, which has the cytoplasmic tail and transmembrane domain of NA attached to the TR ectodomain was targeted predominantly to the apical surface. These results indicate that the cytoplasmic or transmembrane domain of NA possesses a sorting signal which is capable of directing a heterologous protein to the apical side. Therefore, taken together, our data support the notion that both the ectodomain and the cytoplasmic or transmembrane domain of NA possess apical sorting signals and that each signal can direct the protein independently to the apical plasma membrane in the polarized MDCK cells. The precise location of the sorting signal in the cytoplasmic or transmembrane domain remains to be determined. Multiple apical and basolateral polarized sorting signals were also found for H, K-ATPase, and low density lipoprotein receptor (6, 21). Additional chimeric

and mutational analysis of the cytoplasmic and transmembrane domains of NA should provide more details on the structural and functional basis of this apical sorting signal. Such work is in progress.

Even though the ectodomain of NA possesses an apical signal, as evident from its secretion from the apical side, the cytoplasmic/transmembrane domain of TR (TRNA $\Delta 35$) when attached to the NA ectodomain directs the protein to the basolateral surface. This would suggest that the TR basolateral signal is stronger than the apical NA signal. Similar results have been reported for an influenza virus HA-VSV G chimera possessing the HA transmembrane and ectodomain attached to the cytoplasmic tail of VSV G. The chimera was transported predominantly to the basolateral surface, demonstrating that the basolateral signal in the cytoplasmic tail of VSV G was stronger than the apical signal of HA (38). However, little is known about how the multiple sorting signals cooperate or antagonize each other. The presence of two opposing signals in the chimeric TRNA $\Delta 35$ protein would be particularly useful in dissecting this process.

It has been proposed that in polarized epithelial cells the apical pathway is signal mediated, whereas the basolateral transport is by default (37), and the reverse has been proposed by others (25). Although other explanations are possible, the results presented in this report would argue that both apical transport and basolateral transport are signal mediated in MDCK cells, since in the absence of a specific signal the protein (TR $\Delta 57$) is missorted to both surfaces. Work from other laboratories also led to similar conclusions (16).

In conclusion, using chimeric and deletion mutants of two type II proteins (influenza virus NA and human TR) we have shown that the basolateral signal in human TR is present in the cytoplasmic tail and that the apical signals are independently present in both the ectodomain and the cytoplasmic or transmembrane domain of influenza virus NA. Whether this observation is unique to A/WSN/33 NA or is more common to other type II viral and cellular apical proteins remains to be seen. Finally, these chimeric constructs directing a heterologous ectodomain to a specific plasma membrane surface would be useful in further dissection of sorting signals.

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