Cell-free reconstitution of the transport of viral glycoproteins from the TGN to the basolateral plasma membrane of MDCK cells

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SUMMARY

An in vitro system to study the transport of plasma membrane proteins from the TGN to the basolateral plasma membrane of polarized MDCK cells has been developed in which purified cell fractions are combined and transport between them is studied under controlled conditions. In this system, a donor Golgi fraction derived from VSV or influenza virus-infected MDCK cells, in which [35S]-labeled viral glycoproteins were allowed to accumulate in the TGN during a low temperature block, is incubated with purified immobilized basolateral plasma membranes that have their cytoplasmic face exposed and are obtained by shearing-lysis of MDCK monolayers grown on cytodex beads. Approximately 15-30% of the labeled glycoprotein molecules are transferred from the Golgi fraction to the acceptor plasma membranes and are recovered with the sedimentable (1 g) beads. Transport is temperature, energy and cytosol dependent, and is abolished by alkylation of SH groups and inhibited by the presence of GTP-γ-S, which implicates GTP-binding proteins and the requirement for GTP hydrolysis in one or more stages of the transport process. Endo H-resistant glycoprotein molecules that had traversed the medial region of the Golgi apparatus are preferentially transported and their luminal domains become accessible to proteases, indicating that membrane fusion with the plasma membrane takes place in the in vitro system. Mild proteolysis of the donor or acceptor membranes abolishes transport, suggesting that protein molecules exposed on the surface of these membranes are involved in the formation and consumption of transport intermediates, possibly as addressing and docking proteins, respectively. Surprisingly, both VSV-G and influenza HA were transported with equal efficiencies to the basolateral acceptor membranes. However, low concentrations of a microtubular protein fraction preferentially inhibited the transport of HA, although this effect was not abolished by microtubule depolymerizing agents. This system shows great promise for elucidating the mechanisms that effect the proper sorting of plasma membrane proteins in the TGN and their subsequent targeting to the appropriate acceptor membrane.

Key words: Golgi apparatus, Viral glycoprotein, Plasma membrane, In vitro transport

INTRODUCTION

Transport of newly synthesized proteins through the cellular endomembrane system takes place by means of carrier vesicles that bud from the membrane of a donor compartment, move through the cytoplasm, and deliver their contents to a specific acceptor compartment upon fusion with its limiting membrane. During the last few years, considerable progress has been made towards an understanding of the molecular processes involved in vesicular transport between the ER and the Golgi apparatus, between the Golgi cisternae themselves, and between the plasma membrane and endosomes (for reviews see Pryer et al., 1992; Rothman, 1994). To a great extent, this has resulted from the use of in vitro systems employing semi-intact cells or subcellular fractions of defined origin that carry out transport under controlled conditions or allow the production of carrier vesicles that can be purified and analyzed.

The picture that has emerged is one in which the formation of the vesicles involves the regulated assembly of a protein coat from cytosolic components on the donor membrane, and their consumption depends on molecular recognition events between complementary proteins on the surfaces of the vesicle and the acceptor membranes. These studies have led to the identification of coat components necessary for vesicle formation (for reviews see Rothman, 1994; Robinson, 1994; Salama and Schekman, 1995), of regulatory GTP-binding proteins of the arf family that control coat assembly (for review see Donaldson and Klausner, 1994), and of the rab family that, in some still unknown way, play a critical role in transport processes (for reviews see Pfeffer, 1994; Ferro-Novick and Novick, 1993; Nuoffer and Balch, 1994). In addition, a soluble NEM sensitive factor (NSF) and proteins (SNAPS) that mediate its attachment to specific receptors (SNARES) in the membranes themselves have been identified and characterized.
and found to function in nearly all vesicular transport processes in the cell (for reviews see Rothman, 1994; Rothman and Warren, 1994; Bennett, 1995).

Proteins that traverse the Golgi apparatus, and reach the trans-Golgi network (TGN) are sorted into carrier vesicles that mediate their transport to lysosomes (for review see Kornfeld, 1992), to forming secretory granules (for review see Bauerfeind and Huttner, 1993), and to the plasma membrane, which in epithelial cells consists of distinct apical and basolateral regions with specific protein compositions (for review see Rodriguez-Boulan and Powell, 1992). The process of sorting lysosomal hydrolases in the Golgi apparatus involves their receptor-mediated concentration and sequestration at clathrin-coated vesicles (for review see Pfeffer, 1991), but very little is known about the nature, process of formation, and targeting of other vesicles that carry membrane and/or secretory proteins from the TGN to the plasma membrane. Several in vitro systems have been developed that reproduce the process of vesicle formation in the TGN (Tooze and Huttner, 1990; Salamero et al., 1990; Jones et al., 1993; Wandinger-Ness et al., 1990; Simon et al., 1992), and these have yielded valuable information on some of the molecules that participate in this process. One system has also been introduced (Gravotta et al., 1990), and recently exploited (Huber et al., 1993; Pimplikar and Simons, 1993; Pimplikar et al., 1994), that utilizes streptolysin O-perforated MDCK cells and transports proteins from the TGN to the apical or basolateral domains of the cells, while permitting the analysis of the effect of impermeant reagents and probes on transport. This has permitted the demonstration of the role of rab 8 in transport to the basolateral surface, and the participation of stimulatory and inhibitory heterotrimeric G proteins in controlling apical and basolaterally directed vesicular traffic, respectively. However, no cell-free system using defined subcellular fractions has yet been described that reproduces the full transport of protein molecules from the TGN to the cell surface.

In this paper we describe one such in vitro transport system that utilizes a Golgi fraction from virus-infected MDCK cells as a donor of glycoproteins accumulated in the TGN, and purified basolateral membranes from uninfected cells as the acceptor compartment. This system manifests many of the features expected of TGN to cell surface transport from previous studies with intact or perforated MDCK cells and provides evidence that GTP binding proteins and receptor proteins exposed on membrane surfaces are involved in this process, and that proteins associated with microtubule extracts contribute to the specificity of polarized vesicular transport to the cell surface.

MATERIALS AND METHODS

Cell culture, viral infection and preparation of donor Golgi

Monolayer cultures of MDCK cells, strain II from the ATCC, were grown in 150 mm plastic dishes (Gonzalez et al., 1987), and infected with either VSV or influenza (strain A, PR-8) (Gravotta et al., 1990). Four hours (VSV) or 7 hours (influenza) after infection, the cultures were pulse labeled (10 minutes, 3 ml/dish) with 0.5 mCi/ml [35S]methionine (sp. act. 1,200 Ci/m mole) or [35S]-labeling mix in methionine-free medium and chased in medium containing 5 mM methionine for 2 hours at 20°C, to accumulate labeled viral glyco-

protein molecules in the TGN (Matlin and Simons, 1983). Golgi fractions were prepared as described (Balch et al., 1984) and stored frozen at −70°C. Freezing and thawing did not alter their capacity to serve as donors in the transport assay (see below). The vast majority (>80%) of the labelled VSV-G molecules in these fractions were endoH-resistant and contained sialylated oligosaccharides, as indicated by their susceptibility to neuraminidase digestion (not shown). The isolated Golgi cisternae retained their characteristic stacked organization (see Fig. 1A) and fractions were highly enriched in the labelled viral glycoproteins (see Fig. 2A and B), which are represented by major bands in the fluorograms of the SDS gels and, therefore, can be directly quantified by densitometry without the need for immunoprecipitation.

Acceptor plasma membranes

MDCK cells were grown to confluence on dextran beads (Cytodex 2; Pharmacia) as described by Smith et al. (1988). The beads were washed and resuspended in 2 volumes of harvesting buffer, in which the cells were disrupted by multiple (~10) passages through an 18 gauge needle using a hypodermic syringe. The beads with the attached basolateral plasma membrane remnants were collected by sedimentation at unit gravity, washed four times and resuspended (1:1, v/v) in the harvesting medium. The effectiveness of the disruption procedure was monitored by phase contrast microscopy: intact confluent monolayers were highly refractile (see Fig. 1B, left panels) and passage through the needle was continued until all cells were disrupted. At this stage, a polygonal pattern corresponding to the attached basolateral remnants was still observed (see Fig. 1B, right panels).

Cytosolic fractions

A rat liver homogenate (1:1, v/v, in 100 mM MES, pH 6.5, 5 mM MgOAc, 1 mM DTT, 1 mM PMSF, 10 μg/ml leupeptin, 1 μM pepstatin, and 20 U/ml Transthyrol) was centrifuged at 15,000 rpm for 20 minutes in a Sorvall centrifuge (SS34 rotor) and the supernatant was recentrifuged for 90 minutes at 100,000 g (Ti60 rotor in a Beckman centrifuge) to yield a cytosolic fraction that was filtered through a 0.22 mm Millipore filter.

MDCK cytosol was prepared from confluent cells (from 20 dishes of 150 mm diameter) harvested in homogenization buffer and lysed by 50 passages through a 22 gauge needle. The homogenate was centrifuged for 90 minutes at 100,000 g and the supernatant was concentrated to 17-20 mg protein/ml by ultrafiltration with a ctreipurp 10 device from Amicon.

Cell-free transport assay

This assay was carried out by incubating the donor Golgi fraction (30-50,000 cpm, approximately ~1-3 μg protein in 75 μl) and 175 μl (approximately 3,600 beads) of a freshly prepared suspension of acceptor membranes on beads, in a final volume of 350 μl containing 50 mM Tris-HCl, pH 7.6, 5 mM MgOAc, 300 mM sucrose, 0.9 mg rat liver cytosolic protein, and either an ATP-regenerating (1 mM ATP, 10 mM creatine phosphate, 0.5 mM UTP and 50 μg of creatine phosphokinase) or an ATP-depleting (10 mM 2-deoxyglucose and 5 mg hexokinase) system. After the reaction mixtures were incubated at 37°C for 3 hours, or for the times indicated, with or without gentle stirring (1.200 rpm in a rotary shaker), the samples were placed on ice and received 2.5 ml each of washing buffer with 1 M KCl in 10 mM Tris-HCl, pH 7.4. The beads, which sediment at 1 g, were collected and washed five times with that buffer to remove any nonspecifically associated material, and twice with PBS, followed by sedimentation at 1,500 rpm for 5 minutes, before processing for SDS gel electrophoresis, followed by fluorography and densitometry (Gravotta et al., 1990), or by phosphorimager analysis.

To assess the exposure of the luminal domains of the glycoproteins after their transfer to the plasma membrane, beads recovered from the transport mixture, washed twice in 150 mM NaCl, 10 mM Tris-HCl, pH 8.0, and resuspended in 100 μl of this buffer, or Golgi fractions
In vitro Golgi to plasma membrane transport

(100 μl), were incubated with 50 μg of TPCK-treated trypsin for 45 minutes at 4°C. Soybean trypsin inhibitor (100 μg) was added and the samples were analyzed by SDS-PAGE.

Microtubule proteins

Microtubules were purified as described (Shelansky et al., 1973) and stored frozen as a pellet. For use, they were resuspended in 100 mM Pipes-KOH, pH 6.9, containing 0.1 mM EGTA, 1 mM MgCl₂, 1 mM GTP, 2 mM DTT, leupeptin, pepstatin, antipain (5 μg/ml each), benzamidine HCl (1.5 μg/ml), Trasylol (10 k.i.u./ml) and PMSF (0.2 mM). Various amounts of protein in 10 μl of this medium were used in the transport assays.

RESULTS

Characterization of the acceptor plasma membrane fraction derived from MDCK cells grown on dextran beads

When grown on cytodex beads (150 μm median diameter) (Fig. 1B, left panel), MDCK cells reach confluence at a density of ~285 cells/bead. Smith et al. (1988) have shown that bead-attached monolayers are as fully polarized as those obtained on conventional filters, judging from the distribution of plasma membrane specializations, such as junctional complexes on the lateral surfaces and microvilli on the apical ones, and from the exclusive localization of the Na⁺,K⁺-ATPase on the basolateral surfaces. We observed (not shown) that, as is the case with cells grown on other supports (Rodriguez-Boulan and Sabatini, 1978), when cells grown on beads are infected with influenza or VSV, the virions bud exclusively from the apical and basolateral surfaces, respectively. Moreover, GP-135, an endogenous apical plasma membrane marker (Ojakian and Schwimmer, 1988), had the same, exclusively apical, localization in cells grown on beads as in cells grown on filters (Fig. 1D, arrows).

When bead-grown cells were disrupted by shearing, basal portions of their plasma membranes remained attached to the beads covering their surfaces (Fig. 1B, right panel). Electron microscopy (Fig. 1C) showed that those membrane segments had their cytoplasmic aspects facing outwards from the solid support, as required for receiving any Golgi-derived transport vesicles. The shearing method yielded a high recovery of basolateral plasma membrane segments, while leaving only negligible amounts of apical plasma membrane on the beads. Thus, when MDCK monolayers grown on beads were infected with either VSV or influenza, and then labelled with [35S]methionine and chased under conditions that allow accumulation of the viral glycoproteins in the basolateral or apical plasma.
membrane domains, respectively, it was possible to show (Fig. 2B) that >70% of the G protein in VSV-infected cells remained associated with the beads, while no HA was detected on the beads from influenza-infected cells. In addition to the basal regions, significant portions of the lateral regions of the plasma membrane were also retained on the beads, as demonstrated by immunoblotting using antibodies to the junctional proteins E-cadherin and plakoglobin (gift of Dr P. Cowin, NYUMC), of which 29% and 42%, respectively, remained bead-associated after lysis of the monolayers. The beads retained only small amounts of intracellular membranes, as determined by immunoblotting from the recovery of galactosyl transferase (3%) and ribophorin I (6%), markers of the Golgi and rough ER, respectively.

**Cell free-transport of VSV-G to the basolateral plasma membrane**

To reconstitute protein transport from the Golgi apparatus to the plasma membrane, a donor Golgi fraction from VSV-infected MDCK cells containing labelled G protein molecules accumulated in the TGN was incubated at 37°C in the presence of cytosol and an ATP-regenerating system with acceptor MDCK cell basolateral plasma membranes immobilized on beads. During the incubation, up to 30% of the labelled G protein originally present in the Golgi fraction was transferred to the beads (Fig. 3A,B). Naked beads did not serve as acceptors in this assay (not shown). Transport required cytosol, reaching a maximum when ~1.0 mg of cytosolic protein was present (Fig. 3C), and did not occur when incubation was carried out at 20°C (Fig. 3B), or when ATP was depleted from the system (Fig. 3A and C). When the incubation was carried out without stirring, transfer began after a lag of 1 hour (Fig. 3A), but this lag was eliminated and transport reached a plateau after 60 minutes when the incubation was carried out with gentle shaking (Fig. 3B).

As shown in Fig. 4A, during the in vitro incubation endo H-resistant G protein molecules appeared to be transported to the beads much more effectively than the small amounts of endo H-sensitive molecules present in the donor Golgi fraction (compare lanes b and f). The preferential transport of G protein molecules that had reached the trans-Golgi region was clearly apparent from an experiment in which the donor Golgi fraction was obtained from cells that had been pulse-labelled for 10 minutes and chased for only 20 minutes at 37°C and, therefore, contained a much higher proportion (~50%) of Golgi-associated endo H-sensitive G-protein molecules. With these fractions, almost twice as much endo H-resistant G molecules were transferred to the beads as were endo H-sensitive ones.
be present either in docked vesicles that had not fused with the acceptor membranes, or in adsorbed Golgi fragments.

Immunoelectron microscopy (Fig. 5) also demonstrated that the transported G protein molecules were actually incorporated into the acceptor membranes. To facilitate their examination, immobilized basolateral plasma membranes were obtained from monolayers that were grown on polycarbonate filters and disrupted by sonication. Using this procedure, extensive flat areas of basal plasma membrane remained attached to the filter, whereas most other subcellular organelles were removed (Fig. 5a,b,c). Clathrin-coated pits forming on the basal membranes were frequently observed (Fig. 5b,c) and, occasionally, segments of lateral plasma membranes with associated junctional complexes were also present (Fig. 5a). The filters bearing the basolateral plasma membrane remnants were incubated in the transport medium with the Golgi fraction and the transported G protein molecules were detected using an antibody to the G cytoplasmic tail, followed by Protein A conjugated to gold particles. For quantitation, the number of gold particles was counted in 132 randomly chosen segments of plasma membrane, each μm in contour length and the averages of the linear measurements were used to calculate surface densities (number of particles per 100 μm² ± s.e.). When transport was carried out in the presence of an ATP-generating system, gold particles, frequently in clusters, were often found (15.3±3.7 particles/100 μm²) on the cytoplasmic face of the membrane remnants (Fig. 5e,f,g). When the ATP-depleting system was added (Fig. 5d), however, gold particles were present at much lower levels (4.5±1.2 particles/100 μm²). This detection procedure definitely established that the transfer of G protein involves membrane fusion between Golgi derived elements and the acceptor membrane.

The in vitro transport process had several features expected from a vesicular transport event between subcellular compartments. Firstly, transport of the G protein was essentially completely inhibited by the sulfhydryl alkylating reagents, N-ethylmaleimide (NEM) (Fig. 6A) and iodoacetamide (not shown) and was markedly reduced when GTP in the medium was replaced by GTP—S (Fig. 6B). The NEM-sensitive factor necessary for transport was contributed by the cytosol and not by the Golgi fraction which, when treated with the alkylating agent remained nearly as active as untreated Golgi (Fig. 6A). Mild trypsin treatment of either the immobilized basolateral membranes or the donor membranes markedly reduced transport (Fig. 6C). This should be expected if transport requires specific interactions between protein molecules derived from the donor membrane and cognate receptors or docking proteins in the acceptor membrane.

The influenza HA glycoprotein is transported to the basolateral membrane as effectively as the VSV-G

In cultures of MDCK cells infected with VSV or influenza, the corresponding viral glycoproteins, G and HA, are delivered directly from the Golgi apparatus to the basolateral and apical plasma membrane domains, respectively (Rindler et al., 1984, 1985; Matlin and Simons, 1984; Misek et al., 1984). To determine if a similar sorting and specific targetting takes place in the in vitro system, we carried out the transport reaction with bead-immobilized basolateral plasma membranes and a donor labelled Golgi fraction from influenza-infected MDCK cells. To our surprise, the influenza HA was transferred to the
membranes in an ATP (Fig. 7A) and cytosol (not shown) dependent fashion, indistinguishable from that of the VSV-G (Fig. 3A and C). Moreover, endo H-resistant HA molecules were preferentially transported and their transport was inhibited by GTP-\(\gamma\)-S and NEM (not shown). Transport of HA also involved fusion with the acceptor membrane, since it led to exposure of the luminal domain of the glycoprotein, which when the beads were treated with trypsin at 4°C was cleaved to yield subfragments HA1 and HA2 (Fig. 7B, lane e). A large fraction of the influenza neuraminidase (NA) was similarly transported and was completely digested by the protease treatment (lanes d and e, Fig. 7B).

We considered the possibilities that the efficient delivery of apical proteins to the bead-immobilized basolateral plasma membranes reflected either an imperfect polarization of the plasma membrane in cells grown on beads, or the presence on the beads of residual apical membrane fragments, which could serve as acceptors for normally targeted viral glycoproteins. Both possibilities were eliminated by an immunoelectron microscopic analysis which demonstrated that immobilized acceptor membranes obtained from MDCK cells grown on polycarbonate filters, a condition that optimizes plasma membrane polarization, can also serve as acceptors of HA molecules in the in vitro system (Fig. 5h,i). This also showed that the transferred HA molecules actually become incorporated into the basolateral plasma membranes themselves, and not into contaminating apical membrane fragments. Moreover, when an ATP-generating system was present during the incubation, the surface density of gold particles on the acceptor membranes, indicating transported HA molecules, greatly decreased (from 72±10 to 2.6±0.7 particles/100 \(\mu\)m\(^2\)).

The apparently indiscriminate transport of HA and G to the basolateral plasma membrane was not due to the absence of specific factors from the rat liver cytosol, since it was also observed with MDCK cell cytosol (not shown). The in vitro mistargeting of HA did not reflect the presence of an excess of acceptor basolateral membranes with a much lower capacity to accept basolaterally-directed than apically-directed vesicles.
Fig. 6. (A) An NEM-sensitive factor contributed by the cytosol is necessary for transport. NEM (5 mM) was added to either the Golgi or cytosolic fraction and each sample was incubated for 15 minutes at 0°C, before adding a 1.5-fold molar excess of DTT. For each condition, control incubations were carried out in which the fractions had been mock-treated with NEM, i.e., each received the DTT together with NEM. The incubations for in vitro transport were then carried out with either cytosol, Golgi or both components pretreated in this fashion, as indicated below each bar. The extent of ATP-dependent transport is given as a percentage of that in the corresponding control with the mock-treated component(s), which amounted to 20 to 22% of the labelled G protein in the donor Golgi.

(B) Inhibition of the in vitro transport of VSV-G by GTP-\(\gamma\)-S. Three pairs of reaction mixtures lacking the acceptor membranes and the ATP regenerating system were incubated for 5 minutes at 37°C with no additions (none), or with the addition of either GTP (200 \(\mu\)M) or GTP-\(\gamma\)-S (20 \(\mu\)M). All samples then received acceptor membranes and one member of each pair an ATP regenerating system (open bars) and the other an ATP depleting system (filled bars), before incubation for 3 hours at 37°C. (C) The integrity of proteins exposed on the cytoplasmic surface of donor and acceptor membranes is required for in vitro transport. Samples (35 \(\mu\)l) of a Golgi fraction containing labelled VSV-G protein, or of beads bearing basolateral plasma membranes (175 \(\mu\)l), were incubated for 15 minutes at 4°C with trypsin (2 \(\mu\)l, 1 mg/ml), with or without the prior addition of soybean trypsin inhibitor (2 \(\mu\)l, 13 mg/ml). After the preincubations, the inhibitor was added to the samples that had not previously received it, followed by addition of the remaining components necessary for transport and incubation at 37°C for 4 hours. Triplicate samples were incubated for each condition and the extent of ATP-dependent transport is expressed as a percentage of that obtained in triplicate control samples that contained untreated donor and acceptor membranes.

Thus, when mixed Golgi fractions from VSV and from influenza virus-infected cells were incubated with variable amounts of acceptor membranes (Fig. 7C), a reduction in the amount of acceptor membranes limited the transport of both glycoproteins to the same extent (Fig. 7C, lanes d,e).

We also examined the possibility that the absence from the in vitro system of some cellular constituents necessary for the maintenance of polarity was responsible for the indiscriminate transport of both glycoproteins. We have previously shown (Rindler et al., 1987) that the polarized delivery of HA to the apical surface of MDCK cells in vivo is disrupted by microtubule depolymerizing agents, which do not affect the basolateral targeting of VSV-G. The in vitro transport system, did not seem to require microtubules, since depolymerizing agents did not affect transport (not shown). We examined, however, the effect that adding a preparation of microtubular proteins to the in vitro assay had on the targeting of the viral glycoproteins.

As shown in Fig. 8, small amounts of microtubular proteins (<6 \(\mu\)g) completely suppressed HA transport, without inhibiting the transport of VSV-G, which was, however, also diminished at higher concentrations of the added proteins (>12 \(\mu\)g). The capacity of the microtubular proteins to inhibit transport was not affected by the presence of the microtubule inhibitors nocodazole (37 \(\mu\)M) or colchicine (15 mg/ml), but was abolished by heating the microtubule extract for 10 minutes at 95°C (experiments not shown).
We have developed a cell-free system that effects the energy, temperature, and cytosol-dependent transport of viral glycoproteins accumulated in the trans region of a donor Golgi fraction from MDCK cells, to acceptor basolateral plasma membranes immobilized on beads. The transfer involves the fusion of donor-derived closed vesicles with the planar acceptor membranes and leads to the incorporation of the glycoprotein molecules into the acceptor plasma membrane with their normal transmembrane disposition. This was strikingly demonstrated by the immunoelectron microscopic localization of the C-terminal tails of the glycoproteins on the cytoplasmic surface of the acceptor membranes, which are derived from uninfected cells. Electron microscopy also showed that the transferred glycoprotein molecules are frequently present in clusters, as would be expected if they were delivered by vesicular carriers in which the molecules are concentrated. However, we cannot exclude the possibility that transfer involved the formation of tubular extensions, or other appendages of the donor Golgi apparatus, that fused with the acceptor membranes, even though this was not observed in the electron micrographs.

When the transport reaction was carried out without stirring, substantial amounts of the viral glycoprotein began to be detected on the acceptor membranes only after a lag of approximately 60 minutes. This lag could be eliminated by gentle stirring of the incubation reaction, which should increase the frequency of collisions between the donor membranes and the cytosolic components (e.g. coat protein complexes) necessary to form vesicles, as well as between the putative transport vesicles and the acceptor membranes. The lag was also significantly reduced when the Golgi fraction was preincubated with cytosol for 60 minutes (not shown). This would be expected if the lag includes the time that is required to generate transport intermediates from the donor membrane, which in permeabilized cells we have previously shown (Gravotta et al., 1990) is at least 15 minutes. In a separate study (D. Gravotta et al., unpublished observations), we have been able to demonstrate the direct transport to the immobilized basolateral membranes of sialylated G protein contained in TGN-derived vesicles which were isolated from a Golgi fraction preincubated in vitro in the presence of cytosol and an energy supply (Simon et al., 1992). Surprisingly, in this case, the temperature-dependent transport did not require ATP or the addition of cytosol, although it was sensitive to NEM and was eliminated by mild proteolysis of the vesicles.

Our finding that GTP-γ-S inhibited Golgi to plasma membrane transport in the in vitro system is consistent with our previous observations using streptolysin SLO permeabilized MDCK cells (Gravotta et al., 1990), and with the reports that this analogue inhibits post Golgi vesicle formation in semi-intact cells (Xu and Shields, 1993) and in a post nuclear supernatant containing Golgi membranes (Tooze et al., 1990). In fact (Huber et al., 1993; Pimplikar and Simons, 1993) both heterotrimeric G proteins of the G1 class and the small GTP-binding protein rab8 have been implicated in controlling Golgi to basolateral membrane transport. We have found, however, that a 21mer C-terminal peptide of rab 8 added at a concentration more than five times higher than that reported to significantly inhibit transport in SLO-permeabilized cells (Huber et al., 1993) had no measurable effect in our in vitro transport system (not shown).

Recent findings suggest that the vesicular transport systems responsible for the preferential delivery of different sets of carrier vesicles to the apical or basolateral plasma membrane domains of MDCK cells rely on fundamentally different targeting mechanisms. A rab-NSF-SNAP-SNARE-dependent system appears to operate only in the docking and fusion of basolaterally-directed vesicles (Ikonen et al., 1995), whereas a specific protein, annexin 13b, seems to participate only in apical targeting (Fiedler et al., 1995). The fact that in our in vitro system both the VSV-G protein, which is normally addressed to the basolateral surface, and the influenza HA, whose distribution is normally apical, were equally well transported to the immobilized basolateral plasma membranes cannot be explained by what could be considered trivial deficiencies of the system, such as contamination of the beads with apical membranes, or an imperfect polarity of the plasma membranes of cells grown on beads. These possibilities were eliminated by cytochemical studies which showed that MDCK cells grown on beads are as well polarized as those grown on filters, and by biochemical and immunoelectron microscopy analysis, which showed that the transported viral glycoproteins were actually incorporated into the basolateral acceptor membranes.

The incapacity of the in vitro system to discriminate between proteins destined to apical and basolateral domains may, in fact, become a useful attribute for future studies on the identification of the components which, within MDCK cells, confer vectoriality to the delivery of the glycoproteins to opposite surfaces. In this context, it is important to note that in other types of polarized epithelial cells, such as hepatocytes (Bartles
We have previously observed that in MDCK cells, microtubules play a role in determining the proper addressing of HA to the apical surface (Rindler et al., 1987), and subsequent studies have shown that microtubule depolymerizing agents lead to the mistargeting of apical proteins in several types of polarized cells (Eilers et al., 1989; Breitfeld et al., 1990; Achler et al., 1989; Tashiro et al., 1993). Hence, we examined the effect of a microtubular protein extract on the targeting specificity of the in vitro system. The encouraging finding that low concentrations of these proteins preferentially suppressed the delivery of HA to the basolateral surface suggests that, as proposed previously (Rindler et al., 1987), in vivo, microtubules may serve to direct the transport of apical vesicles to the apical surface, thus restricting their access to the basolateral membrane. Since in confluent MDCK cells many microtubules are oriented along the basoapical axis, with their minus ends towards the apical surface (Bacallao et al., 1989), within the cell, the transport of vesicles containing HA would normally occur along those microtubules in the retrograde direction and involve dynein motor molecules. In contrast, it is possible that in the in vitro system, which lacks apical membranes and their putative associated microtubule organizers, assembly of the microtubules from the added protein is initiated by microtubule organizing centers (MOC) associated with the Golgi apparatus. In this case, the inhibitory effect would be explained, since the minus ends of the microtubules would be oriented towards the Golgi, and the HA-containing vesicles would be transported away from the acceptor membranes. On the other hand, the transport of the VSV-G protein-containing vesicles, which have been reported to utilize the plus-directed microtubule motor kinesin (Lafont et al., 1994), would be inhibited only at higher microtubule protein concentrations, at which assembly of randomly oriented microtubules could occur independently of the MOCs. We must point out, however, that we found that addition of colchicine or nocodazole to the in vitro system did not eliminate the inhibitory capacity of the microtubule protein fraction. This leads us to conclude that either stable microtubules are formed in the system that are resistant to the depolymerizing agents, as is the case with microtubules containing acetylated tubulin (Piperno et al., 1987; Billger et al., 1991), or that proteins in the microtubule extract exert their effect without the need for microtubule polymerization. Further experiments focusing on the purification of the inhibitory component(s) are required to resolve this question.

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