Different Biosynthetic Transport Routes to the Plasma Membrane in BHK and CHO Cells

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Abstract. The question of how membrane proteins are delivered from the TGN to the cell surface in fibroblasts has received little attention. In this paper we have studied how their post-Golgi delivery routes compare with those in epithelial cells. We have analyzed the transport of the vesicular stomatitis virus G protein, the Semliki Forest virus spike glycoprotein, both basolateral in MDCK cells, and the influenza virus hemagglutinin, apical in MDCK cells. In addition, we also have studied the transport of a hemagglutinin mutant (Cys543Tyr) which is basolateral in MDCK cells. Aluminum fluoride, a general activator of heterotrimeric G proteins, inhibited the transport of the basolateral cognate proteins, as well as of the hemagglutinin mutant, from the TGN to the cell surface in BHK and CHO cells, while having no effect on the surface delivery of the wild-type hemagglutinin. Only wild-type hemagglutinin became insoluble in the detergent CHAPS during transport through the BHK and CHO Golgi complexes, whereas the basolateral marker proteins remained CHAPS-soluble. We also have developed an in vitro assay using streptolysin O–permeabilized BHK cells, similar to the one we have previously used for analyzing polarized transport in MDCK cells (Pimplikar, S.W., E. Ikonen, and K. Simons. 1994. *J. Cell Biol.* 125:1025–1035). In this assay anti-NSF and rab-GDI inhibited transport of Semliki Forest virus spike glycoproteins from the TGN to the cell surface while having little effect on transport of the hemagglutinin. Altogether these data suggest that fibroblasts have apical and basolateral cognate routes from the TGN to the plasma membrane.

LITTLE is known of how newly synthesized proteins destined for the plasma membrane or for export are delivered from the TGN to the fibroblast cell surface. The current working hypothesis postulates that surface delivery occurs by a bulk pathway. No sorting signal seems to be required for cargo proteins (Pfeffer and Rothman, 1987). Inclusion into the vesicular carrier is postulated to operate by a default mechanism. The evidence for this view is meager and is mostly based on the fact that most mutations affecting cargo proteins do not result in inhibition of surface delivery from the TGN although exceptions are known (Low et al., 1994). Usually mutant proteins that have passed the quality control mechanisms in the ER will be transported to the plasma membrane.

In comparison to fibroblasts, epithelial cells have to deliver newly synthesized proteins to the apical and the basolateral plasma membrane domains of the polarized cell surface. Most of the studies analyzing how this polarized surface delivery is accomplished have been carried out in MDCK cells. Sorting takes place in the TGN and at least two vesicular carriers exist for apical and basolateral delivery (Ikonen et al., 1995; Lafont et al., 1994; Pimplikar and Simons, 1993, 1994). The basolateral sorting signals have been well characterized and consist of either tyrosine or dileucine containing motifs in the cytoplasmic regions of basolateral proteins (Hunziker et al., 1991; Matter and Mellman, 1994; Mostov et al., 1987). Apical sorting signals, on the other hand, are of at least two types. Glycosylphosphatidyl inositol (GPI)[1]–anchored proteins use their GPI anchors as sorting signals (Brown et al., 1989; Lisanti et al., 1989). Another sorting signal is constituted by the mannose rich-core part of N-glycans. This signal is used by apical secretory proteins and probably by apical transmembrane proteins as well (Scheiffele et al., 1995). The machinery decoding these different sorting signals in the TGN has not yet been identified. However, it is known that the basolateral route uses the rab/NSF/SNAP/SNARE mecha-

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1. *Abbreviations used in this paper*: AlF, aluminum fluoride; CM, chase medium; GPI, glycosylphosphatidyl inositol; HA, hemagglutinin; LDH, lactate dehydrogenase; LB, lysis buffer; NSF, N-ethylmaleide-sensitive factor; rab-GDI, rab-GDP dissociation inhibitor; SFV, Semliki Forest virus; SLO, streptolysin O; STI, soybean trypsin inhibitor; TM, transport medium; TX100, Triton X-100; VSV-G, vesicular stomatitis virus glycoprotein.

nism for delivery (Ikonen et al., 1995). In contrast the apical pathway seems not to involve rabs nor NSF (Ikonen et al., 1995) and may use a completely new mechanism employing glycolipid rafts, VIPs (VIP21/caveolin [Dupree et al., 1993], VIP36 [Fiedler et al., 1994] and VIP17/MAL [Zacchetti et al., 1995]), and annexin XIIIb (Fiedler et al., 1995).

In this paper we have analyzed how the membrane trafficking routes in BHK and CHO cells compare with those in epithelial cells. Is the post-Golgi surface delivery in these cells completely different from the apical and the basolateral pathways? Or do they use apical and/or basolateral cognate routes to deliver proteins to the plasma membrane (Rodriguez-Boulan and Powell, 1992)? This is the hypothesis that Mellman et al. (1993) have formulated on the basis of the fact that basolateral targeting determinants are present on proteins expressed both in polarized and nonpolarized cells. Typical examples of such proteins are viral glycoproteins (Simons and Fuller, 1985). We have therefore used as our surface protein probes for the transport studies in BHK and CHO cells the vesicular stomatitis virus (VSV-G) protein, the Semliki Forest virus spike glycoprotein (SFV E1+E2), both basolateral in MDCK cells, and the influenza virus hemagglutinin (HA), apical in MDCK cells (Fuller et al., 1985; Rodriguez-Boulan and Pendergast, 1980; Roman and Garoff, 1986). In addition, we have analyzed the surface transport of an HA mutant which has a single amino acid change in its cytoplasmic tail (Cys543Tyr) converting it from an apical protein to a basolateral protein in MDCK cells (Brewer and Roth, 1991). We have developed an in vitro assay using streptolysin O (SLO)–permeabilized BHK cells similar to the one that we have used for studying polarized cell surface transport in MDCK cells (Pimplikar et al., 1994). With this assay we have analyzed the effects of anti-NSF (Whiteheart et al., 1994) and rab-GDI (Sasaki et al., 1991) on the surface transport of the virus marker proteins. Our results suggest that fibroblasts have apical and basolateral cognate routes from the TGN to the cell surface.

Materials and Methods

Materials

Media and reagents for cell culture were purchased from GIBCO BRL (Eggestein, Germany). NHS-LC-Biotin and streptavidin-agarose were obtained from Pierce (Oud-Beijerland, Netherlands); TPCK-treated trypsin and soybean trypsin inhibitor (STI) from Worthington (Freehold, NJ); creatine phosphate, creatine kinase, hexokinase, and endoglycosidase H from Boehringer (Mannheim, Germany); CHAPS, ATP, and protease inhibitors from Sigma (Deisenhofen, Germany); protein A–Sepharose CL-4B from Pharmacia (Uppsala, Sweden); and [^{35}S]methionine from Amersham (Braunschweig, Germany). The mAb against NSF, 6E6 (Tagaya et al., 1993), was obtained from M. Tagaya (Tokyo College of Pharmacy, Japan). Chromatographically purified SLO was obtained from S. Bhakdi (University of Mainz, Germany). A vector encoding His_6-tagged rab-GDI (Ullrich et al., 1993) was a kind gift of M. Zerial (European Molecular Biology Laboratory, Heidelberg, Germany). Recombinant His_6-tagged rab-GDI was purified as described (Ullrich et al., 1993). A rabbit antiserum to SFV spike glycoproteins was raised and affinity-purified as previously described (de Curtis and Simons, 1988).

Cell Culture, Virus Preparation, and Viral Infection

BHK 21 cells were cultured on 11-mm-diam coverslips in either 35-mm-diam cell culture dishes or in 24 multi-well plates (both NUNC) for 2 d to ~90% confluency as previously described (de Curtis et al., 1988). A CHO cell line stably expressing a mutant of the A/Japan/305/57(H2) HA (HA Tyr543) was prepared as previously described for MDCK cells (Brewer and Roth, 1991). This cell line (CHO-Y543) and the parent CHO-K1 cell line were cultured in 35-mm-diam cell culture dishes for 2 d to ~90% confluency in RPMI 1640 medium containing 10% FCS.

Stocks of the influenza virus N and Japan strains, whose HA proteins are not cleaved by proteases of the host cell, and of SFV were prepared as described before (Bennett et al., 1988; Kääriäinen et al., 1969; Matlin and Simons, 1983).

Cells were infected for 1 h at 37°C with the viruses in infection medium (Earle's MEM, 0.2% BSA, 10 mM Hepes [pH 7.3]). Then the infection medium was aspirated, fresh medium without virus was added, and the cells were incubated for 4 (influenza virus) or for 3 h (SFV) at 37°C in the presence of 5% CO_2.

Metabolic Labeling and Accumulation of Viral Proteins in the TGN

After infection, BHK cells in 35-mm-diam dishes were washed three times with labeling medium (Earle's MEM without methionine and with low bicarbonate [0.35 g/liter], 10 mM Hepes [pH 7.3], 0.2% BSA), and labeled for 5 min at 37°C with 7–35 μCi [^{35}S]methionine in 0.28 ml labeling medium. The pulse was terminated by adding chase medium (CM; labeling medium containing 40 μg/ml cycloheximide and 0.15 mg/ml methionine) and incubating for an additional 3 min at 37°C. Ice-cold CM was then added and the cells were incubated for 75 min in a 19.5°C waterbath to accumulate the viral glycoproteins in the TGN (Matlin and Simons, 1983).

CHO-K1 cells infected with the influenza Japan strain or CHO-Y543 cells were labeled for 12 min at 37°C with 380 μCi [^{35}S]methionine in 0.4 ml labeling medium and then chased for 75 min at 19.5°C.

BHK cells for in vitro transport assays (see below) were labeled for 7 min at 37°C with 60–70 μCi [^{35}S]methionine in 0.2 ml labeling medium, chased for 3 min at 37°C, and further incubated for 75 min at 19.5°C.

TGN-to-Surface Transport in Intact Cells

Virus-infected BHK or CHO-K1 cells were pulse-labeled at 37°C and chased at 19.5°C as described above. After the 19.5°C block the cells were incubated in CM containing 50 μM ammonium aluminum sulphate and 10 mM potassium fluoride for 10 min on ice, followed by an incubation for 45 min at 37°C in fresh prewarmed medium containing the same reagents. As a control, CM without the reagents was used.

To detect surface arrival of influenza N HA, trypsin cleavage (Matlin and Simons, 1983) was used. All steps were performed on ice. After two washes with PBS+ (Dulbecco's PBS, containing 0.9 mM Ca^{2+} and 0.5 mM Mg^{2+}), 1 ml of 0.1 mg/ml trypsin in PBS+ was added to the cells. After 30 min, the reaction was stopped by adding 0.1 ml of 1 mg/ml STI in PBS+ for 5 min. Then the cells were washed twice, 5 min each, with 50 μg/ml STI in PBS+ and solubilized in 150 μl lysis buffer (LB, PBS– containing 2% NP-40 and 0.2% SDS) supplemented with a protease inhibitor cocktail (CLAP; 25 μg/ml each of chymostatin, leupeptin, antipain, and pepstatin A). Insoluble material was removed by centrifugation for 10 min at 4°C. A 15-μl aliquot of the lysate was analyzed by SDS-PAGE on a 10% acrylamide gel (Laemmli, 1970). Radioactivity in the individual bands was determined using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA) and the amount of HA transported was calculated: percent of transport = $2 \times HA2/[HA + 2 \times HA2] \times 100$ (Matlin and Simons, 1983).

The amount of SFV E1+E2 transported was determined by surface biotinylation on ice. The cells were washed twice with PBS–, and then they were incubated for 30 min in 0.5 ml PBS– with 1 mg/ml NHS-LC-Biotin. After a PBS– wash, the cells were incubated twice, 5 min each, in PBS– containing 0.1 M glycine, 0.3% BSA. Finally the cells were washed twice with PBS– and solubilized in 500 μl of LB as described above. For determination of total SFV glycoproteins, a 20-μl aliquot was removed, and to 450 μl of the remaining lysate, 20 μl of streptavidin-agarose was added. After an overnight incubation at 4°C, the agarose was pelleted by centrifugation, washed twice with LB, and then incubated for 5 min at 4°C in 50 mM Tris-HCl (pH 7.5) containing 25 mM DTT by rotating end-over-end. After washing with the same solution, streptavidin-bound material (i.e., surface SFV glycoproteins) was eluted by boiling in Laemmli sample buffer. Both total and surface SFV glycoproteins were analyzed by SDS-PAGE on a 10% acrylamide gel. Note that the two subunits E1 and E2 of the SFV glycoproteins comigrate under these electrophoresis conditions. Radioactivity in the individual bands was determined using a Phosphor-

Imager and the amount of SFV E1+E2 transported, after normalizing for recoveries, was calculated as the amount of them bound to streptavidin-agarose. The equation for this is as follows: E1+E2 on surface/(P97/2 + P62/1.5 + [E1+E2] in total).

To determine the amount of HA Tyr543 transported to the surface in CHO-Y543 cells, a modified trypsinization protocol (Brewer and Roth, 1991) was used, since this mutant of HA is expected to be rapidly endocytosed after its arrival at the cell surface (Brewer and Roth, 1991; Ktistakis et al., 1990; Lazarovits and Roth, 1988). After pulse-labeling and a block at 19.5°C, the CHO-Y543 cells were chased for 45 min at 37°C in 0.5 ml BSA-free CM containing 10 μg/ml trypsin. 100 μl 1 mg/ml STI in PBS+ were then added and the cells were incubated for 5 min on ice. As this treatment detached the cells, they were collected by centrifugation. After a wash with PBS+ containing 50 μg/ml STI, the cells were lysed and HA was immunoprecipitated as described (Brewer and Roth, 1991). The extent of cleavage was determined by SDS-PAGE and PhosphorImager analysis, and the amount of HA Japan transported was calculated: percent of transport = 1.5 × HA2/(HA + 1.5 × HA2) × 100. As a control, parent CHO-K1 cells infected with the wild-type influenza Japan strain were treated the same way.

CHAPS Insolubility

CHAPS insolubility of viral proteins was assayed as described before (Fiedler et al., 1993). Pulse-labeling was 5 min for virus-infected BHK or CHO-K1 cells and 15 min for CHO-Y543 cells. HA Japan and HA Tyr543 were immunoprecipitated as described above.

Preparation of Bovine Brain Cytosol

Bovine brain cytosol was prepared as described by Miller and Moore (1991) except for using KOAc buffer (115 mM potassium acetate, 25 mM Hepes [pH 7.4], 2.5 mM $MgCl_2$) supplemented with 1 mM EGTA, 1 mM $MgCl_2$, 1 mM DTT, 0.1 mM PMSF, and CLAP. The protein concentration (BioRad Labs, Richmond, CA) of this cytosol preparation was 4.8 mg/ml.

In Vitro Transport in SLO-Permeabilized Cells

The TGN-to-surface transport of HA and SFV E1+E2 was reconstituted in SLO-permeabilized BHK cells based on the assay in MDCK cells described by Pimplikar et al. (1994) and Ikonen et al. (1995). BHK cells grown on 11-mm-diam coverslips in 24 multi-well plates were infected with the viruses, pulse-labeled, and incubated at 19.5°C as described above. The coverslips were then washed by dipping four times in ice-cold KOAc buffer and placed cell side up on a sheet of parafilm in a moisture box. 50 μl of KOAc buffer containing 10 mM DTT and 15–20 μg/ml preactivated SLO (Pimplikar et al., 1994) were added onto the coverslips. After incubation for 7 min on ice, the coverslips were washed in ice-cold KOAc buffer as described above, and, in order to deplete endogenous cytosol, incubated for 30 min at 19.5°C in 500 μl of transport medium (TM; KOAc buffer containing 1 mM DTT, 5 mM EGTA, 2.5 mM $CaCO_3$) in 24 multi-well plates.

After cytosol depletion the coverslips were washed in ice-cold TM, placed on a sheet of parafilm in a moisture box, and incubated for 30 min on ice with 50 μl of cytosol supplemented with an ATP-regeneration system (Pimplikar et al., 1994) or an ATP-depletion system (195 U/ml of hexokinase, 0.8 mM D-glucose). TM was used instead of cytosol in the cytosol (−) samples. Then the moisture box containing the coverslips was transferred to a 37°C waterbath for 45 min. Transport was terminated by transferring the coverslips on ice.

The amount of HA transported to the surface was determined as in intact cells (see above) except that the cells were washed only once with 50 μg/ml STI in PBS+. SFV E1+E2 transport was determined by surface immunoprecipitation using an affinity-purified polyclonal antibody against the exoplasmic portion of the spike glycoproteins of SFV (de Curtis and Simons, 1988). The coverslips were washed in CM containing 10% FCS, and placed on a sheet of parafilm in a moisture box. 50 μl of the antibody (1:100 dilution in CM containing 10% FCS) were added and after an incubation for 60 min at 4°C, the coverslips were transferred to 24 multi-well plates and washed, 10 min each, four times with 500 μl of CM containing 10% FCS, and then once with infection medium containing an excess of cold SFV. The coverslips were gently shaken during these washes. The cells were then lysed in 200 μl of LB as described above. After centrifugation to remove insoluble material, an 8-μl aliquot was taken for the determination of total SFV glycoproteins, and 10 μl of protein A–Sepharose was added to the rest. The mixture was rotated end-over-end for 60 min at 4°C, and then the resin was washed four times with LB. Protein A–bound material (i.e., surface SFV E1+E2) was eluted by boiling in Laemmli sample buffer. Total and surface viral proteins were analyzed as in intact cells (see above). Nonspecific binding was determined by (*a*) adding no antibody, (*b*) combining lysates of two samples, one of which was not infected, not labeled, but was incubated with the antibody, and one that was not incubated with the antibody, but was infected and labeled, and (*c*) combining lysates of two samples, one of which was not labeled, but was infected and incubated with the antibody, and one that was not incubated with the antibody, but was infected and labeled. Binding of radiolabeled SFV E1+E2 to protein A–Sepharose was not detected in these controls (data not shown).

As a small part of the cells detached from the coverslips during the washing steps, they were recovered by centrifugation and processed in parallel with the cells remaining on the coverslips. Lysates of the detached and remaining cells were combined.

Lactate Dehydrogenase Release Assay

BHK cells grown on coverslips were treated with SLO and cytosol was depleted as described above. Lactate dehydrogenase (LDH) activity in the supernatant and the cell lysate was determined as described by Schnaar et al. (1978).

In Vitro Transport in the Presence of Anti-NSF Antibodies or Rab-GDI

The mAb against NSF, 6E6 (Tagaya et al., 1993), was added to the exogenous cytosol as described previously (Ikonen et al., 1995). His_6-tagged rab-GDI (4.6 mg/ml in TM) was added to both the cytosol depletion buffer (TM) and the exogenous cytosol at 2 or 5 μM. Note that higher concentrations of rab-GDI could not be used since they disturbed the assay. Control samples were diluted with TM accordingly.

Results

AlF Inhibits the TGN-to-Surface Transport in BHK Cells of SFV E1+E2, but Not of HA

In MDCK cells, after de novo synthesis in the RER, HA is preferentially transported to the apical surface, whereas the spike glycoproteins of SFV are targeted to the basolateral surface. Aluminum fluoride (AlF), a general activator of heterotrimeric G proteins, but not small GTP-binding proteins (Kahn, 1991), stimulated transport of HA and retarded that of another basolateral marker, VSV-G (Pimplikar and Simons, 1994).

To explore the possible existence of apical and basolateral cognate pathways in fibroblasts, we have treated BHK cells with AlF (Fig. 1). BHK cells infected with influenza N virus or SFV were pulse-labeled with [^{35}S]methionine and incubated at 19.5°C to accumulate the viral marker proteins in the TGN (Griffiths et al., 1985; Hughson et al., 1988; Matlin and Simons, 1983). The cells were then allowed to transport the viral markers to the surface, either in the presence or absence of AlF. Surface arrival of HA was detected by trypsin treatment, which cleaved only surface-bound HA into HA1 and HA2, but not intracellularly retained HA (Matlin and Simons, 1983). SFV spike proteins are generated from one single precursor, which in the ER is cleaved into E1 and P62. The latter subunit is further cleaved into E2 and E3, either during or shortly after the formation of transport vesicles from the TGN (de Curtis and Simons, 1988). The amount of E1 and E2 protein (SFV E1+E2) on the cell surface was determined by surface biotinylation. When the cells were incubated at 4°C instead of 37°C, transport of HA and SFV E1+E2 to the cell surface did not occur (Fig. 1 *A*, lanes 5 and 6; Fig. 1 *B*,

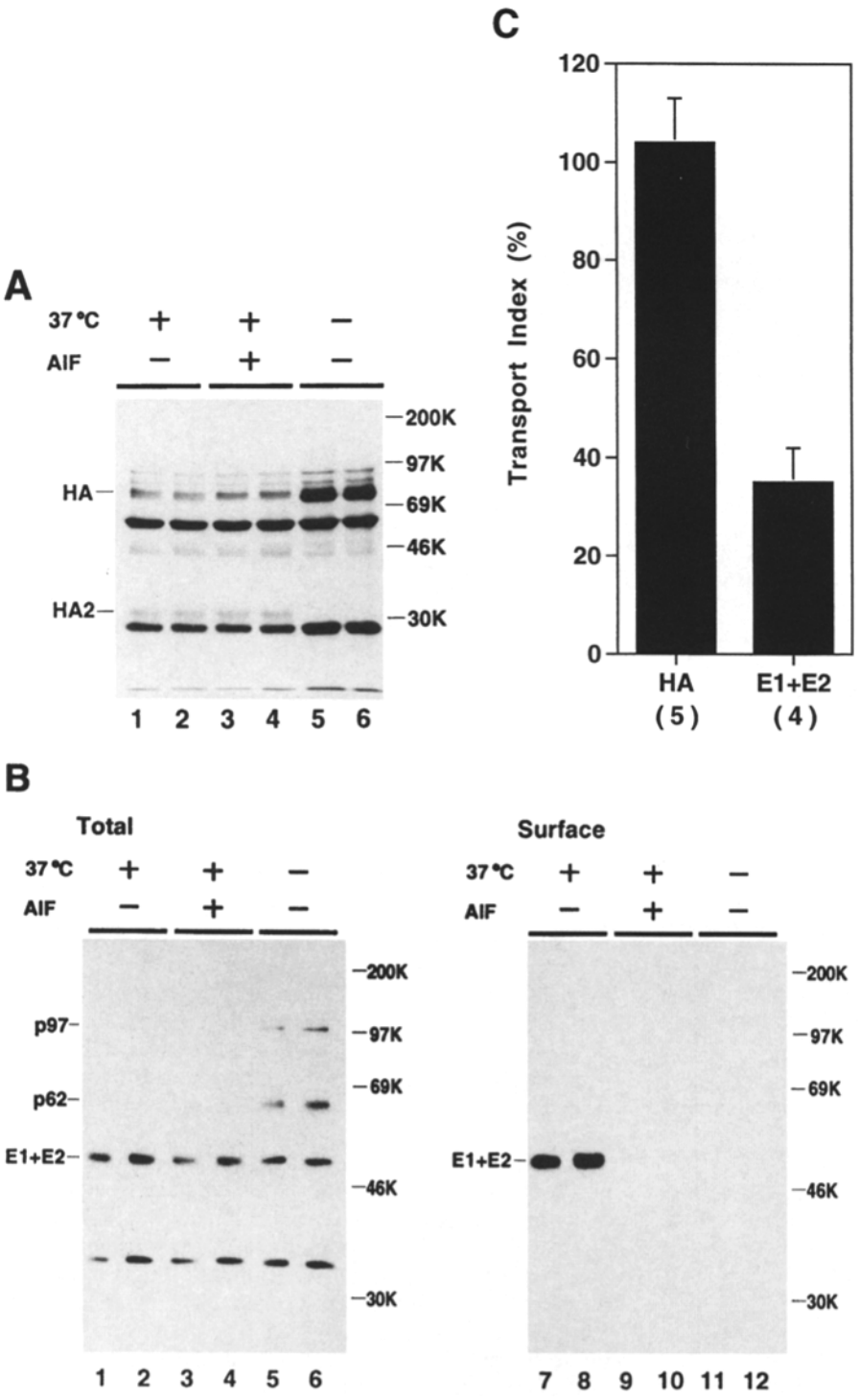

Figure 1. AlF treatment inhibits TGN-to-surface transport in BHK cells of SFV E1+E2, but not of HA. (*A*) Influenza N virus–infected BHK cells were pulse-labeled with [^{35}S]methionine and viral glycoproteins were accumulated in the TGN by a 19.5°C block. The cells were then incubated for 10 min on ice in the absence (lanes *1*, *2*, *5*, and *6*) or presence of AlF (lanes *3* and *4*) followed by 45 min at 37°C (lanes *1–4*) or on ice (lanes *5* and *6*). Arrival of HA at the cell surface was determined by trypsinization. HA2 denotes the cleaved fragment of HA. (*B*) SFV-infected BHK cells were processed as in *A*. The surface delivery of SFV E1+E2 was determined by surface biotinylation. Cell lysates (*left panel*) and material bound to streptavidin-agarose (*right panel*) were analyzed by SDS-PAGE. P62 denotes the precursor form generating the E2 and E3 subunits. Note that the E1 and E2 subunits comigrate under our electrophoresis conditions while E3 is not seen. P97 is a form that contains all spike subunits. (*C*) TGN-to-surface transport of HA and SFV E1+E2 in the presence of AlF was quantitated by PhosphorImager analysis and the transport index was calculated as described in Materials and Methods. The values are expressed relative to transport in the absence of AlF (100%) and to that of the samples left on ice (0%). Each column represents the mean ± SEM of several experiments (number indicated in parentheses) done with duplicate dishes as depicted in *A* and *B*.

lanes *11* and *12*). AlF significantly inhibited SFV E1+E2 transport (Fig. 1, *B*, and *C*), but did not affect HA transport (Fig. 1, *A*, and *C*). The inhibition of SFV E1+E2 transport was observed not only at 45 min of chase (Fig. 1 *B*), but also at all other times studied (Fig. 2). On the other hand, the transport of HA was hardly affected at any time (Fig. 2).

These results suggest that in BHK cells, similarly as in MDCK cells, HA and SFV E1+E2 could be differentially sorted in the TGN. As in MDCK cells (Pimplikar and Simons, 1994), the transport of SFV E1+E2-containing vesicles could be regulated by the G_i class of heterotrimeric G proteins, whereas the transport vesicles for HA are regulated in a different way.

HA, but Not SFV E1+E2 and VSV-G, Becomes CHAPS-Insoluble during Transport in BHK Cells

As judged by the different sensitivity to AlF, there might be a segregation of HA and SFV E1+E2 into distinct vesi-

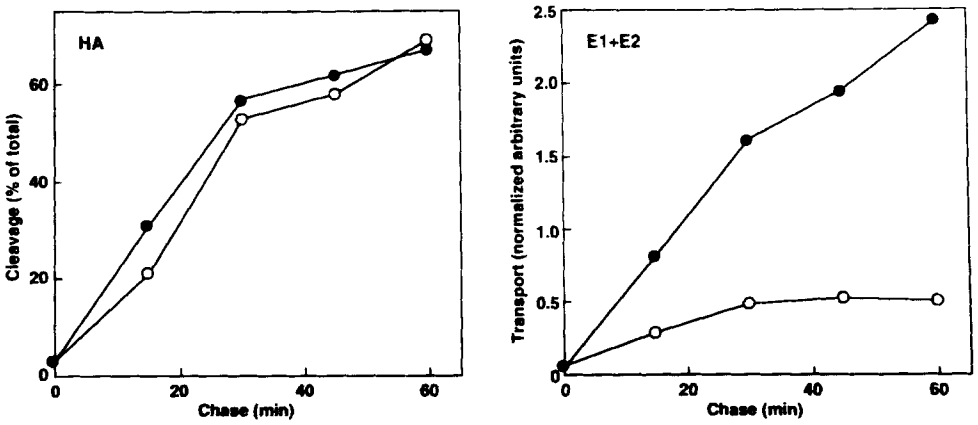

Figure 2. Kinetics of TGN-to-surface transport in BHK cells of HA and SFV E1+E2 in the presence or absence of AlF. Transport of HA (*left panel*) and SFV E1+E2 (*right panel*) in BHK cells was performed in the absence (*filled circles*) or presence (*open circles*) of AlF for various periods after the "TGN block" as described in Fig. 1.

cle populations. If so, at what stage does this separation occur? In MDCK cells, HA becomes CHAPS-insoluble only after it has reached the late Golgi (Fiedler et al., 1993; Kurzchalia et al., 1992), probably in the TGN by coclustering with apically sorted proteins and glycosphingolipid microdomains (for review see Simons, 1995). Basolateral marker proteins are efficiently excluded from these complexes, suggesting that sorting into different membrane domains takes place at this stage.

As shown in Fig. 3, the same behavior can be observed in BHK cells. Virus-infected BHK cells were pulse-labeled with [^{35}S]methionine, chased for 2 or 30 min at 37°C, and extracted with 20 mM CHAPS. Immediately after synthesis the core-glycosylated ER form of HA (HA0) was completely soluble (Fig. 3, lanes *1* and *2*), whereas after 30 min of chase most of it was found in the CHAPS-insoluble fraction (Fig. 3, lanes *3* and *4*). CHAPS-insoluble HA had a slightly slower mobility than CHAPS-soluble HA0 (compare lanes *3* and *4*) and corresponded to the complex glycosylated Golgi form of HA. On the other hand, the basolateral marker proteins, SFV E1+E2 (Fig. 3, lanes *5–8*) and VSV-G (Fig. 3, lanes *9–12*), were CHAPS-soluble even after 30 min of chase. At 30 min of chase cleavage of SFV P62 into E2 (decrease of P62 accompanied by an increase of SFV E1+E2) and conversion of VSV-G from the core-glycosylated ER form (VSV-G0) to the complex glycosylated Golgi form (VSV-G) were apparent (Fig. 3, lanes *7* and *11*, respectively). Thus, although the majority of both viral proteins had reached the *trans*-Golgi, they remained completely CHAPS-soluble. These results indicate that, as in MDCK cells, apical cognate and basolateral cognate marker proteins are segregated from each other only after complex glycosylation has occurred.

Change of Transport Behavior of HA by a Single Amino Acid Exchange in Its Cytoplasmic Tail

It was previously shown that a single amino acid exchange in the cytoplasmic tail of HA (Cys543Tyr) caused missorting in MDCK cells (Brewer and Roth, 1991). HA containing this mutation (HA Tyr543) was no longer targeted to the apical surface but instead was delivered to the basolateral membrane. The sorting signal in basolaterally targeted membrane proteins is generally known to reside in their cytoplasmic tails (for review see Matter and Mellman, 1994). Some of these signals closely resemble the Tyr-containing signals involved in rapid endocytosis (coated-pit localization signals). Since the mutant HA, unlike wild-type HA, was rapidly internalized in CV-1 (Lazarovits and Roth, 1988) and in MDCK cells (Brewer and Roth, 1991), this Cys543Tyr mutation introduced a basolateral sorting signal of this type into HA. This sorting information must be dominant over the putative apical signal in HA.

To see whether such sorting signals also operate in nonpolarized cells, we used a stably transfected CHO cell line (CHO-Y543), expressing the same HA Japan mutant that was used to obtain the MDCK cell line described above (Brewer and Roth, 1991). When the parent cell line (CHO-K1) was infected with either influenza virus (Japan strain), or SFV and then treated with AlF, only the transport of SFV E1+E2 was affected (Fig. 4 *A*). This suggests that the sorting behavior observed in BHK cells is not a peculiarity of this cell line, but that it is common to other cell lines as well.

We then measured TGN-to-surface transport in CHO-Y543 cells of HA Tyr543 in either the presence or absence of AlF. To circumvent potential detection problems caused by possible endocytosis of HA Tyr543 (Brewer and Roth, 1991), we used a modified trypsin treatment, which cleaved all HA arriving at the surface. Both trypsinization protocols yielded essentially the same results for wild-type–infected CHO-K1 cells (compare left columns in Fig. 4, *A* and *B*). Transport of HA Tyr543, unlike wild-type HA, was significantly inhibited by AlF (Fig. 4 *B*). The kinetics

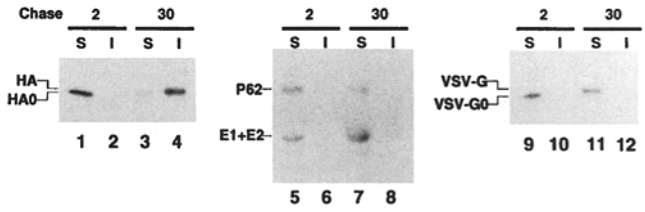

Figure 3. HA, but not SFV E1+E2, acquires CHAPS-insolubility late in transport in BHK cells. BHK cells were infected with influenza N virus (*left panel*), SFV (*middle panel*), or VSV (*right panel*), pulse-labeled with [^{35}S]methionine for 5 min, and chased for 2 or 30 min at 37°C. After extraction of cells with 20 mM CHAPS, a soluble (*S*) and insoluble (*I*) fraction was generated. HA0 and VSV-G0 denote core-glycosylated ER forms, whereas HA and VSV-G denote the Golgi forms, respectively.

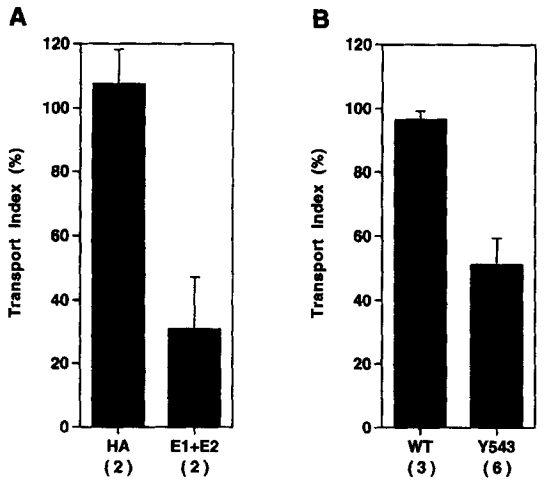

Figure 4. TGN-to-surface transport of HA Tyr543 is inhibited by AlF. (*A*) TGN-to-surface transport of influenza Japan HA and SFV E1+E2 in CHO-K1 cells in the presence or absence of AlF (see Fig. 1 *C*). (*B*) The effect of AlF treatment on the TGN-to-surface transport of wild-type (WT; influenza Japan virus–infected CHO-K1 cells) or mutant HA (Y543; stable transfectant CHO-Y543). Surface arrival of HA was quantitated by a modified trypsinization protocol (see Materials and Methods). Data represent mean ± SEM for several assays with duplicate dishes. The number of experiments is indicated in parentheses.

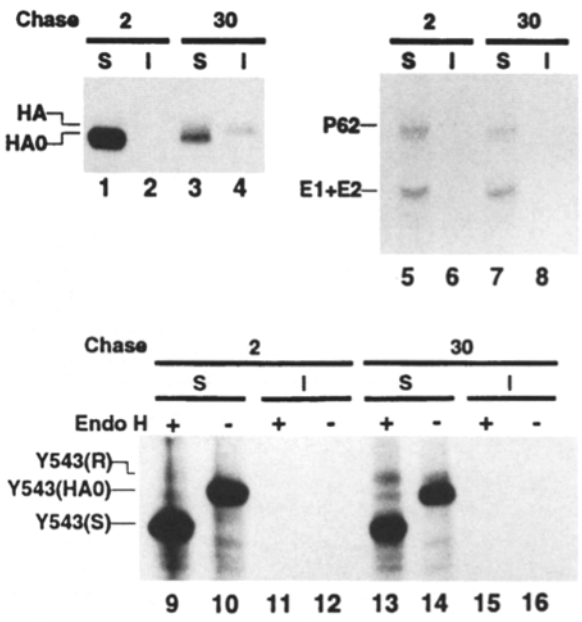

Figure 5. HA Tyr543 does not acquire CHAPS insolubility in CHO-Y543 cells. CHO-K1 cells infected with influenza Japan virus (lanes *1–4*) or SFV (lanes *5–8*) and CHO-Y543 cells expressing the mutant HA Tyr543 (lanes *9–16*) were pulse-labeled with [^{35}S]methionine, and chased for 2 or 30 min. After extraction of cells with 20 mM CHAPS, a soluble (*S*) and insoluble (*I*) fraction was generated. HA Tyr543 samples were also digested with endoglycosidase H (*Endo H*). Y543(R), Y543(HA0), and Y543(S) denote the endoglycosidase H–resistant Golgi form, the ER form, and the endo H–digested ER form of HA Tyr543, respectively.

of Golgi-to-surface transport of HA Tyr543 was the same as that of the wild-type HA (data not shown). As for SFV E1+E2 in BHK cells (Fig. 2), AlF inhibited the transport of HA Tyr543 at all times (data not shown).

As in BHK cells (Fig. 3), wild-type HA became rapidly CHAPS-insoluble in CHO-K1 cells (Fig. 5, lanes *1–4*), whereas SFV E1+E2 remained CHAPS-soluble (Fig. 5, lanes *5–8*). Although HA Tyr543 acquired complex glycosylation more slowly than wild-type HA, as judged by endoglycosidase H treatment (Fig. 5, lanes *9* and *13*), the endoglycosidase H resistant form remained completely CHAPS-soluble (Fig. 5, lanes *9–16*). This was the case for up to 150 min of chase (data not shown). A considerable fraction of HA Tyr543 was endoglycosidase H–sensitive after such a long time, indicating that mutant HA was retained in the ER as shown earlier (Brewer and Roth, 1991).

In Vitro Reconstitution of the TGN-to-Surface Transport of HA and SFV E1+E2 Using SLO-permeabilized BHK Cells

The results described above are consistent with our working hypothesis that nonpolarized fibroblasts would have, in analogy to MDCK cells, a sorting and transport machinery that allows separation of apically from basolaterally sorted proteins. To get further information on the machinery involved, we have reconstituted the TGN-to-surface transport of HA and SFV E1+E2 in vitro by using SLO-permeabilized BHK cells. This system proved to be useful in MDCK cells as it allowed us to study transport and sorting by manipulation of single components involved (Ikonen et al., 1995; Lafont et al., 1994; Pimplikar et al., 1994; Pimplikar and Simons, 1994).

In BHK cells the use of 15–20 ng/μl SLO produced optimal release of endogenous cytosol (Fig. 6). Higher concentrations of SLO did not release more than 60% of LDH activity, so that we used 15–20 ng/μl SLO for our transport assays. Fig. 7 clearly shows that the TGN-to-surface transport of HA and SFV E1+E2 in SLO-permeabilized BHK cells is dependent upon the addition of exogenous cytosol, energy (ATP), and temperature (37°C). The transport efficiency of HA was approximately half of that in intact cells (Fig. 2, *left panel*, 45 min chase), while the transport efficiency of SFV E1+E2 was ~65% of that of nonpermeabilized cells (data not shown). Addition of exogenous cytosol stimulated HA transport about twofold, which is comparable to that previously reported for MDCK cells (Ikonen et al., 1995; Pimplikar et al., 1994). Transport of SFV E1+E2 was, however, more dependent on exogenous cytosol. We do not know the reason for this difference in cytosol dependency between HA and SFV E1+E2 transport, but it might reflect the involvement of different cytosolic factor(s) in apical and basolateral cognate pathways.

Anti-NSF and Rab-GDI Inhibit the TGN-to-Surface Transport of SFV E1+E2, but Not of HA

Recently, we have shown that basolateral transport in MDCK cells was inhibited by anti-NSF antibodies, rab-GDI, tetanus and botulinum neurotoxins, and that it was stimulated by α-SNAP, whereas apical transport was in-

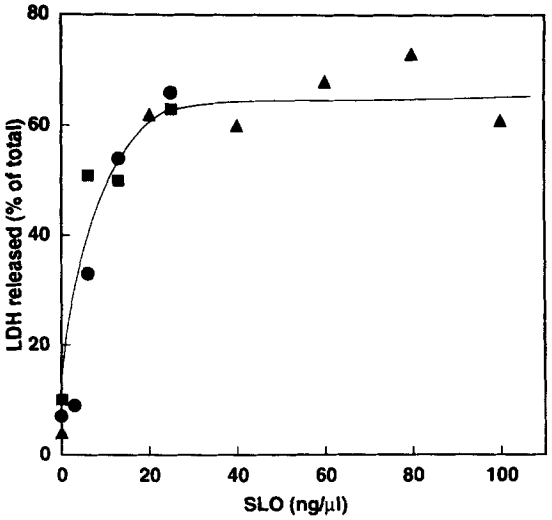

Figure 6. LDH release from BHK cells depends on the concentration of SLO used. BHK cells were treated with SLO for 7 min on ice at the indicated concentrations. Unbound SLO was washed away and the cells were incubated for 30 min at 19.5°C. LDH released into the incubation buffer and LDH remaining in the cells was assayed. Data from three independent experiments are shown.

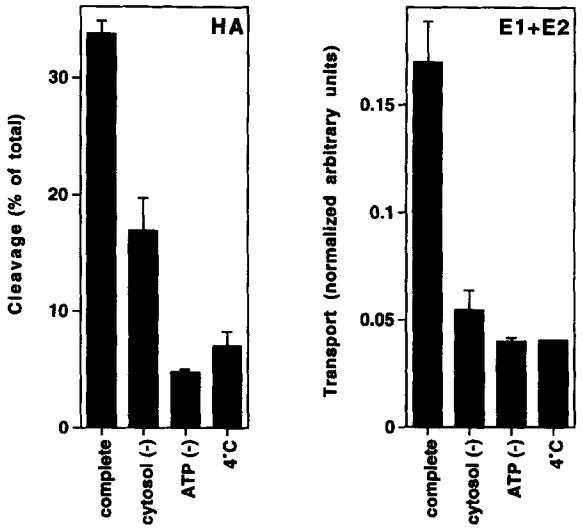

Figure 7. Reconstituted TGN-to-surface transport in SLO-permeabilized BHK cells of HA and SFV E1+E2. Virus-infected BHK cells were pulse-labeled with [^{35}S]methionine and incubated at 19.5°C, and were then treated with 15 ng/μl of SLO. The cells were incubated with bovine brain cytosol and an ATP-regeneration system (complete) or K-TM buffer and an ATP-regeneration system [cytosol(−)] or cytosol and an ATP-depletion system [ATP(−)] for 15 min on ice, and then for 45 min at 37°C. Surface arrival of HA (*left panel*) and SFV E1+E2 (*right panel*) was detected by trypsinization and by surface immunoprecipitation, respectively. 4°C denotes transport under complete conditions at 4°C instead of 37°C. Data represent mean ± SEM of duplicate coverslips for each condition.

sensitive to all these reagents, suggesting that a rab-NSF-SNAP-SNARE mechanism operates in basolateral transport, while other components constitute the apical transport machinery (Ikonen et al., 1995).

A mAb (6E6) that recognizes an epitope in the ATPase domain of NSF (Whiteheart et al., 1994) and is inhibitory in a cell-free intra-Golgi transport assay (Tagaya et al., 1993) has also been shown to inhibit ER-to-Golgi transport of HA and basolateral transport of VSV-G but not apical transport of HA in MDCK cells (Ikonen et al., 1995). In SLO-permeabilized BHK cells, addition of this antibody to the exogenous cytosol inhibited transport of SFV E1+E2, but not of HA (Fig. 8). This 40% inhibition was in the same range as it previously was reported for ER-to-Golgi and basolateral transport in MDCK cells (Ikonen et al., 1995). Rab-GDI extracts rab proteins from membranes (Ullrich et al., 1993). By adding an excess of rab-GDI, it is possible to inhibit transport steps involving rab proteins. This inhibitory effect has been shown in several systems (Elazar et al., 1994; Peter et al., 1994). In MDCK cells rab-GDI inhibited the basolateral transport of VSV-G but had no effect on apical transport of HA (Ikonen et al., 1995). In BHK cells, 5 μM rab-GDI inhibited the TGN-to-surface transport of SFV E1+E2 by ~50%, but hardly affected HA transport at all (Fig. 9). The efficient extraction of rab proteins by 5 μM rab-GDI in the individual experiments was verified by probing Western blots prepared from an aliquot of the cell lysates for rab5 (data not shown). SFV E1+E2 transport was not affected by 2 μM rab-GDI, suggesting that the rab protein(s) involved in the basolateral cognate pathway is fairly abundant.

These results indicate that, as in MDCK cells (Ikonen et al., 1995), only the basolateral cognate pathway relies on NSF and rab proteins.

Discussion

Our data clearly demonstrate that apical and basolateral viral glycoproteins when expressed in BHK and CHO cells can be distinguished by similar criteria that characterized their behavior in MDCK cells. The delivery of the basolateral marker proteins to the BHK or CHO cell surface was inhibited by AlF to the same extent as in MDCK cells suggesting that a G_i protein is regulating plasma membrane transport in both these cells and in MDCK cells (Pimplikar and Simons, 1993). Strong inhibition by AlF has recently been reported on SFV glycoprotein delivery in BHK cells (Sariola et al., 1995). The similarities in the delivery mechanisms of VSV-G and SFV E1+E2 in both BHK and MDCK cells were further strengthened by the findings that anti-NSF in SLO-permeabilized cells inhibited surface delivery to the same extent in the two cell types (Ikonen et al., 1995). Furthermore, introduction of 5 μM rab-GDI into the permeabilized BHK cells led to decreased surface delivery as observed previously in MDCK cells (Ikonen et al., 1995). We also analyzed whether tetanus toxin affected transport (Keller, P., unpublished data). However, in contrast to MDCK cells (Ikonen et al., 1995) no effect was seen. Altogether these data suggest that VSV-G and SFV E1+E2 seem to use a similar transport mechanism as they use to reach the basolateral surface in MDCK cells. Since we have not isolated the carrier vesicles responsible for transport in BHK and CHO cells, we do not know whether one or more carriers are used to

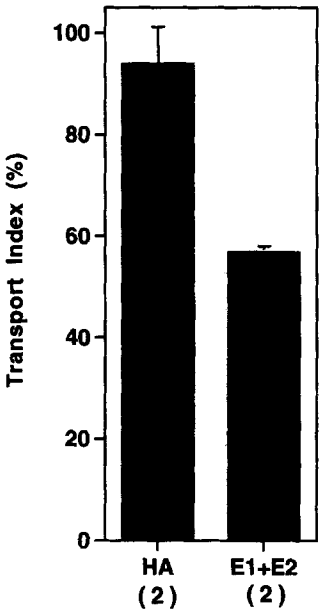

Figure 8. Antibodies against NSF inhibit TGN-to-surface transport in SLO-permeabilized BHK cells of SFV E1+E2, but not of HA. The monoclonal anti-NSF antibody 6E6 was added to virus-infected, SLO-permeabilized BHK cells (see Materials and Methods), and transport of HA and SFV E1+E2 was quantitated as detailed in Fig. 7. Data are expressed as in Fig. 1 *C* and represent mean ± SEM of two experiments performed with duplicate coverslips.

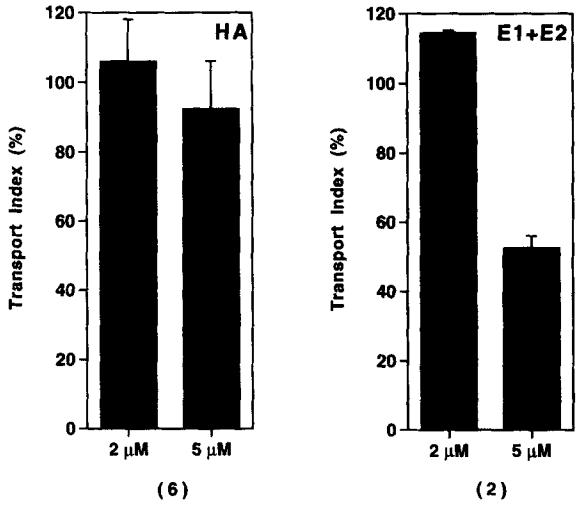

Figure 9. Rab-GDI inhibits TGN-to-surface transport in SLO-permeabilized BHK cells of SFV E1+E2, but not of HA. Rab-GDI was added to virus-infected, SLO-permeabilized BHK cells as indicated in Materials and Methods. Transport of HA and SFV E1+E2 was quantitated as detailed in Fig. 7. Data are expressed as in Fig. 1 *C* and represent mean ± SEM for each transport with duplicate coverslips. The number of experiments is indicated in parentheses.

transport these proteins from the TGN to the plasma membrane. Recent results in *Saccharomyces cerevisiae* suggest that two vesicular carriers are involved in transport from the Golgi complex to the yeast bud (Harsay and Bretscher, 1995). Both are sec4-dependent. The rab8 protein which seems to be the mammalian homologue of ypt2p in *S. pombe* (Craighead et al., 1993) and sec4p in *S. cerevisiae* (Haubruck et al., 1990) is regulating the surface delivery of VSV-G in MDCK cells (Huber et al., 1993) and probably in BHK cells as well (Peränen, J., P. Auvinen, R. Wepf, and K. Simons, manuscript in preparation). Recent results suggest that the basolateral transport route of the asialoglycoprotein receptor traverses endosomes and that this pathway could involve transport vesicles different from those delivering glycosaminoglycans to the plasma membrane (Leitinger et al., 1995).

The behavior of the apical marker influenza HA in BHK and CHO cells was in striking contrast to that of the basolateral marker proteins. AlF had no effect on its surface delivery in infected cells. Neither did anti-NSF nor rab-GDI inhibit transport in SLO-permeabilized BHK cells. The latter results are similar to what we observed for HA transport to the apical membrane in SLO-permeabilized MDCK cells (Ikonen et al., 1995). However, we have previously demonstrated by several criteria that a G_s protein regulates apical delivery in MDCK cells (Pimplikar and Simons, 1993). The lack of effect of AlF argues against such an involvement in BHK and CHO cells. Clearly further analysis will be needed to demonstrate how HA transport is regulated in nonpolarized cells. Nevertheless all the data suggest that apical and basolateral proteins use different carriers for transport to the BHK and CHO plasma membrane. Strong support for the working hypothesis that apical and basolateral cognate transport vesicles exist in nonpolarized cells comes from our analysis of the influenza HA mutant (Cys543Tyr). This single amino acid substitution converts the protein from an apical to a basolateral protein (Brewer and Roth, 1991). First, we found that AlF inhibited transport of the mutant protein, but not of wild-type HA, from the TGN to the CHO cell surface. Second, the wild-type HA became CHAPS-insoluble after being terminally glycosylated during transport through the Golgi complex as has previously been demonstrated in MDCK cells (Fiedler et al., 1993; Skibbens et al., 1989) while the mutant HA remained soluble in CHAPS as the basolateral markers VSV-G and SFV E1+E2 did. These findings suggest that HA transport in these cells also involves incorporation into glycolipid rafts mediated by unidentified protein linkers which load cargo onto the rafts in the TGN. We have recently repeated these experiments in a third fibroblast line, canine A72 cells, with similar results. Altogether these data suggest the presence of apical and basolateral cognate routes in A72, BHK, and CHO cells. Whether this is also the case for fibroblasts in vivo remains to be seen. Nevertheless, none of the three cell lines have distinct domains of VSV-G and HA on their plasma membranes. After delivery to the cell surface these two protein species spread all over the surface.

If there were apical and basolateral cognate routes from the TGN to the fibroblast cell surface, then the same signals that operate in epithelial sorting would probably also function in fibroblasts (Mellman et al., 1993). Proteins with basolateral signals would use the basolateral carriers as in MDCK cells. If the basolateral signal were inactivated by posttranslational modifications such as phosphorylation (Mostov and Cardone, 1995) or by mutation, then the potential lumenal apical signal would come into play and allow loading into the apical carriers (Matter and Mellman, 1994). The promiscuity of the apical signals would easily be explained if the recognition determinants were N-glycans (Scheiffele et al., 1995). This propensity for switching from one vesicular carrier type to another so typical for many basolateral proteins in MDCK cells would at least partially explain why mutagenesis of cell

surface proteins usually did not interfere with post-Golgi delivery (Pfeffer and Rothman, 1987). Inactivation of the basolateral signal in the cytoplasmic tail of transmembrane proteins would according to this view lead to activation of the apical signal in the extra-cytoplasmic domain of the protein. However, it should be noted that some basolateral proteins such as VSV-G (Thomas et al., 1993) may become randomized in their delivery to the MDCK cell surface after mutagenesis so that intermediate situations are possible. The question whether bulk flow carriers exist is still open. Our results only point to an alternative explanation. We cannot exclude the possibility that a separate bulk pathway operates both in fibroblasts and epithelial cells (Mays et al., 1995). Alternatively, carrier vesicles could be constructed such that signal mediated inclusion of cargo proteins lead to the assembly of a membrane meshwork in which space is available for cargo enclosure by default (Simons and Fuller, 1985). Only elucidation of the sorting machinery involved will allow us to answer these key issues in post-Golgi membrane transport.

If fibroblasts have separate carriers for apical and basolateral cognate proteins, then the two routes could be differentially regulated to transport proteins to different sites on the cell surface. For instance, different proteins could be delivered to the leading edge of a migrating fibroblast as compared to the rest of the cell surface (Singer and Kupfer, 1986; Skibbens et al., 1989). The protein would of course become distributed all over the cell surface by lateral diffusion, if not fixed at the delivery site by cytoskeletal interactions or by binding to extracellular matrix components. However, membrane subdomains could be established in this way. They would specifically be generated and maintained by membrane traffic routes.

The existence of apical and basolateral routes in epithelial cells, as well as in mesenchymal cells such as fibroblasts would greatly simplify the interconversions of cell types during embryonic development. In mesenchymal-epithelial transitions the problem of polarized surface delivery could be reduced to moving the transport vesicles in the correct direction and to fixing the docking sites for cargo delivery apically and basolaterally. Because vesicular movement requires motors for translocation along microtubules (Cole and Lippincott-Schwartz, 1995; Lafont et al., 1994) the differentiating cell will have to remodel its microtubular networks to facilitate the task of segregating apical and basolateral cognate vesicles from each other. This process involves the reorganization of the microtubule asters nucleated by the microtubule-organizing centers in mesenchymal cells into apical-basal bundles with the microtubular minus ends apically in epithelial cells (Bacallao et al., 1989). When the tracks for segregated delivery are installed during the differentiation process, polarization of the docking sites will be facilitated by the segregated delivery of membrane proteins involved in apical and basolateral docking. Such segregation has in fact been observed in single attached cells that are beginning to polarize (Rodriguez-Boulan et al., 1983). We assume that epithelial differentiation includes the differential expression of proteins, such as annexin XIIIb in MDCK cells (Fiedler et al., 1995), employed to construct apical and basolateral docking machineries specific to epithelial cell types. The validity of this working hypothesis will be testable for instance during the induction of the metanephric mesenchyme to form kidney tubules (Ekblom, 1989). The conversion from a nonpolarized to a polarized mode of surface delivery may be further complicated by the fact that apical delivery of proteins not only takes place directly from the TGN but can also occur by an indirect route over the basolateral surface, followed by transcytosis to the apical side (Mostov et al., 1992). The simplest solution on how to construct the indirect route to the apical surface would be to direct the apical transport vesicles from the TGN to the basolateral surface by appropriate microtubule tracks to lateral docking sites. Delivery to the apical side would then follow by endocytosis and transcytosis. It has been shown that sorting by the indirect transport route precedes the differentiation of the direct route from the TGN to the apical surface in thyroid development (Zurzolo et al., 1992). However, nothing is known about the machinery involved.

This excursion into development only illustrates how important it will be to elucidate how the traffic routes in eukaryotic cells are organized to understand how cell morphogenesis is patterned. Eukaryotic cells seem to have a fairly similar overall organization of membrane traffic between the ER and the Golgi complex. The differences concern the differentiation of the post-Golgi routes. Our findings that nonpolarized cells already possess two putative post-Golgi vesicles for surface delivery opens up new perspectives on polarized membrane trafficking and gives experimental evidence for the hypothesis previously formulated (Mellman et al., 1993; Rodriguez-Boulan and Powell, 1992).

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