A Single Amino Acid Change in the Cytoplasmic Domain Alters the Polarized Delivery of Influenza Virus Hemagglutinin

Colleen B. Brewer and Michael G. Roth

Department of Biochemistry, University of Texas Southwestern Medical Center, Dallas, Texas 75235-9038

Abstract. In the polarized kidney cell line MDCK, the influenza virus hemagglutinin (HA) has been well characterized as a model for apically sorted membrane glycoproteins. Previous work from our laboratory has shown that a single amino acid change in the cytoplasmic sequence of HA converts it from a protein that is excluded from coated pits to one that is efficiently internalized. Using trypsin or antibodies to mark protein on the surface, we have shown in MDCK cells that HA containing this mutation is no longer transported to the apical surface but instead is delivered directly to the basolateral plasma membrane. We propose that a cytoplasmic feature similar to an endocytosis signal can cause exclusive basolateral delivery.

THE question of how epithelial cells synthesize and maintain domains of the plasma membrane that differ in protein composition has received abundant attention in the last ten years. Studies of the transport of both endogenous and exogenous proteins in cultured epithelial cell lines have yielded information about the final destinations of the proteins, have identified the intracellular site where sorting was likely to occur and have eliminated the possibility that differences in glycosylation are responsible for the sorting of cell surface proteins (reviewed by Simons and Fuller, 1985; Caplan and Matlin, 1989; Eaton, 1989). However, what features of these proteins are recognized by the cellular mechanism responsible for sorting them have not been identified.

In the attempt to identify determinants of sorting, several workers have recently compared pairs or groups of proteins that are structurally similar but are sorted differently. Included among these are proteins from which a region has been deleted, chimeras formed from a portion of an apical protein fused with another part of a basolateral protein, and naturally varying receptor isoforms. Two groups have redirected basal proteins to the apical surface by replacing the transmembrane and cytoplasmic domains of basolateral proteins with the attachment sequences for a glycosyl phosphatidylinositol (GPI)[1] anchor (Brown et al., 1989; Lisanti et al., 1989*a*). For each of these basal proteins there is evidence suggesting that information in the external domain is sufficient for delivery to the basal surface (Lisanti et al., 1989*a*; McQueen et al., 1987), indicating that the GPI anchor either prevents sorting to the basal surface or has a dominant effect of targeting the proteins to the apical surface. A role in sorting for cytoplasmic amino acid sequences has been reported for Fc receptor isoforms that contain or lack a functional signal for internalization through coated pits (Hunziker and Mellman, 1989). When expressed in MDCK cells, at steady state the isoform unable to be efficiently internalized is preferentially located at the apical surface and the internalization-competent form is found at the basolateral surface. These authors suggest that some basolateral proteins may be initially delivered to the apical surface and then be selected for transcytosis to the basolateral surface based only upon their ability to be internalized. On the other hand, deletion studies using the polymeric immunoglobulin receptor suggest that separate segments of the cytoplasmic domain of that protein may be responsible for initial basolateral delivery, endocytosis through coated pits, and transcytosis to the apical surface (Mostov and Deitcher, 1986; Mostov et al., 1986; Breitfeld et al., 1990). Apparently contradictory results have been obtained from some domain-deletion experiments (Roman and Garoff, 1986; Mostov et al., 1986; Stephens and Compans, 1986), even from experiments in which almost identical sequences have been deleted from the same protein (Roth et al., 1987; McQueen et al., 1986, 1987; Compton et al., 1989; Gonzalez et al., 1987; Puddington et al., 1987). Whereas some of these studies suggest that proteins can be sorted on the basis of information found in their extracellular domains, others disagree. A potential problem with the interpretation of results from all of these lines of research is that two similar proteins that differ by the addition or deletion of several amino acids or a glycolipid moiety may have unknown differences in the conformation of the rest of the protein.

To minimize the possibility that changes in protein conformation might occur at a distance from the site of mutation, we have studied a pair of proteins which differ by a single amino acid in the cytoplasmic domain. We report that the polarity of initial surface delivery of influenza hemagglutinin

1. *Abbreviations used in this paper*: A, apical; B, basolateral; C, control; GPI, glycosyl phosphatidylinositol; HA, hemagglutinin; STI, soybean trypsin inhibitor.

(HA) can be changed by the alteration of a single cytoplasmic residue.

Materials and Methods

Cell Culture and Transfection

MDCK cells were a high-passage uncloned line from American Type Culture Collection (Rockville, MD). They were routinely maintained in plastic dishes in DME supplemented with 5% FBS (HyClone, Logan, UT), and 5% Serum Plus (Hazleton Systems, Lenexa, KS) in a 37°C incubator with a humidified environment of 5% CO_2, 95% air. All experiments were performed with cells grown on Costar Transwells (Cambridge, MA), 24.5-mm diam, 3 μm pore size, in the same medium. The polycarbonate filters were seeded with 0.5 to 1 million cells each, with 2 ml medium above each filter and 3 ml in the chamber below. Monolayers were fed on the second, third, and fourth days after seeding and were used for experiments on the fifth day.

For transfection, cDNAs were subcloned between the human cytomegalovirus immediate early promoter and the human growth hormone terminator in a plasmid vector (pCB6) which also contains the Tn5 neomycin-resistance gene between the SV-40 early promoter and the SV-40 polyadenylation sequence. One day after transfection using polybrene, as described by Chaney et al. (1986), cells were trypsinized and replated at $\sim 10^5$ per 10-cm dish in the presence of 0.25 mg/ml active G418 sulfate (Gibco Laboratories, Grand Island, NY) to select cells expressing the neomycin-resistance gene. After 18–23 d, surviving colonies were cloned with glass cylinders and passed once or twice, then the selective drug was omitted in subsequent passages. Clones were screened by immunoprecipitating metabolically labeled protein from cell lysates. All experiments were performed using cell lines of passage numbers 5 through 29 after cloning.

Measurement of Resistance and Polarity of Methionine Uptake and Transferrin Uptake

Resistances were measured using a Millicell ERS voltohmmeter (Millipore Continental Water Systems, Bedford, MA) in the Transwells with fresh aliquots of the growth medium at room temperature (23–26°C). Corrected values in ohm cm^2 were calculated by subtracting 150 ohms (the resistance of a filter with no cells) from the resistance of a monolayer-covered filter, then multiplying the resistance due to the monolayer by 4.7 cm^2 (the area of a filter).

Polarity of methionine uptake was measured essentially as described by Balcarova-Ständer et al. (1984). After four washes with Dulbecco's PBS + Ca^{2+} + Mg^{2+} at room temperature, monolayers were prestarved at 37°C for 1 h with DME lacking methionine and cysteine and then were labeled from the apical or basal side for 10 min at 37°C with 10–15 μCi/ml Tran ^{35}Slabel (ICN Radiochemicals, Irvine, CA) or Expre^{35}SS (New England Nuclear, Boston, MA) in prestarve medium. After four washes with complete DME, cells were lysed in 1-ml ice-cold NP40 lysis buffer (composition described below in "Pulse-chase assays" section), and gently scraped. Lysates were centrifuged in a microfuge at 10,000 *g* for 10 min to pellet debris and intact nuclei. Aliquots of supernatants (70 μl each) were spotted onto filter strips, which were allowed to dry and then soaked in ice-cold 10% TCA for 30 min, washed 5 min each in 1:1 ether/ethanol, then ether, and then allowed to dry. Filter strips were cut and counted in a scintillation counter. The number of TCA-soluble counts adhering to filter strips were determined by including filter strips with no lysate in each TCA incubation, and then treating them in parallel with the others. Counts for these control strips were subtracted from counts for the sample strips and typically amounted to 10–20% of counts for the apical samples. Results were expressed as basolateral: (apical + basolateral) ratios.

Dog transferrin (apo form; Sigma Chemical Co., St. Louis, MO) was iron saturated using nitriloacetic acid by the method of Bates and Schlabach (1973) and separated from unincorporated iron on a Sephadex G-25 column (Pharmacia Fine Chemicals, Piscataway, NJ). It was then labeled using Na^{125} I (New England Nuclear) and Iodobeads (Pierce Chemical Co., Rockford, IL) and separated from unincorporated radioactivity on another G-25 column. Monolayers on filters were first washed three times with neutral PBS + Ca^{2+} + Mg^{2+} at room temperature and then twice for 15 min each with DME + 20 mM Hepes (pH 7.4) + 0.5% BSA at 37°C. Next iodinated holotransferrin in the same medium was allowed to bind and internalize at 37°C for 30 min, from either the apical or the basal surface. A volume of 0.15 ml was used on the bottom of the filter and 0.38 ml on top, with the filter unit resting on Parafilm. After this incubation, cells were washed exhaustively (at least seven times) with alternate washes of PBS + Ca^{2+} + Mg^{2+} (pH 5) + 0.5% BSA and the same solution at pH 8, all at 4°C. Filters were cut out of filter units and counted in a gamma counter. Nonspecific binding/uptake was estimated by incubation with a 100-fold excess of unlabeled holotransferrin on the same side of the monolayer as the labeled protein. Counts from these samples were subtracted from total counts to give specific counts, and basolateral: (apical + basolateral) ratios were calculated.

Several cell lines were tested, and in every case the results of the transferrin uptake assay agreed with the results of methionine uptake measurements (not shown). Because the transferrin uptake experiment was inconvenient and expensive, it was not used for cell lines determined to be polarized by all other criteria.

Virus Infection

5-d-old monolayers of 0.5–1 million cells on Transwells were washed four times with Dulbecco's PBS + Ca^{2+} + Mg^{2+} at room temperature to remove serum components. The cells were then incubated on the apical surface with influenza virus at multiplicity of infection of ~10–20 pfu/cell in 0.5 ml serum-free DME, with 1.5 ml of the same medium on the basal side of the filter. After 1-h incubation at 37°C, the inoculum was replaced with fresh DME and the cells were incubated 3 h at 37°C to allow infection to proceed and then washed and subjected to a pulse-chase protocol as described below.

Pulse-Chase Assays Using Trypsin

Cells were washed four times with Dulbecco's PBS + Ca^{2+} + Mg^{2+} at room temperature to remove excess methionine and cysteine, and then were prestarved in DME lacking these amino acids for 30 min to 1 h at 37°C. Cells were labeled from the basal side with ^{35}S amino acids by placing the filter directly on a drop of 0.15 ml of the labeling medium on a piece of Parafilm, using 0.38 ml of the same medium without label to keep the apical side wet. Labeling was for 10 min in a humidified environment at 37°C with 0.3 mCi/ml for virus-infected cells and 1.2 to 2.4 mCi/ml for others. After labeling, cells were subjected to one of three chase and trypsinization procedures.

(*a*) Virus-infected cells were chased at 37°C in DME for various periods of time and then trypsinized for 30 min on ice with ice-cold trypsin (TPCK-treated, Sigma Chemical Co.) at 100 μg/ml on one side of the cells and with soybean trypsin inhibitor (STI, Sigma Chemical Co.) at 100 μg/ml on the other side. Then the trypsinized side of the cells was incubated with STI (same concentration) on ice for 10 min to inhibit further trypsinization and cells were lysed on ice in 3 ml NP40 lysis buffer (50 mM Tris, pH 8, 10 mM EDTA, 0.1 U/ml Aprotinin, 1% vol/vol NP40, 0.1% SDS) containing 100 μg/ml STI.

(*b*) Cells were incubated for 2 h in a 20°C water bath in 10 mM Hepes, pH 7.3 + DME containing only 1/3 the usual bicarbonate, and then chased at 37°C and trypsinized and lysed as above.

(*c*) Cells were chased for various periods of time at 37°C in DME containing 10 μg/ml trypsin on one side and 20 μg/ml STI on the other. At the end of the chase period, the cells were placed on ice, and the trypsinized side of the cells was incubated with 20 μg/ml STI for 10 min. Cells were then lysed as above.

Control experiments were performed to determine whether monolayer integrity was maintained under these conditions of trypsin and temperature. Monolayers on filters were incubated in DME with ^{3}H-inulin added to the apical medium and trypsin in the apical or basal medium. Samples of basal medium were counted at 30-min intervals. No leakage of ^{3}H-inulin into the basolateral medium was observed until after 1.5 h with either 10 μg/ml trypsin at 37°C or 100 μg/ml trypsin at 4°C.

Immunoprecipitation, Electrophoresis, Fluorography, and Densitometry

After 15-min lysis on ice, cells were gently scraped, and lysates were centrifuged at 4°C for 30 min at 12,000 *g* in a fixed-angle rotor to remove cell debris and nuclei. Aliquots of supernatants (1.5 or 2 ml) were diluted to 3 ml with NET gel (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.05% NP40, 0.25% gelatin, 5 mM EDTA, 0.02% NaN_3), and then the protein of interest was immunoprecipitated as follows: for Aichi HA - 0.5 μl anti-Aichi-HA goat serum with 100 μl of 10% protein A - Sepharose (Pharmacia Fine Chemicals, Piscataway, NJ) or for Japan HA - 0.3 μl anti-Japan-HA rabbit serum with 40 μl of 10% protein A - Sepharose. Immunoprecipitates were washed successively with NET gel, NET gel + 0.5 M NaCl, NET gel, NET

gel + 0.1% SDS, NET gel and then washed with Tris-Cl, pH 7.4 (each wash 3 ml). Precipitated proteins were eluted by boiling 2 min with sample buffer containing 2% SDS and 2% mercaptoethanol, Sepharose was repelleted, and aliquots of supernatant were subjected to discontinuous SDS polyacrylamide electrophoresis (separating gel of 12.5% polyacrylamide). Within each experiment, equal volumes of all samples were loaded on gels. After fixation, gels were soaked in 1 M salicylic acid with 1% glycerol for fluorography, dried, and allowed to expose preflashed X ray film (Kodak XAR 5; Eastman Kodak Co., Rochester, NY). Bands on the film were quantitated by densitometry using a laser scanning densitometer (model 300K; Molecular Dynamics, Sunnyvale, CA). Care was taken to ensure that all bands quantitated were within the range of linear response of the film.

Surface Antibody Binding or Uptake

For these assays the antiserum to Japan HA was first preadsorbed to fixed cells of the parent MDCK line to remove antibodies which bind to endogenous MDCK proteins. Immediately before use, the antiserum was heated at 55°C for 25 min to inactivate complement. Monolayers of Tyr 543 cells were washed, prestarved, and pulse labeled as described under "Pulse-chase assays using trypsin" and then incubated for 2 h at 20°C.

For surface antibody capture experiments, cells were washed with low-bicarbonate DME containing 10 mM Hepes, pH 7.3, and 1% BSA and then antiserum at a final dilution of 1:70 to 1:170 was applied to one side of the monolayer, and the cells were incubated at 37°C for 30 min. In some experiments, 10 μg/ml trypsin was included in the medium opposite the antiserum during this 30-min incubation and for a further 30 min at 37°C after removal of antiserum and then the trypsin was inactivated with medium containing 20 μg/ml of STI before cells were lysed. In other experiments the incubation with antiserum was for 60 min at 37°C followed by 30 min on ice. In all cases, cells were then washed three times with the BSA solution and lysed as above.

For antibody internalization experiments, after the 2 h chase at 20°C, an additional chase of 30 min at 37°C was used to allow the labeled proteins to reach the surface. Cells were washed in BSA solution as above, antiserum was applied for 30 min on ice, and internalization was permitted during short chases at 37°C. Cells were returned to ice for 45 min for treatment with 100 μg/ml trypsin in low-bicarbonate medium buffered with Hepes and then trypsin was inactivated by a 10-min treatment with medium containing 100 μg/ml STI. Cells were washed three times with BSA solution and lysed.

Biotinylation and Recovery of Biotinylated Tyr 543

The method was similar to that described by Le Bivic et al. (1989). After cells were washed five times with ice-cold PBS + Ca^{2+} + Mg^{2+}, NHS-LC-biotin (Pierce Chemical Co.) was applied at 0.5 mg/ml in PBS + Ca^{2+} + Mg^{2+} three times successively for 15 min each time at 4°C in 1.5 ml in the basal chamber of the Transwell, using 1 ml of the same solution without NHS-LC-biotin in the apical chamber to keep cells wet. The reaction was quenched with two washes of ice-cold DME. After chases of various times, the lysis of cells, immunoprecipitation of Tyr 543, and washing of immunoprecipitates were performed as described above. Tyr 543 was eluted from protein A - Sepharose using 40 μl of 10% SDS. For avidin-agarose precipitation of biotinylated Tyr 543, the SDS was diluted to a final concentration of 0.12% with NET gel, BSA was added to a concentration of 0.17%, and 30 μl of 50% avidin-agarose (Sigma Chemical Co.) were used in a final volume of 3 ml. Washing and subsequent treatment of the avidin-agarose precipitates were as described above for immunoprecipitates.

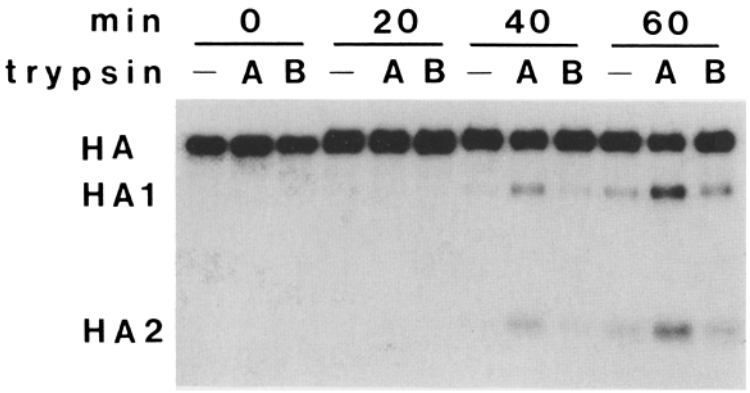
Results

Cell Lines Expressing Tyr 543

Polarized epithelial cells of the Madin-Darby canine kidney (MDCK) line are easily grown on permeable filter supports, where they form monolayers of sufficient tightness to separate the media which bathe the apical and the basolateral cell surfaces (Misfeldt et al., 1976; Cereijido et al., 1978). We have constructed MDCK cell lines stably expressing a mutant of the A/Japan/305/57(H2) HA in which cysteine 543 of the cytoplasmic domain has been changed to tyrosine. This protein, Tyr 543, has no detectable change in overall structure, but differs from the wild-type protein in that Tyr 543 is efficiently incorporated into clathrin-coated pits (Lazarovits and Roth, 1988), whereas HA is not (Roth et al., 1986; Gottlieb et al., 1986). To be assured that the transfected cell lines were still properly differentiated, we tested for several common characteristics of polarized MDCK cells. One test was to infect the cell lines with A/Aichi/2/68(H3) influenza virus, which has an HA that is antigenically distinct from Tyr 543, and to monitor the appearance of metabolically labeled Aichi HA at the apical or basal cell surface. Because HA monomers of H2 and H3 subtypes do not form mixed trimers (Boulay et al., 1988), the Aichi HA could be immunoprecipitated with no contamination from the transfected Japan HA. Cells grown on permeable supports were infected and pulse labeled, and the proteins were chased for various times at 37°C. The cells were then cooled and trypsin was added at 4°C to medium on either the apical or basal side of the cells to cleave the HA present at each cell surface into HA1 and HA2. The extent of cleavage was measured in cell lysates by immunoprecipitation, PAGE, and densitometry. Fig. 1 shows the result of one such experiment. The time course of appearance of labeled HA at each surface in these cells is comparable to the result originally published for this assay (Matlin and Simons, 1984), with apical expression exceeding basolateral expression at every time point. The percent of HA cleaved by endogenous proteases during the experiment was determined at each time point for a sample to which no trypsin was added. These values varied from 0 to 13% and were subtracted from the corresponding percent cleaved on the apical or basal surface. After 1 h of chase, 85% of the surface HA was apical, compared with values of 75 and 85% in the Matlin and Simons report (1984). Along with the ratios of apical to total surface HA at the end of 60 min of chase, measurements of transmonolayer electrical resistance and results of other assays of polarity for our cell lines are reported in Table I. In addition to the assay for delivery of HA to the plasma membrane, which shows correct polarity of an apical marker, the polarity of [^{35}S]methionine uptake was used as a measure of correct sorting of an endogenous basolateral protein (Balcarova-Ständer et al., 1984). Using this assay, we again obtained results comparable with published values for untransfected cells. In some of our cell lines, we also verified correct (basolateral) polarity of uptake of ^{125}I-labeled transferrin by the endogenous, basolateral transferrin receptor. All of our transfected cell lines had transmonolayer resistances appropriate for MDCK cells. Since our parent cell line was a mixture of high- and low-resistance cells (Barker and Simmons, 1979), it is not surprising that we obtained cloned transfectants of both high and low resistance.

Endocytosis of Tyr 543

Unlike wild-type HA, Tyr 543 is rapidly endocytosed after its arrival at the cell surface in CV-1 cells (Lazarovits and Roth, 1988; Ktistakis et al., 1990). To verify that Tyr 543 is also efficiently endocytosed in MDCK cells, we assayed the rate of internalization of metabolically labeled Tyr 543 that had bound antibodies at the cell surface. Cells on filters were pulse labeled and after proteins were chased to the surface, antiserum was added to the medium at 4°C, and excess antibodies were removed. The cells were rapidly warmed to 37°C and allowed to internalize proteins for various intervals. The cell monolayers were returned to ice, and



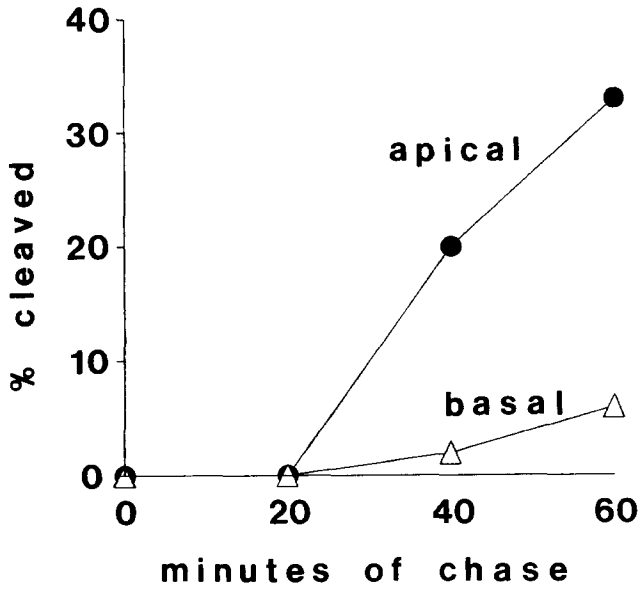


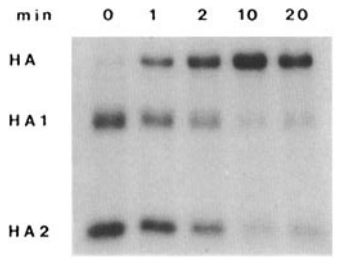
Figure 1. Surface arrival of HA during influenza infection. 3 h after infection, Tyr 543 c5 cells were pulse labeled, HA was chased to the surface for the indicated times at 37°C, and then trypsin was applied apically (*A*) or basolaterally (*B*) on ice to cleave HA into HA1 and HA2. After cell lysis, immunoprecipitation was with antiserum which recognized the virus HA (Aichi) but not the transfected HA (Japan). Control samples (–) were treated the same except that trypsin was omitted during incubation on ice. Immunoprecipitated proteins were analyzed by PAGE, fluorography, and densitometry. The graph shows values of (HA1 + HA2)/(HA + HA1 + HA2) for apically and basally trypsinized samples after correction for the endogenous cleavage in controls.

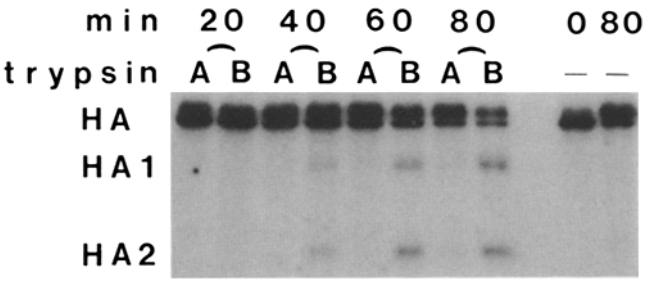
ice-cold trypsin was applied to cleave Tyr 543 remaining at the cell surface. The results of such an experiment are shown in Fig. 2 with a graph displaying individual values from 3 separate experiments. The apparent first-order rate constant of endocytosis of surface Tyr 543 is about 20% per minute under these conditions. The amount of internalized Tyr 543 leveled off at <100%, probably as a result of recycling to the surface. Although our estimate of the internalization rate constant exceeds that calculated for CV-1 cells expressing Tyr 543 via a recombinant SV-40 virus system (Ktistakis et al., 1990), the experimental differences are so great that it is impossible to infer that the rate constants are significantly

Table I. Characteristics of Cell Lines

Cell line	R	Surface HA from infection	Methionine uptake	Transferrin uptake
	(Ω cm^2)	(% *Apical*)[a]	(% *Basal*)[b]	(% *Basal*)[b]
MDCK parent	360 ± 100 (7)	ND	95 ± 2 (11)	99 ± 0 (3)
Tyr543 c5	1650 ± 460 (4)	85	96 ± 0 (4)	100,99 (2)
Tyr543 c15	340 ± 70 (4)	81	95 ± 1 (3)	ND

Values are given as mean ± SD (n) if $n > 2$. For $n = 2$, both values are shown. Other values represent one experiment. *ND*, Not done. *a*, Values are apical/(apical + basal) expressed as percent. *b*; Values are basal/(apical + basal) expressed as percent. The polarity of surface expression of HA in cells infected with influenza virus was measured by trypsinization of metabolically labeled HA at the cell surface. Uptake of methionine was measured by TCA precipitating proteins from lysates of monolayers that had been allowed to take up radiolabeled methionine. The polarity of transferrin receptors was measured as cell-associated radioactivity remaining after ^{125}I-transferrin was allowed to be internalized and surface transferrin was removed.





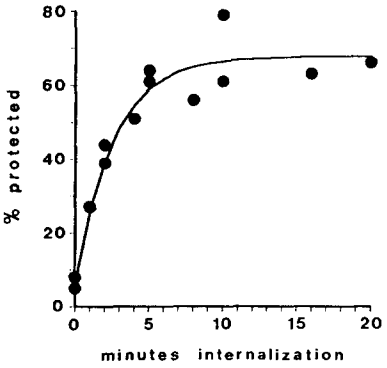


Figure 2. Endocytosis of Tyr 543 in MDCK cells. Tyr 543 c15 cells were pulse labeled, Tyr 543 was chased to the surface, and then polyclonal antiserum to Japan HA was applied to the basolateral side of the cells on ice. After being washed, cells were allowed to internalize Tyr 543 at 37°C for intervals as shown. Cells were returned to ice and trypsin was applied to the surface to cleave any HA which was not protected by internalization. Protein A-Sepharose was added to cell lysates to precipitate antibody-bound proteins, which were then analyzed by PAGE, fluorography, and densitometry. The percent of HA protected was calculated as HA/(HA + HA1 + HA2). The graph shows values from three experiments.

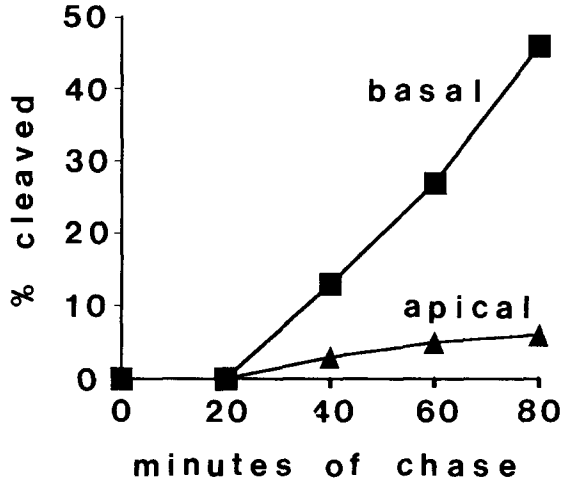


Figure 3. Cumulative surface arrival of Tyr 543. Tyr 543 c5 cells were pulse labeled, then Tyr 543 was chased to the surface for the indicated times at 37°C with trypsin on the apical (*A*) or basolateral (*B*) surface, to cleave all of the Tyr 543 which arrived on the surface during the chase. After cell lysis, Tyr 543 was immunoprecipitated with anti-Japan-HA and analyzed by PAGE, fluorography, and densitometry. Control samples were chased for 0 or 80 min without trypsin. Values on the graph are uncorrected. A similar result was obtained using the Tyr 543 c15 cell line.

different. However we have clearly demonstrated that, in our transfected MDCK cells, Tyr 543 exhibits its characteristic behavior of efficient endocytosis.

Delivery of Tyr 543 to the Surface, Trypsin Assays

Because of the rapid endocytosis of Tyr 543, we modified the surface-arrival assay shown in Fig. 1 to allow detection of Tyr 543 that might appear only transiently on the cell surface. Instead of adding trypsin to the cell surfaces on ice at the end of the 37°C chase, we included a low concentration of trypsin in the chase medium on either side of the cells. Hence, trypsin was continuously present in the external medium and in the fluid filling all endocytic vesicles derived from the plasma membrane facing the compartment containing trypsin. Since internalized trypsin is active in the early endosome (D. Zwart and M. Roth, unpublished results), any Tyr 543 appearing on the side of the cell exposed to trypsin would be in contact with active trypsin for 10 min or more. At the end of the chase period, further trypsin activity was inhibited with soybean trypsin inhibitor, and the cells were lysed, and immunoprecipitated using anti-Japan–HA antiserum. The result of such an experiment is shown in Fig. 3. At every time point when Tyr 543 was detected at the cell surface, the cleavage of Tyr 543 was much greater when trypsin was on the basolateral side of the cells than when trypsin was present at the apical surface. The endogenous cleavage of Tyr 543 (4% of total HA in this experiment) was much less than that of the Aichi HA seen in Fig. 1, so the values shown in Figs. 3 and 4 were not corrected. In two experiments, the percent of Tyr 543 cleaved when trypsin was present at the apical cell surface was not significantly different from the percent cleaved in the absence of added trypsin. In a similar experiment (not shown), during a chase of 160 min, Tyr 543 was still not detected at the apical surface.

The time course of arrival of Tyr 543 on the basolateral surface is similar to such data for several endogenous proteins (Lisanti et al., 1989*b*) and is completely consistent with intracellular sorting directly to the basolateral domain. However, it might be argued that this protein had been delivered first to the apical surface but was internalized very rapidly and transcytosed. Therefore we wished to eliminate the possibility that a significant amount of Tyr 543 appeared at the

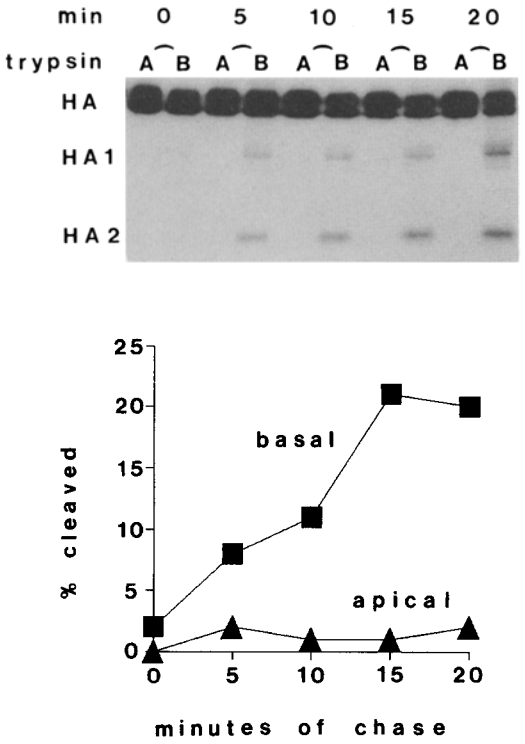


Figure 4. Surface arrival of Tyr 543 after 20° block. Tyr 543 c5 cells were pulse labeled, Tyr 543 was chased into the Golgi for 2 h at 20°C and then chased to the surface for the indicated times at 37°C. Apical (*A*) or basolateral (*B*) cell surfaces were then treated with trypsin on ice to cleave the Tyr 543 which occupied the surface at the end of the chase. After cell lysis, samples were treated as in Fig. 3. Similar results were obtained with Tyr 543 c15 cells.

apical surface but was endocytosed too rapidly to be detected by our assay. We performed a control experiment (not shown) using virus-infected cells to determine whether HA which came into contact with trypsin for only a short time would be cleaved. In 1 min or less, 40% of HA on the apical surface was cleaved by exposure to 10 μg/ml trypsin at 37°C. This result indicates that if, for example, only 50% of Tyr 543 visited the apical surface and stayed for only 1 min before being transcytosed to the basolateral surface, then apical trypsin would cleave 20% of the surface Tyr 543 (above background cleavage), in contrast with the background level actually observed.

The next assay of surface arrival was designed to avoid any possible problem caused by intracellular cleavage of Tyr 543 because of trypsin internalization during the chase. Cells were pulse labeled and then chased for 2 h at 20°C to block the progress of labeled glycoproteins from the Golgi complex to the surface, causing them to accumulate in the *trans*-Golgi network (Matlin and Simons, 1983). This accumulation permitted the use of short chases to the surface, augmenting the size of the cohort of Tyr 543 which reached the surface at any one of the early time points. This signal-enhancing technique was desirable not only because of the rapid endocytosis of Tyr 543 but also because the level of expression of transfected proteins is much lower than that of HA during influenza infection. After 2 h at 20°C followed by short chases at 37°C, monolayers were trypsinized at 4°C from the apical or basal side. The results from such an experiment are shown in Fig. 4. Again, cleavage of Tyr 543 was detected exclusively when trypsin was present at the basal surface, implying that the protein was transported directly to the basolateral surface without first appearing at the apical surface.

The data shown in Fig. 3 leave open the possibility that a significant portion of Tyr 543 is delivered to the apical surface but is very rapidly degraded by a protease that is active only on the apical side. In that experiment, approximately one-fourth of the labeled Tyr 543 has been degraded between the 20 and 80-min time points. However, the data shown in Fig. 4 cannot be explained in this way. At time points of 15 and 20 min, ~20% of the labeled Tyr 543 is at the basolateral surface and <3% is at the apical surface, but no degradation has occurred during this chase period, i.e., the total amount of labeled Tyr 543 has not declined between 0 and 20 min. Therefore the preferential appearance at the basolateral surface cannot be explained by an apical-specific loss of the protein.

Surface Delivery of Tyr 543 Measured by Antibody Binding

Because of the previous suggestion that endocytosed proteins might be delivered to the apical surface and then internalized and transcytosed to the basolateral surface (Hunziker and Mellman, 1989), we used an additional assay, antibody capture, which was not dependent upon accessibility to a single region of the HA external domain, to verify that Tyr 543 was not first delivered to the apical surface. Tyr 543 was pulse labeled at 37°C and chased for 2 h at 20°C as before. Then polyclonal anti-HA antibodies were included in culture medium on either the apical (A) or basolateral (B) side of the cells for 30 min at 37°C. Unbound antibodies were removed, and complexes of antibody and Tyr 543 were precipitated from cell lysates with protein A-Sepharose and analyzed as shown in Fig. 5. In agreement with the trypsin-cleavage results, in two experiments, 97–99% of Tyr 543 that bound antibodies during the 30-min chase was basolateral. To test whether Tyr 543-antibody complexes were transcytosed to the opposite cell surface, we included trypsin on the side opposite the anti-HA during the antibody incubation and for 30 min thereafter (at 37°C). Transcytosis was not detected during this time period, since no HA1 or HA2 is seen in Fig. 5 *a*. This negative result does not eliminate the possibility of transcytosis, since antibodies may dissociate from Tyr 543 during passage across the cell or after arrival at the opposite surface in the presence of trypsin at 37°C. Lanes *A* (control; *c*) and *B* (*c*) contain controls indicating that nonspecific binding of anti-HA antibodies did not occur during the experiment. Each of these two samples represents the combined lysates of two monolayers, one of MDCK parent cells that were not labeled but were treated with anti-HA in parallel with the experimental cells, and one of cells expressing Tyr 543 that were not treated with antibody but were labeled in parallel with the experimental cells. No Tyr 543 band is seen in Fig. 5 *a*, lanes *A*(*c*) or *B*(*c*), indicating that no antibody bound nonspecifically to the cell surface or to the polycarbonate filters and contributed to the observed signal by binding to labeled Tyr 543 proteins after cell lysis. For verification that individual cultures were expressing similar levels of Tyr 543 during this experiment, the supernatants from immunoprecipitation with surface-bound anti-HA were reprecipitated with additional anti-HA antiserum and protein A-Sepharose

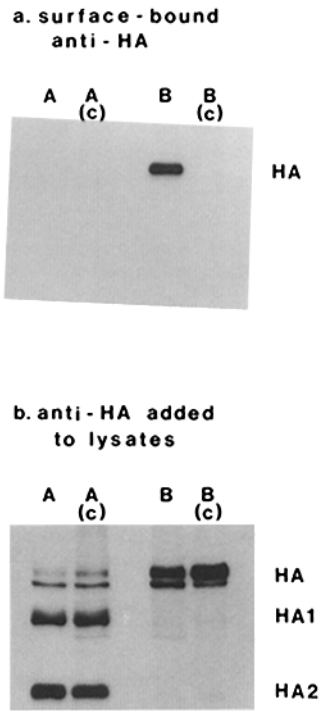


Figure 5. Surface arrival of Tyr 543 measured by antibody capture. Tyr 543 cl5 cells were pulse labeled and labeled proteins were allowed to migrate to the Golgi complex for 2 h at 20°C in the absence of radioactive label. Then Tyr 543 was chased to the surface at 37°C for 30 min with polyclonal antibodies to HA present in the medium on either the apical (*A*) or basolateral (*B*) side of the cells. Trypsin was simultaneously present in the medium on the opposite side of the cells. The trypsin incubation was continued for an additional 30 min at 37°C after the removal of the medium containing antiserum in an attempt to detect Tyr 543 which might have been transcytosed after binding antibody. Controls (*c*) for nonspecific antibody binding were performed as described in the text (see Results). After cell lysis, surface-bound antibody–HA complexes were immunoprecipitated with protein A-Sepharose, and the resulting supernatant solution was reimmunoprecipitated to recover the remaining Tyr 543. Precipitated proteins were analyzed by PAGE, fluorography, and densitometry. Of the Tyr 543 detected at the surface, 99% was basolateral, and none of this was cleaved by trypsin on the apical side of the monolayer.

to detect the remaining Tyr 543 proteins, which had not bound antibody during the initial chase. The fluorograph shown in Fig. 5 *b* indicates that all of the samples were labeled to approximately the same extent. In addition, lanes *A*(*c*) and *B*(*c*) contain levels of immunoprecipitated protein similar to those in lanes *A* and *B*, indicating that the presence of antibody did not result in significant loss of Tyr 543; thus, Tyr 543 with antibody bound was not preferentially routed to lysosomes. In the cells incubated with antibody on the apical surface and trypsin on the basolateral surface (*A* and *A*[*c*]), the vast majority of Tyr 543 is trypsinized, while cleavage is not seen in the other two samples, in accord with the previous trypsin assays. As expected, Fig. 5 *a* lane *B* shows that only the form of Tyr 543 containing complex oligosaccharides was precipitated by antibodies present at the cell surface, while Fig. 5 *b*, lane *B* has also the faster-migrating, high-mannose form which does not appear on the cell surface. This observation confirms that none of the Tyr 543 precipitated in the surface-binding assay was derived from association with antibodies after cell lysis.

As a further test of whether basal-to-apical transcytosis of Tyr 543 occurs after its initial basal delivery, we performed an additional experiment (not shown). Metabolically labeled protein was chased to the cell surface, biotinylated on ice on the basolateral side, and then chased for periods up to 2 h with trypsin in the apical chase medium. Tyr 543 molecules were immunoprecipitated with anti-HA on protein A - Sepharose and then eluted by boiling with an SDS solution. The biotinylated molecules were reprecipitated with avidin–agarose and electrophoresed. The fluorogram indicated that the biotinylated Tyr 543 was not cleaved by trypsin while control samples showed that recovery of biotinylated Tyr 543 was not biased against the trypsin-cleaved form. This experiment confirms the lack of basal-to-apical transcytosis and agrees with the results of the pulse–chase experiments which indicate no significant apical delivery of Tyr 543 during 160 min of chase. The amounts of Tyr 543 which could be detected at the apical surface were too small to permit testing for apical-to-basal transcytosis in the same type of experiment.

Discussion

Our results can be summarized as follows: the replacement of cysteine 543 in the cytoplasmic domain of Japan HA with a tyrosine residue causes the protein to be sorted directly to the basolateral surface, in contrast to the apical sorting of the original protein. We interpret our results as indicating the existence of an efficient cytoplasmic signal for protein delivery to the basolateral surface.

In comparison with previous studies of polarity of mutagenized proteins, our work used a single amino acid mutation to reduce the possibility of long-range effects on the structure of the protein. In fact, the Tyr 543 mutant of HA has previously been characterized with respect to overall structure and found to be very similar to its parent molecule. In CV-1 cells, Tyr 543 has been shown to resemble closely the wild-type HA in kinetics of exocytosis and in adopting a folded conformation which prevents degradation by added trypsin (Lazarovits and Roth, 1988). These properties are not shared by several HA mutants which do not assume a native structure (Doyle et al., 1985; Copeland et al., 1986; Lazarovits et al., 1990). Also, Tyr 543 is recognized at the cell surface by a trimer-specific mAb and not by a polyclonal antiserum for unfolded HA, in common with wild-type HA (Lazarovits and Roth, 1988; Gething et al., 1986). We cannot entirely rule out the possibility that Tyr 543 has a small conformational change in the transmembrane domain or the extracellular domain that causes the change in sorting. However, it is much more straightforward to attribute the dramatically altered sorting to a change in the structure of the cytoplasmic domain itself, especially in view of the previous reports that cytoplasmic sorting signals exist in some membrane proteins. Two naturally occurring isoforms of a murine macrophage-lymphocyte Fc receptor (FcRII), which differ only in their cytoplasmic regions, are sorted differently when they are expressed in MDCK cells (Hunziker and Mellman, 1989). The polymeric immunoglobulin receptor is sorted directly to the apical surface when its cytoplasmic domain has been removed, although the complete receptor is delivered first to the basolateral surface (Mostov et al., 1986; Mostov and Deitcher, 1986). In the case of the pIg receptor, it appears that the external domain may contain an apical sorting signal, which is overridden by a dominant cytoplasmic basolateral signal. This may parallel the case of Tyr 543, since previous work from our laboratory has suggested the presence of an apical sorting signal in the luminal domain of HA (Roth et al., 1987).

The fact that there is a cytoplasmic tyrosine-containing signal for endocytosis in several proteins (Davis et al., 1987; Lobel et al., 1989; Jing et al., 1990; McGraw and Maxfield,

1990) prompted us to search for a correlation between endocytosis and initial delivery to the basolateral surface in MDCK cells. There are multiple examples of proteins which are delivered directly to the basolateral surface of MDCK and are efficiently endocytosed, such as the transferrin receptor (Fuller and Simons, 1986), rat liver asialoglycoprotein receptor (Graeve et al., 1990), chicken hepatic lectin (Graeve et al., 1989), cation-independent mannose-6-phosphate receptor (Prydz et al., 1990), and polyimmunoglobulin receptor (Mostov et al., 1986). Although there appear to be membrane-anchored proteins which are delivered to the basolateral surface and not efficiently endocytosed, such as cell-substrate-adhesion molecules, we know of no case in which a protein that undergoes endocytosis through coated pits has been shown to be delivered directly to the apical surface in MDCK cells. Therefore we propose that there are similar cytoplasmic structures which can mediate basolateral exocytic delivery in MDCK cells and which can mediate internalization through coated pits. Perhaps a structure which causes cell-surface association with clathrin-coated pits is related to one which causes association with coated areas of the *trans*-Golgi network. These specialized areas of the *trans*-Golgi network could ultimately be segregated to the basolateral surface, carrying along the bound proteins. The adaptation of a pre-existing internalization feature to form a sorting feature seems to be a plausible mechanism for the evolution of a signal upon which to sort an important class of proteins to the appropriate surface domain. We are investigating the possible relationship between signals for endocytosis and signals for polarized surface delivery.

A simple working model for sorting is briefly as follows. In the *trans*-Golgi network, sorting receptors for basolateral proteins cluster in a patch of membrane (perhaps clathrin coated) destined to bud off as a basal vesicle; apical sorting receptors also cluster together in separate membrane domains. Proteins in the sorting compartment distribute themselves according to their relative efficiencies of binding to apical and basal receptors. Proteins which do not bind either type of receptor may nonetheless be vectorially delivered if they preferentially associate with a specific type of lipid domain that envelops apical or basal sorting receptors, or if one pathway simply has a larger protein-carrying capacity than the other.

Another model (Simons and Wandinger-Ness, 1990) is that apical transmembrane proteins have a signal for sorting and that all basolateral delivery is simply by default. According to that hypothesis, the introduction of a tyrosine into the cytoplasmic domain of HA might interfere with the functioning of the luminal HA apical signal rather than contribute to a cytoplasmic signal for the basolateral surface. This hypothesis is attractive for its simplicity, but there are no data showing a default pathway to only the basolateral surface. However, we have preliminary data that a point mutant of a normally basolateral protein (VSV G protein) expressed in permanent polarized MDCK cell lines is transported approximately equally to the apical and basolateral surfaces, an observation most easily interpreted as indicating the existence of unsorted transport to the apical surface. For this reason, we think that the simplest interpretation of the effect of the tyrosine 543 mutation on sorting of HA is the one that we favor.

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