Transport of Influenza HA from the *trans*-Golgi Network to the Apical Surface of MDCK Cells Permeabilized in their Basolateral Plasma Membranes: Energy Dependence and Involvement of GTP-binding Proteins

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Abstract. A procedure employing streptolysin O to effect the selective permeabilization of either the apical or basolateral plasma membrane domains of MDCK cell monolayers grown on a filter support was developed which permeabilizes the entire monolayer, leaves the opposite cell surface domain intact, and does not abolish the integrity of the tight junctions. This procedure renders the cell interior accessible to exogenous macromolecules and impermeant reagents, permitting the examination of their effects on membrane protein transport to the intact surface.

The last stages of the transport of the influenza virus hemagglutinin (HA) to the apical surface were studied in pulse-labeled, virus-infected MDCK cells that were incubated at 19.5°C for 90 min to accumulate newly synthesized HA in the *trans*-Golgi network (TGN), before raising the temperature to 35°C to allow synchronized transport to the plasma membrane. In cells permeabilized immediately after the cold block, 50% of the intracellular HA molecules were subsequently delivered to the apical surface. This transport was dependent on the presence of an exogenous ATP supply and was markedly inhibited by the addition of GTP-γ-S at the time of permeabilization. On the other hand, the GTP analogue had no effect when it was added to cells that, after the cold block, were incubated for 15 min at 35°C before permeabilization, even though at this time most HA molecules were still intracellular and their appearance at the cell surface was largely dependent on exogenous ATP. These findings indicate that GTP-binding proteins are involved in the constitutive process that effects vesicular transport from the TGN to the plasma membrane and that they are charged early in this process. Transport of HA to the cell surface could be made dependent on the addition of exogenous cytosol when, after permeabilization, cells were washed to remove endogenous cytosolic components. This opens the way towards the identification of cell components that mediate the sorting of apical and basolateral membrane components in the TGN and their polarized delivery to the cell surface.

THE intracellular transport of membrane, secretory and lysosomal proteins synthesized in the ER involves a sequence of steps in which membrane vesicles containing these proteins are formed in one compartment, move within the cytoplasm, and fuse with a specific acceptor membrane (Palade, 1975). There is now intense interest in the mechanisms and sorting processes that first effect the segregation of specific classes of proteins into the forming vesicles and then restrict the fusion of these vesicles to the appropriate acceptor membrane, since they are responsible for the characteristic distribution of proteins within cells.

The trans region of the Golgi apparatus, now designated the *trans*-Golgi network (TGN[1]; Griffiths and Simons, 1986), is a major site of sorting in which proteins are segregated into vesicles that are destined to lysosomes, to forming secretory granules, or for constitutive delivery to the plasma membrane. Of particular interest are the mechanisms which, in some polarized epithelial cells, operate in the TGN to generate different classes of vesicles that transport proteins vectorially to the distinct apical or basolateral plasma membrane domains characteristic of those cells (Rindler et al., 1984, 1985; Matlin and Simons, 1984; Misek et al., 1984).

It is widely recognized that a thorough analysis of vesicular transport requires the use of cell-free or permeabilized cell systems that, by eliminating the barrier imposed by the plasma membrane, permit the manipulation of the environment surrounding the intracellular membranes. Employing such systems, it has been shown that the nonhydrolyzable GTP analogue, GTP-γ-S, can block vesicular transport from

1. *Abbreviations used in this paper*: HA, hemagglutinin; HU, haemolytic unit; SLO, streptolysin O; TGN, *trans*-Golgi network; VSV, vesicular stomatitis virus.

the ER to the Golgi apparatus (Beckers et al., 1989) and between Golgi cisternae (Melançon et al., 1987; Malhotra et al., 1988). In the latter case it was found that the GTP analogue led to the accumulation of carrier vesicles in the acceptor membrane, as if an activated GTP-binding protein regulating transport blocked a step between vesicle attachment to the acceptor membrane and the subsequent membrane fusion. These findings, which implicate GTP-binding proteins and GTP hydrolysis in vesicular transport, are in accord with results obtained with temperature-sensitive yeast mutants in which defects in GTP-binding proteins have been shown to be responsible for blocks in specific steps along the secretory pathway, including transport from the TGN to the cell surface (Schmitt et al., 1986; Salminen and Novick, 1987; Schmitt et al., 1988; Goud et al., 1988; Segev et al., 1988; Walworth et al., 1989; Baker et al., 1990; Haubruck et al., 1990).

The molecular mechanism by which GTP-binding proteins regulate vesicular transport has not yet been elucidated, but there is now considerable support (Walworth et al., 1989; Salminen and Novick, 1989; Plutner et al., 1990) for the notion proposed by Bourne (1988) that they function as molecular switches associated with the cytoplasmic surface of the vesicle membrane. GTP hydrolysis would take place after the vesicle docks in the receiving membrane and the resulting conformational change would lead to release of the GTP-binding protein, leaving the vesicle in a state in which it can undergo fusion with the acceptor membrane. Although studies with mutants in the *Sec4* gene, which encodes a protein that is required for constitutive secretion and is associated with the surface of secretory vesicles (Salminen and Novick, 1987, 1989; Goud et al., 1988; Walworth et al., 1989), have implicated this GTP-binding protein in Golgi to cell surface transport, no direct biochemical demonstration of the participation of a GTP-binding protein in that stage of intracellular transport has yet been provided.

The intracellular sorting of plasma membrane proteins to the distinct apical or basolateral surface domains of epithelial cells has been extensively studied in polarized monolayers of cultured MDCK cells which, when infected with enveloped viruses or transfected with genes encoding the viral glycoproteins, address these proteins to one or the other plasma membrane domain (Rodriguez-Boulan and Sabatini, 1978; Compton et al., 1989; Stephens et al., 1986; McQueen et al., 1987; Roth et al., 1987; Puddington et al., 1987). Transport of the newly synthesized viral glycoproteins from the TGN to the respective cell surface can be synchronized by incubating the cells at a low temperature (19.5°C), that leads to the accumulation of the proteins in the TGN, and then raising the temperature to allow their movement to the cell surface (Matlin and Simons, 1983; Rindler et al., 1985).

We have developed a procedure that utilizes the bacterial toxin streptolysin O (SLO) to selectively permeabilize either the apical or basolateral surfaces of MDCK monolayers formed on a permeable support. This toxin binds to cholesterol within the plasma membrane and, when applied to mast cells (Howell et al., 1987; Howell and Gomperts, 1987), was shown to produce plasma membrane discontinuities sufficiently large to permit the entrance of macromolecules into the cytoplasm. Using this permeabilization procedure, which we show maintains the integrity of intercellular junctions, we have been able to modify intracellular conditions from the permeabilized side of the monolayer, while monitoring the effect of these modifications on vesicular transport to the intact surface. It was found that GTP-γ-S blocked transport of the influenza hemagglutinin (HA) glycoprotein from the TGN to the apical plasma membrane domain, but only when it was added immediately after the cells were released from the cold block and not at a later time, even though nearly all the HA molecules were still inside the cell. This indicates that GTP-binding proteins are involved in the constitutive process that affects vesicular transport of viral envelope glycoproteins from the TGN to the plasma membrane and that two stages can be recognized in this process. GTP binding would take place during the first stage, which probably corresponds to vesicle formation, and GTP hydrolysis during the subsequent delivery of the vesicle to the plasma membrane. Since removal of cytosolic components from the permeabilized cells was shown to render transport dependent on the addition of exogenous cytosol, it should be possible in future studies to identify the specific cytosolic proteins that play essential roles in the delivery of membrane proteins in a polarized fashion from the TGN to the cell surface.

Materials and Methods

Cell Culture Viral Infection and Metabolic Labeling

MDCK cells, strain II, were cultured as previously described (Cereijido et al., 1978). After reaching confluence, cells were detached with trypsin-EDTA and plated at a density of 5×10^3 cells/cm^2 in 24-well dishes or, when indicated, at 1–2 $\times$ 10^5 cells/cm^2 on 24.5-mm Polycarbonate Transwell™ filters of 0.4-μm pore size (Costar Data Packaging Corp., Cambridge, MA). Cells grown in plastic wells were used 2–3 d after they reached confluence once again and those grown on filters after they developed a transmonolayer electrical resistance of 200–300 Ω·cm^2, generally 4–7 d after plating.

For viral infection, cells rinsed once in prewarmed, serum-free, DME were incubated with 10 p.f.u/cell of vesicular stomatitis virus (VSV; Indiana strain, grown in HeLa cells) or 15 p.f.u/cell of influenza virus (strain A PR-8, grown in 18-d-old chicken embryos) in medium, which for VSV infection also contained 100 μg/ml DEAE-dextran (Pharmacia Fine Chemicals, Piscataway, NJ). After 60 (VSV) or 90 (influenza) min at 37°C, the inoculum was removed, the medium replaced with fresh DME containing 7.5% serum, and incubation continued for 3.5 h (VSV) or 6 h (influenza).

For metabolic labeling, monolayers were rinsed once with prewarmed HBSS-Ca,Mg (HBSS containing 0.45 mM $CaCl_2$ and 1 mM $MgCl_2$) and then incubated for 15 min at 37°C in methionine-free RPMI medium containing HEPES-Na buffer (15 mM, pH 7.4). Subsequently, the cells were pulse labeled for 10 min at 37°C with ^{35}S-methionine (300–500 μCi/ml, 1,000 Ci/mMole) in 250 μl of methionine-free RPMI containing the Hepes buffer. To terminate the pulse, the medium was removed and the cells were quickly rinsed with cold serum-free DME containing 300 μg/ml methionine. Filter-grown cells were labeled by adding the radioactive precursor to the basolateral surface. The filters were first inverted in a wet chamber, and 250 μl of the ^{35}S-methionine-containing medium was added to the bottom of the filter, which was then incubated at 37°C for 10–15 min. To terminate the pulse, the filters were returned to the normal orientation and washed once in cold DME containing unlabeled methionine. For experiments in which Golgi to plasma membrane transport was studied, after the pulse the cells were incubated at 19.5°C for 90 min in bicarbonate-free MEM to accumulate the labeled viral glycoproteins in the trans Golgi region (Matlin and Simons, 1983; Griffiths et al., 1985; Fuller and Simons, 1985).

Preparation and Activation of SLO

The content of one vial (10–12 haemolytic units [HU]) of freeze-dried SLO (VWR Scientific, South Plainfield, NJ) was dissolved in 10 ml of distilled water and the preparation was concentrated ~10-fold in a Centriprep™ 30 device (Amicon Corp., Division of W. R. Grace & Co., Danvers, MA). The

concentrated toxin was activated by addition of 2 mM DTT and incubation at 37°C for 10 min. The haemolytic activity was titrated as described (Bhakdi et al., 1984) with minor modifications. The toxin activity was adjusted to a titer of 120 HU/ml.

Selective Permeabilization of Apical or Basolateral Plasma Membranes of MDCK Cells with SLO

MDCK cells cultured on 24-well dishes or Polycarbonate Transwell™ filters were washed once at 0°C with HBSS-Ca,Mg. Permeabilization was then accomplished by a two-step procedure. In the first step, the cells were incubated for 5 min at 0°C with 300 μl (for each well in a 24-well dish) or 600 μl (for apical or basolateral sides of cells on filters) of a prechilled solution of activated SLO. After this toxin-binding step, excess toxin was removed and the cells were rinsed twice with HBSS-Ca,Mg at 0°C. At this stage, the cells are still intact and any desired medium can be added before the temperature is raised to 35°C to effect the permeabilization. Perforation was carried out in a very small volume of medium (50 μl), which minimized the dilution of released cytosolic protein. To this effect, after applying the medium to the desired surface, this was covered with a polystyrene disk that had the same diameter as the chamber. This insured that the small volume of fluid homogeneously wetted the filter and minimized evaporation.

Labeling of Cells with Impermeant Fluorescent Probes

MDCK cells grown on 6.5-mm diameter Polycarbonate Transwell™ filters were permeabilized with SLO from the apical or basolateral surface, as just described but a proportionally smaller volume of SLO solution was used (150 μl). After the toxin was washed out, the cells were incubated at 35°C for 15 min with 50 μl of "transport medium" (see below) added to the apical or basolateral side containing either rhodamine-labeled phalloidin (TRITC-phalloidin, 1 μg/ml final dilution) or rabbit anticytokeratin antibodies (gift of Dr. T. T. Sun, NYU School of Medicine; 1:80 final dilution). Unbound reagents were removed by three successive washes at room temperature with buffer A (80 mM PIPES-KOH buffer, pH 7.0, 25 mM NaCl, 25 mM KCl, 1 mM $MgCl_2$, 0.6 mM $CaCl_2$, and 1 mM EGTA to buffer Ca^{2+} ions to an approximately free concentration of 0.67 μM [Bers, 1982]) and the monolayers were then fixed with 4% paraformaldehyde in PBS. To detect the keratin-bound antibodies, samples were permeabilized by incubation with 0.2% Triton X-100 in PBS for 10 min at room temperature, incubated for 1 h in PBS containing albumin (0.8%) and Rhodamine-conjugated affinity-purified goat anti-rabbit IgG (Cappel Lab., Cochranville, PA) at 1:80 final dilution, and washed three times with PBS. Both the phalloidin-treated and antibody-labeled filters were dehydrated in graded ethanol at room temperature, mounted in Eukitt (Calibrated Instruments, Inc., Ardsley, NY) and observed in a Zeiss photoscope equipped with epifluorescence optics.

Incubation for Protein Synthesis

SLO-treated cells were rinsed once in pre-chilled buffer A and then incubated with 50 μl of "synthesis medium" made by mixing (in one volume) the following stock solutions: 0.1 vol of 300 mM PIPES-KOH buffer (pH 7.0), 300 mM KOAc, 100 mM NaCl, 30 mM $MgOAc_2$, 10 mM DTT, 6 mM $CaCl_2$, 10 mM EGTA; 0.1 vol of a freshly prepared energy-regenerating system containing 10 mM ATP-Na, 5 mM GTP-Na, 50 mM creatine phosphate, 150 μg/ml rabbit muscle creatine phosphokinase; 0.2 vol methionine free-RPMI media; 0.02 vol of 0.4 mM hemin, 0.02 vol of total liver tRNA (Boehringer Mannheim Biochemicals, Indianapolis, IN; 10 mg/ml); 0.01 vol RNAsin (Promega Biotec, Madison, WI; 4,000 U/ml); 10–30 μCi ^{35}S-methionine (final concentration 200–600 μCi/ml, 1,000 Ci/mMole). When desired, the energy-regenerating system was replaced with 0.05 vol of apyrase (grade VIII, 100 U/ml; Sigma Chemical Co., St. Louis, MO). For pulse labeling, the cells were incubated with the labeling medium for 10 min. The medium was then removed and the monolayers were rinsed with prechilled buffer A containing 300 μg/ml methionine. At this point, the cells were lysed and processed for TCA precipitation, immunoprecipitation, and/or SDS-gel electrophoresis, or were subjected to a chase incubation in an appropriate medium (see below).

Incubation for ER to the Golgi Transport

MDCK cells infected with VSV or influenza were either pulse labeled with ^{35}S-methionine for 10 min and then treated with SLO, or were first treated with SLO and then labeled (as indicated above). For transport experiments, the cells were incubated at 35°C for 45 min, or for the indicated times, with 50 μl of "transport medium" containing (in 1 vol) 0.1 vol of 300 mM PIPES-KOH buffer (pH 7.0), 300 mM KOAc, 100 mM NaCl, 30 mM $MgOAc_2$, 10 mM DTT, 6 mM $CaCl_2$, 10 mM EDTA; 0.1 vol of a freshly prepared energy-regenerating system containing 10 mM ATP-Na, 2 mM GTP-Na, 0.5 mM UTP-Na, 50 mM creatine phosphate, rabbit muscle creatine phosphokinase (150 μg/ml); 0.2 vol methionine free-RPMI medium; 0.1 vol of a mixture of 100 μM CMP-NAN, 500 μM UDP-GlcNAc$_2$, and 500 μM UDP-Gal. Transport was stopped by addition of buffer A at 0°C, or lysis buffer. In some experiments, the transport cocktail lacked the energy-regenerating system but contained, instead, either apyrase (5 U/ml to destroy ATP) or deoxyglucose (6 mM) and antimycin A (5 μM; Boehringer Mannheim Biochemicals) to prevent its endogenous accumulation.

Incubation for Golgi to Plasma Membrane Transport

Virus-infected MDCK cells grown on Polycarbonate Transwell™ filters were pulse labeled and then incubated in a chase medium at 19.5°C for 90 min to allow transport from the ER through the Golgi apparatus and accumulation in the *trans*-Golgi region (Fuller and Simons, 1985; Griffiths et al., 1985). After permeabilizing the apical or basolateral surface with SLO, 50 μl of transport medium were added to each side of the filters which were then incubated at 35°C for 45 min. Transport was stopped by addition of 1.5 ml of HBSS-Ca,Mg at 0°C or lysis buffer (see below).

Preparation of MDCK Cytosol

Confluent MDCK cells were washed with warm (37°C) and cold (0°C) HBSS, harvested in the cold by scraping with a rubber policeman, collected by centrifugation (1,500 rpm for 5 min at 4°C), and homogenized in 2 vol of buffer H (0.2 M Sucrose, 15 mM PIPES-KOH, pH 7.0, 2 mM $MgCl_2$, 0.1 mM EDTA, 10 μg/ml antipain, 10 μg/ml leupeptin, 100 U/ml Trasylol, 0.2 mM PMSF) with 20 strokes in a type B Dounce homogenizer. A post-nuclear supernatant was prepared (3,000 *g* for 15 min) and stored in frozen aliquots which, when needed, were quickly thawed in a 37°C water bath, centrifuged at 100,000 *g* for 45 min to remove aggregates, and used within 3 h.

Cell Lysis and Immunoprecipitation

Cells were scraped off the plastic or filters with a rubber policeman and solubilized with 300 μl of lysis buffer (25 mM Tris-HCl, pH 7.5, 1% SDS, 10 mM EDTA, 1 mM PMSF, 100 U/ml Trasylol). Lysates were transferred to a 1.5-ml Eppendorf tube, sonicated for 15 s, and the insoluble material was removed by sedimentation for 10 min in an Eppendorf centrifuge. An aliquot (30–60%) of the lysate was added to 1 ml of immunoprecipitation buffer (50 mM Tris-HCl, pH 7.4, 190 mM NaCl, 6 mM EDTA, 2.5% Triton X-100, 1 mM PMSF, 100 U/ml trasylol) containing 1–2 μl of either anti-HA mAb (obtained from a hybridoma generously provided by Dr. P. Palese, Mt. Sinai School of Medicine, NY) or rabbit polyclonal anti-VSV-G antiserum (provided by Dr. M. Rindler, NYU School of Medicine, New York, NY). After 8 h incubation at 4°C, 20 μl of packed goat anti-mouse IgG Sepharose beads (Cappel Lab., Cochranville, PA) or 25 μl of packed protein A–Sepharose beads (Sigma Chemical Co.; for precipitation with rabbit antiserum) was added to the mixtures which were then incubated for 6 h at 4°C. Bead-absorbed immunocomplexes were sedimented and rinsed three times in immunoprecipitation buffer and once in distilled water. The beads bearing the immunoprecipitates were then either processed for SDS gel electrophoresis or glycosidase digestion.

Endoglycosidase H Digestion

Immunoprecipitated VSV-G or influenza HA was eluted from washed beads by boiling for 5 min in Na-citrate buffer (100 mM, pH 5.5) with 1% SDS. The supernatant obtained after a brief centrifugation in an Eppendorf centrifuge was added to an equal volume of water and divided into two aliquots which were incubated at 37°C for 16 h with (2/3 of the sample), or without (1/3 of the sample) 2–5 mU of Endoglycosidase H (Boehringer Mannheim Biochemicals). Samples were dried in a speed vacuum concentrator (model SVC 100H; Savant Instruments, Inc., Farmingdale, NY) before processing for gel electrophoresis.

Trypsin Digestion of HA Molecules Delivered to the Cell Surface

After incubation for Golgi to plasma membrane transport, filters were incubated for 60 min at 4°C with 1 ml of HBSS-Ca,Mg containing 300 μg/ml of Tosylamide phenylethylchloromethylketone-treated trypsin (Worthington Biochemical Corporation, Freehold, NJ) added to the apical side. Digestion was stopped by addition of 2,000 U soybean trypsin inhibitor (Sigma Chemical Co).

Gel Electrophoresis and Fluorography

VSV-G or influenza HA immunoabsorbed on washed beads or dried glycosidase-treated samples were analyzed by SDS-PAGE (10%) (Laemmli, 1970) and fluorography using EN^{3}HANCE (New England Nuclear, Boston, MA). The dried gels were exposed to X-OMAT AR film (Eastman Kodak Co., Rochester, NY) at −70°C for 2–5 d and fluorograms obtained, after different exposure times, were scanned with a transmission scanning densitometer (model GS300 connected to a computer with a GS370 integrator; Hoefer Instruments, San Francisco, CA) to determine the area under each peak.

Electrical Resistance Measurements

Cultures of MDCK cells on 6.5-mm diameter polycarbonate Transwell™ filters of 0.4-μm pore size (Costar Data Packaging Corp.), which were maintained for 3 d after confluency to allow the development of maximal transepithelial electrical resistance (200 Ω·cm^2), were treated for 5 min at 0°C with 150 μl of SLO solution (120 HU/ml) added to the apical, basolateral, or both surfaces. After the toxin was removed, the transport medium was added (50 μl to the apical side and 500 μl to the basolateral side) and incubation continued for various times at 35°C. The electrical conductance was measured with a microammeter (Simpson Electric Co., Elgin, IL) using Ag/AgCl electrodes. The values were converted into Ω·cm^2, as described by Cereijido et al. (1978). The value of the electrical resistance of the filter without cells was subtracted from the values measured.

Results

Selective Permeabilization of the Apical or Basolateral Domain of the Plasma Membrane of MDCK Cells

SLO is a convenient reagent to affect the permeabilization of the plasma membrane without damaging intracellular membranes, because the toxin can be first bound at 0°C to the surface of intact cells without causing permeabilization and the excess toxin can then be removed before pore formation is induced by simply raising the temperature to 35°C (Alouf, 1980; Hugo et al., 1986). In this two-step procedure, therefore, the permeabilizing agent never has access to the cell cytoplasm. Using this method, it was found that an SLO concentration of 120–150 HU/ml was required to yield maximal release of labeled cellular proteins from MDCK cells that had been incubated with ^{35}S-methionine during a preceding 60-min pulse (Fig. 1). In several experiments of this type, ~35–40% of the total radioactive protein was released into the medium after 20 min of incubation at 37°C. The pores produced by SLO were sufficiently large to permit release of proteins with M_r as large as 200 KD (Fig. 1, *inset*).

With this two-step procedure, SLO could be used to selectively permeabilize either apical or basolateral plasma membrane domains of epithelial cell monolayers. Thus, when the toxin was applied to only one surface of MDCK monolayers grown on filters, impermeant probes, such as fluorescent-labeled phalloidin, which binds to actin filaments, and antibodies to keratins, entered the cells only when applied to the same surface as the toxin (Fig. 2). Under these conditions the permeabilization was highly effective, since the entire cell population became labeled.

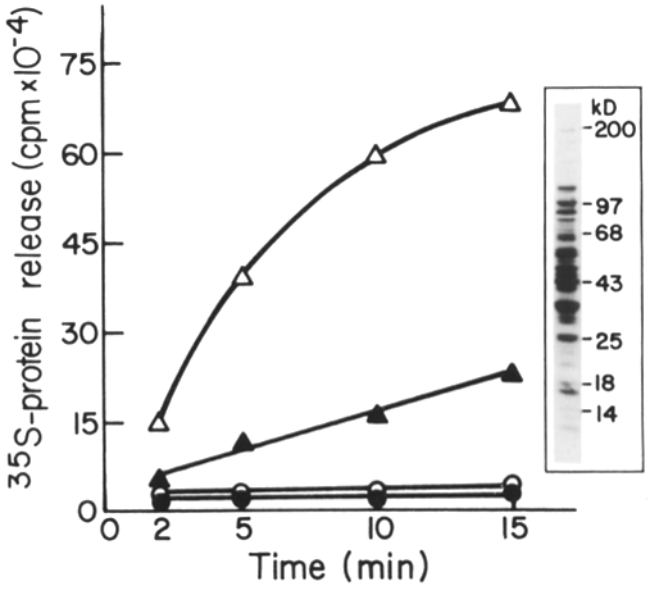

Figure 1. Release of prelabeled proteins from MDCK cells upon treatment with SLO. MDCK cells grown in wells of a 24-well dish were labeled with ^{35}S-methionine for 60 min and incubated in chase medium for the same period of time. The cells were then incubated at 0°C for 5 min with solutions containing different concentrations of preactivated SLO (△, 150 HU/ml; ▲, 40 HU/ml; ○, 10 HU/ml; ●, 0 HU/ml). After removing the toxin, the cells were rinsed twice and incubated with 250 μl of buffer A prewarmed to 35°C. At different times, 25-μl aliquots of the medium were removed to determine the TCA-precipitable radioactivity released from the cells and at the end of the incubation the remaining cell-associated radioactivity was determined. The maximum radioactivity release obtained in cells treated with 150 HU/ml SLO was 40% of the total incorporated ^{35}S-methionine. An aliquot of medium taken after 20 min of incubation with 150 HU/ml was analyzed by electrophoresis in a 7.5 to 20% polyacrylamide SDS gradient gel (*inset*).

Maintenance of a Transepithelial Resistance in Monolayers with Only One Permeabilized Surface

The immunofluorescence experiments just described indicated that the impermeant probes, although capable of entering the cells through the permeabilized plasma membrane, were unable to cross the tight junctions. Measurements of the transepithelial resistance established that the junctional barrier had not been eliminated, since only when the toxin was applied from both sides of the monolayers was the electrical resistance totally abolished (Fig. 3). Although treatment of the monolayers with SLO from one side caused a substantial reduction in the resistance, this was not due to a disruption of the junctions, since a drop in resistance, albeit less pronounced, also occurred in control monolayers not treated with SLO but submitted to successive incubations at 0°C and 35°C in normal medium (Fig. 3).

Protein Synthesis and Transport of Viral Glycoproteins from the ER to the Golgi Apparatus in SLO-permeabilized Cells

Permeabilized MDCK monolayers were capable of synthesizing both cytosolic and membrane proteins, as was demonstrated using VSV-infected cells (Fig. 4). After SLO

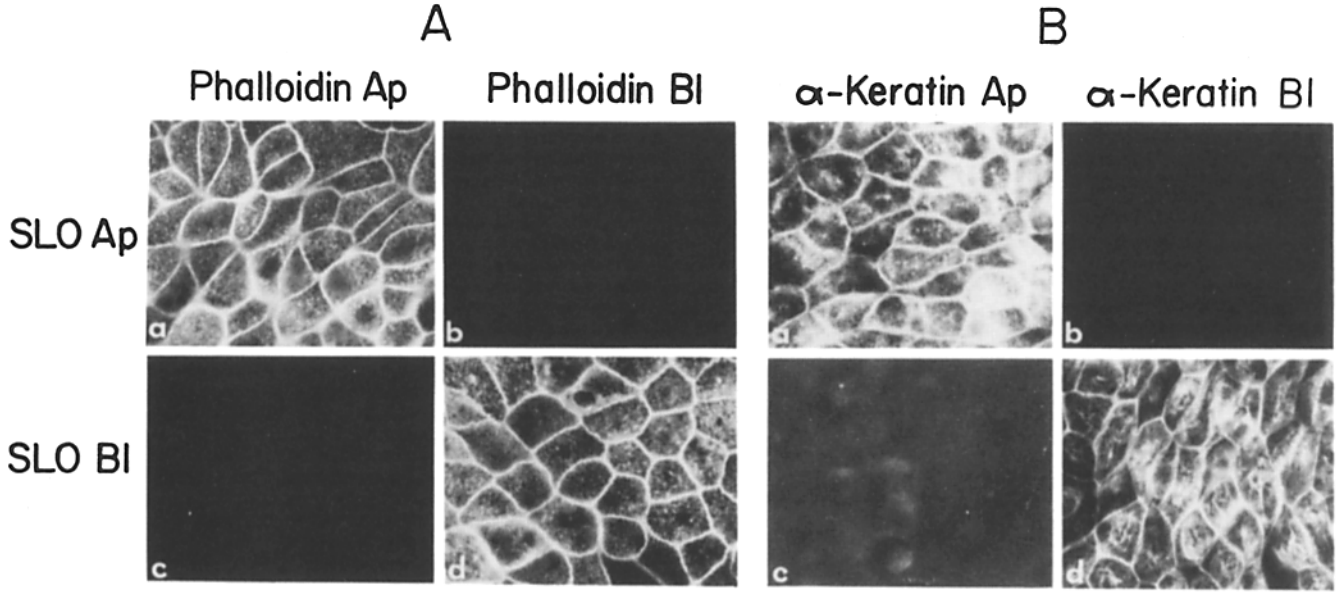

Figure 2. Selective permeabilization of the apical or basolateral surfaces of filter-grown MDCK cells. 3 d after reaching confluence, MDCK cells grown on filters were permeabilized with SLO, as described in Materials and Methods. The toxin was added to either the apical (panels *a* and *b* in *A* and *B*) or basolateral (panels *c* and *d* in *A* and *B*) surfaces. After the toxin was removed, the warming step of the permeabilization procedure was carried out for 15 min in a medium containing rhodamine-labeled phalloidin (*A*) or rabbit anticytokeratin antibodies (*B*) applied only to the apical (panels *a* and *c* in *A* and *B*), or basolateral (panels *b* and *d* in *A* and *B*) surfaces. The filters were then examined by fluorescence microscopy.

permeabilization, the extent of ^{35}S-methionine incorporation was ~40% of that in nonpermeabilized monolayers, but the various viral proteins, including the membrane glycoprotein G, were synthesized in approximately the same ratios observed in nonpermeabilized cells. In the permeabilized cells, the synthesis of all proteins was inhibited to a high extent by both apyrase (Fig. 4 *A*, lane *b*), an enzyme that hydrolases ATP, and gelonin (Fig. 4 *A*, lanes *d* and *e*), a 35-kD plant polypeptide that inhibits protein synthesis as a consequence of its binding to the small ribosomal subunit (Stirpe et al., 1980). The effect of these impermeant reagents, which do not affect protein synthesis in intact cells (Fig. 4 *B*), demonstrates that the bulk of the ^{35}S-methionine incorporation observed, indeed, takes place in the permeabilized cells.

Using the permeabilized cell system, it was possible to demonstrate that the transfer of a glycoprotein from the ER to the Golgi apparatus requires an energy supply. When VSV-infected labeled cells were permeabilized and then incubated for transport, a large fraction of the prelabeled G molecules (varying from 45 to 75% in different experiments) became endo H resistant (Fig. 5, lanes *c* and *d*), but in the presence of apyrase, which destroys ATP, ~80% of the G protein molecules remained endo H sensitive (Fig. 5, lanes *e* and *f*). As expected, apyrase had no effect on the maturation of G protein in nonpermeabilized cells (Fig. 5, *g–j*). Similar experiments with influenza virus-infected cells, showed that transport of HA from the ER to the Golgi apparatus also takes place after permeabilization and is nearly totally abolished when ATP is depleted by the addition of apyrase (see Fig. 7 *B*, compare lanes *c* and *d* with *e* and *f*).

Since apyrase destroys GTP as well as ATP, we determined whether both nucleotides were required for ER to Golgi transport. Pulse-labeled, influenza virus–infected MDCK cells were permeabilized and then incubated for transport in a medium that contained GTP but lacked the energy-regenerating system and contained, instead, antimycin A and 2-deoxyglucose, to prevent the endogenous generation of ATP. It was found that, whereas in the presence of ATP ~65% of the HA molecules became endo H resistant during the 45-min chase (Fig. 6, *e* and *f*), in the absence of ATP, GTP was unable to support the transport of HA (Fig. 6, *c* and *d*).

To determine if GTP plays any role in ER to Golgi transport in this system, we examined the effect of the nonhydrolyzable GTP analogue, GTP-γ-S, on the capacity of the permeabilized cells to convert the high mannose form of the G and HA glycoproteins to endo H–resistant forms. It was found that 100 μM GTP-γ-S (Fig. 7, *A* and *B*, lanes *g* and *h*) blocked the acquisition of endo H resistance in both proteins to nearly the same extent as apyrase (Fig. 7, *A* and *B*,

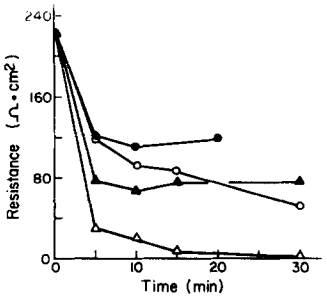

Figure 3. Maintenance of a transepithelial electrical resistance in MDCK cell monolayers with only one permeabilized surface. Filter-grown monolayers were treated with SLO added to the the apical (▲), basolateral (○), or both surfaces (△) and the transepithelial resistance was measured at different times after raising the temperature to 35°C. A control monolayer (●) was preincubated at 0°C without SLO before the temperature shift.

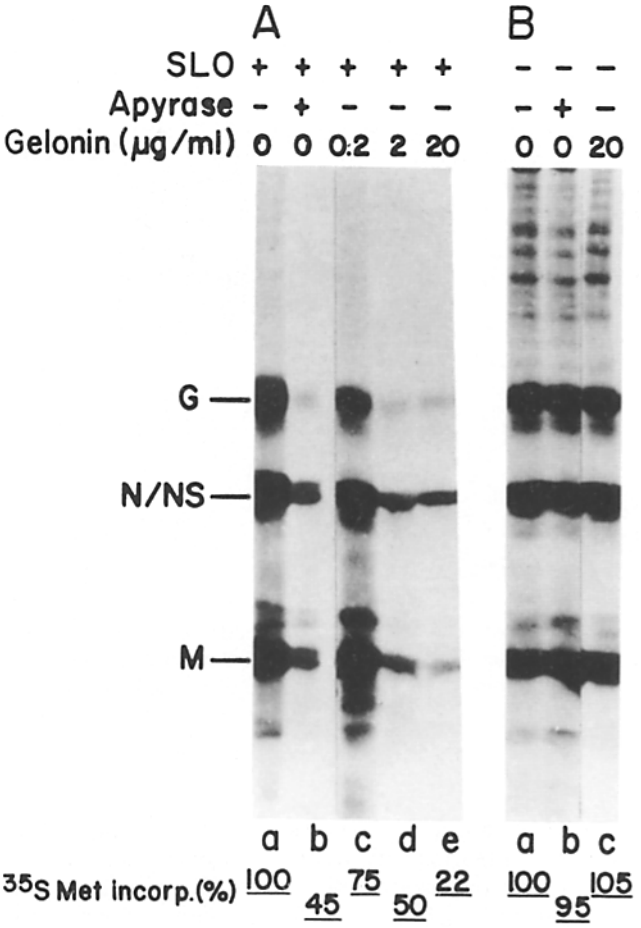

Figure 4. Viral protein synthesis in VSV-infected MDCK cells permeabilized by SLO treatment. Confluent MDCK cells infected with VSV were treated with (*A*) or without (*B*) SLO and then incubated at 35°C for 15 min with the protein synthesis medium containing the energy-regenerating system, in the presence (*A*, lanes *c–e*; *B*, lane *c*) or in the absence (*A*, lanes *a* and *b*; *B*, lanes *a* and *b*) of gelonin, or in a medium without the energy-regenerating system but containing apyrase (*A*, lane *b*; *B*, lane *b*). The bands corresponding to the various viral proteins are indicated. The number at the bottom of each lane indicates the ^{35}S-methionine incorporated into acid precipitable material in each sample, expressed as a percentage of the respective control (*A*, lane *a*; *B*, lane *b*) in the absence of inhibitors.

lanes *e* and *f*). Some G protein molecules, however, were transported to the medial Golgi apparatus even in the presence of GTP-γ-S and were only partially deglycosylated by endo H (Fig. 7 *A*, lane *h*, *upper band*).

Transport from the TGN to the Apical Surface of MDCK Cells Is Energy Dependent and Requires GTP Hydrolysis

The possibility of permeabilizing polarized monolayers from only one surface without affecting the integrity of the opposite surface permitted us to study the effect of impermeant metabolic inhibitors on the vectorial delivery of HA to the apical surface. For these experiments, pulse-labeled, influenza virus–infected cells were incubated at 19.5°C for 90 min to induce the accumulation of HA molecules in the *trans*-region of the Golgi apparatus (Matlin and Simons,

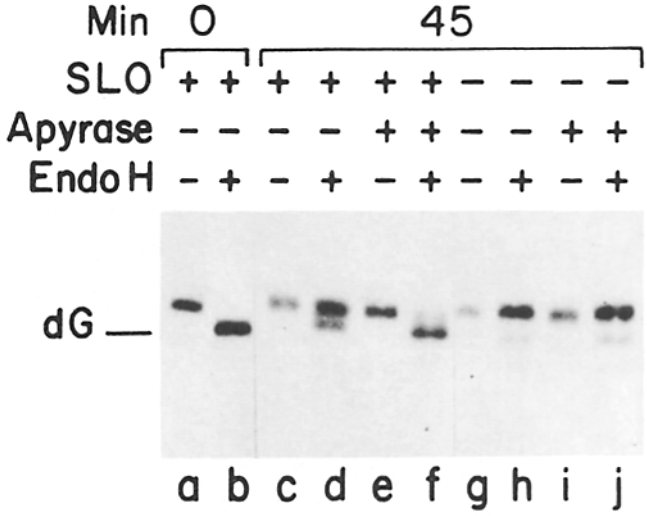

Figure 5. Transport of G protein from the ER to the Golgi apparatus is energy dependent. Confluent MDCK cells infected with VSV virus and pulse labeled with ^{35}S-methionine were incubated with (*a–f*) or without (*g–j*) SLO and then with transport medium containing the energy-regenerating system (*a–d*, *g* and *h*) or apyrase (*e*, *f*, *i*, and *j*) at 0°C (*a* and *b*) or at 35°C for 45 min (*c–j*). The cells were lysed and the immunoprecipitated VSV G protein was analyzed by SDS-gel electrophoresis and fluorography with or without prior incubation with endoglycosidase H, as indicated.

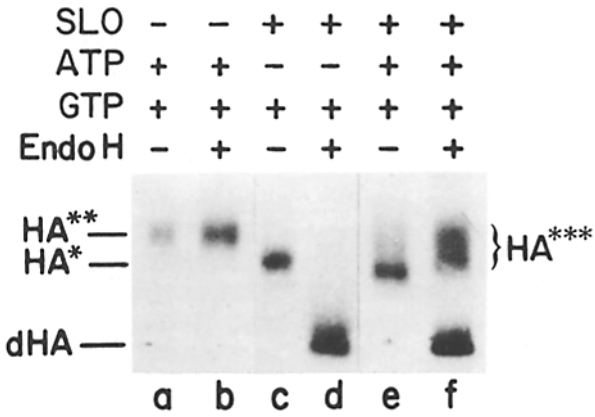

Figure 6. ER to Golgi transport in SLO-permeabilized MDCK cells requires ATP and cannot be supported by GTP alone. Confluent MDCK cells infected with influenza virus and pulse-labeled with ^{35}S-methionine were pretreated with (*c–f*) or without (*a* and *b*) SLO and then incubated at 35°C for 45 min with transport medium containing the complete energy-regenerating system (*a*, *b*, *e*, and *f*) or with a system lacking ATP but containing 200 μM GTP, 2-deoxy-glucose, and Antimycin A (*c* and *d*). The cells were lysed and the immunoprecipitated HA analyzed as in Fig. 5. Lanes *b*, *d*, and *f* were loaded with twice as much material as the corresponding control lanes (*a*, *c*, and *e*). HA* represents the ER form of HA containing high mannose oligosaccharides that is converted by endo H digestion to dHA, the deglycosylated protein. HA** is the endo H–resistant form of HA that has reached the medial region of the Golgi apparatus. HA*** refers to a heterogenous population of endo H–resistant molecules containing oligosaccharides of various degrees of processing.

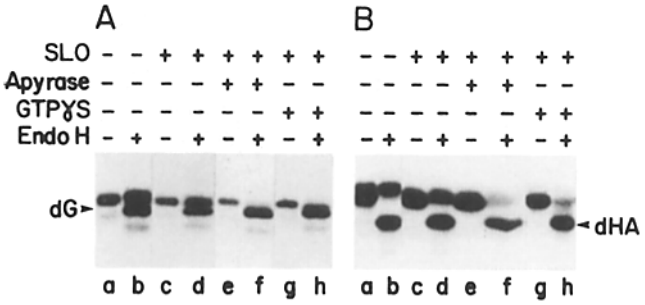

Figure 7. GTP-γ-S prevents the conversion of the viral G and HA glycoproteins to endoglycosidase H resistant forms. Confluent MDCK cells infected with either VSV (*A*) or influenza virus (*B*) and pulse labeled with ^{35}S-methionine were incubated with (*c–h*) or without (*a* and *b*) SLO and then at 35°C for 45 min with transport medium containing the energy-regenerating system with (*g* and *h*) or without (*a–d*) 100 μM GTP-γ-S, or without the energy-generating system but containing apyrase (*e* and *f*). Cells were lysed and the immunoprecipitated glycoproteins analyzed as in Fig. 5. dG and dHA represent the deglycosylated forms of the two glycoproteins generated by endo H digestion. The mobilities of products in pairs of adjacent lanes representing endo H–treated samples and their untreated controls should be compared to each other and not to those in other lanes. In *A*, lane *h*, no fully endo H–resistant G molecules are present, although a poorly resolved band of mobility intermediate between that of the starting material (lane *g*) and the fully deglycosylated molecule can be observed. This band most likely results from the removal of a single oligosaccharide chain from the G protein by endoglycosidase H. In both *A* and *B*, lanes *b*, *d*, *f*, and *h* were loaded with twice as much material as the corresponding non-endo H–treated controls in lanes *a*, *c*, *e*, and *g*.

1983; Rindler et al., 1985), before permeabilization and incubation for transport to the cell surface. We have confirmed by IEF (results not shown) that most of the viral glycoprotein molecules accumulated intracellularly during the 19.5°C block display the same sensitivity to *Clostridium perfringens* neuraminidase treatment as those molecules found at the surface of intact cells after a subsequent 45-min chase period at 35°C. This established that most of the HA molecules that accumulated intracellularly during the cold block contained sialic acid and, therefore, had reached the *trans*-region of the Golgi apparatus.

The influenza HA is synthesized as a precursor of M_r ~76 kD which, in many cell types, is cleaved by cell surface proteases to the subunit polypeptides HA_1 and HA_2 of M_r 50 and 27 kD, respectively (Lazarowitz et al., 1971; Klenk et al., 1974). In MDCK cells infected with the influenza A virus strain PR-8, however, HA does not undergo such cleavage. Nevertheless, the viral glycoprotein molecules that reach the cell surface can be cleaved to the HA_1 and HA_2 polypeptides by exogenous trypsin applied to the apical surface of intact cells (Lazarowitz et al., 1971; Klenk et al., 1975; Matlin and Simons, 1983). This provides a convenient assay for the extent to which newly synthesized intracellular HA molecules reach the apical cell surface during a period of incubation for transport in cells permeabilized from the basolateral surface. Using this assay (Fig. 8) it was found that a substantial fraction (an average of 52% in 10 experiments, Table I) of the HA molecules that had accumulated in the Golgi apparatus of intact cells during the cold block (Fig. 8, *A* and *B*, lanes *a*) were delivered to the cell surface when, after permeabilization, the cells were incubated for 45 min at 35°C (Figs. 8, *A* and *B*, lanes *b*). Measurements of the overall transport that takes place after 45 min of incubation demonstrated that, as is the case with ER to Golgi transport, both ATP and GTP are required for transport from the Golgi apparatus to the cell surface. Thus, in the presence of an ATP-regenerating system, GTP-γ-S markedly inhibited transport (Fig. 8 A, lanes *b* and *d*), but in the absence of ATP, GTP alone could not support transport (Fig. 8 *A*, lane *c*). Furthermore, when the ATP-regenerating system was omitted and ATP-γ-S was added in the presence of GTP, transport was also halted indicating that ATP hydrolysis was required (see Fig. 10 *B*, lane *b*). As expected, the addition

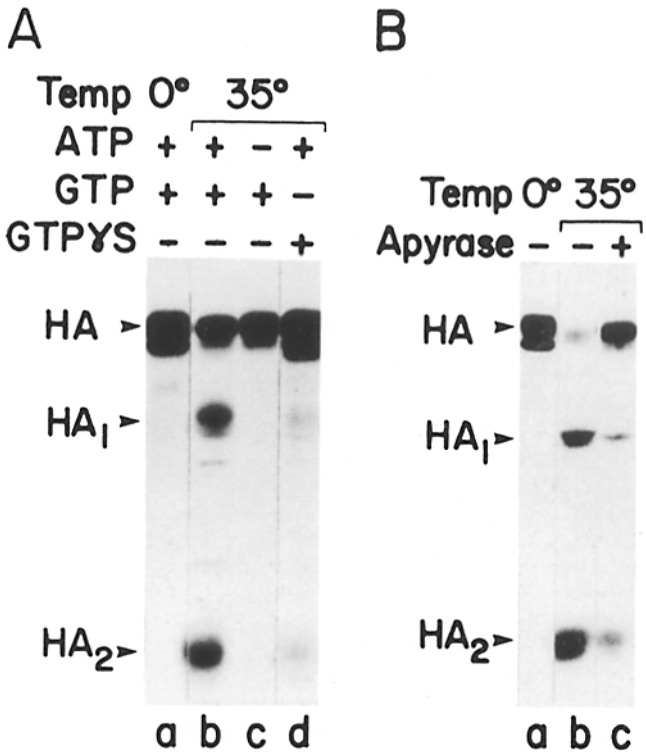

Figure 8. Transport of the influenza HA glycoprotein from the Golgi apparatus to the apical surface requires both ATP and GTP. Confluent MDCK cells grown on filters were infected with influenza virus, pulse labeled with ^{35}S-methionine, and then incubated under chase conditions for 60 min at 19.5°C, to allow for the accumulation of HA molecules in the *trans*-Golgi network. (*A*) Monolayers treated with SLO added to the basolateral surface were incubated at 0°C (*a*) or at 35°C (*b–d*) for 45 min in transport medium containing the complete energy-regenerating system (*a*, *b*, and *d*) or a system lacking ATP but containing GTP (200 μM), as well as 2 deoxyglucose and antimycin A (*c*). The incubation mixture in *d* contained 100 μM GTP-γ-S instead of GTP. After cell lysis, HA and its cleavage products were recovered by immunoprecipitation and analyzed by gel electrophoresis and fluorography. (*B*) Monolayers were treated with SLO added to the basolateral surface and incubated for 45 min with transport medium containing the energy-regenerating system (*a*, *b*) or apyrase (*c*) at 0°C (*a*) or at 35°C (*b*, *c*). The cells were then incubated with trypsin added to the apical surface. Samples were analyzed for apical surface arrival of HA as in *A*.

Table I. Effect of Various Inhibitors on the Transport of Influenza HA from the TGN to the Apical Plasma Membrane

*Transport medium**		*No preincubation*		*15 min preincubation at 35°C*	
Removed	Added	Transport‡	Inhibition§	Transport‡	Inhibition§
		%	%	%	%
None	None	52.2 ± 15.2(10)	–	72.3 ± 4.0 (3)	–
GTP	GTP-γ-S(100 μM)	13.5 ± 7.5(7)	68.1 ± 13.3	64.0 ± 1.0 (3)	11.3 ± 5.1
ATP	2-deoxyGlc(6 mM)	6.0 ± 3.5(3)	83.0 ± 12.1	45.5 ± 6.3 (2)	38.0 ± 12.7
Glc	Antimycin (3 μM)				
ATP	ATP-γ-S	13.0(1)	69	ND	–
ATP,GTP	apyrase(5 U/ml)	20.3 ± 8.6(3)	66.6 ± 11.5	33.0 ± 4.2 (2)	52.0 ± 8.5
None	O-vanadate(100 μM)	11.0 ± 11.3(3)	77.3 ± 26.2	30.5 ± 3.5 (2)	57.0 ± 9.9
None	okakaic acid(0.2 μM)	49.0(1)	4	ND	–
	(2.0 μM)	51(1)	0	ND	–
None	Gly-Gly(4 mM)	42(1)‖	0	66	6
None	Gly-Phe(4 mM)	13(1)‖	69	57	19
None	H-8(100 μM)	39(1)‖	5	67	4
None	AMG-C16(1 mM)	40(1)‖	3	70	0

Influenza virus-infected MDCK cells were pulse labeled for 10 min with ^{35}S-methionine and then incubated under chase conditions at 19.5°C for 90 min, to accumulate the viral glycoprotein in the TGN. After the 19.5°C incubation, the cultures were either immediately permeabilized with SLO with the inhibitors present during the warming step of the permeabilization procedure (no preincubation), or preincubated for 15 min at 35°C before permeabilization and addition of the inhibitors.

* Incubation for transport was carried out in the medium described in Materials and Methods. The items in the "Removed" column represent reagents normally present in the transport medium but omitted in the particular experiment. Those in the "Added" column are the inhibitors whose effect in transport were being investigated.

‡ Percent transport $= \frac{(HA_1 + HA_2)}{(HA_1 + HA_2 + HA)} \times 100$ and represents the proportion of the total radioactivity in HA molecules that were cleaved to HA_1 and HA_2, the proteolytic products generated when trypsin was added to the apical surface of the monolayers. The relative amounts of radioactivity in HA and its cleavage products (HA_1 and HA_2) were assessed from the areas of the respective peaks in each lane, measured by densitometry. The values given are the averages obtained from the number of experiments indicated in parenthesis.

§ For each individual experiment, the percentage of inhibition of transport was calculated as:

$$\frac{\%\text{transport}_{\text{control}} - \%\text{transport}_{\text{experimental}}}{\%\text{transport}_{\text{control}}} \times 100$$

Percent $\text{transport}_{\text{control}}$ represents the value in a control carried out in parallel. The values given are the averages obtained from all the experiments included in the percent transport column.

‖ In these experiments the percent transport in the control was 41%.

of apyrase, an enzyme which destroys all nucleoside triphosphates, also inhibited transport (Fig. 8 *B*, lanes *b* and *c*). In seven independent experiments in which GTP-γ-S was added to cells at the time of permeabilization, immediately after the release from the cold block, the GTP analogue caused a 68% inhibition of transport (Table I).

Given the fact that GTP-γ-S would not be expected to replace GTP molecules that had already bound to GTP-binding proteins during incubation at 19.5°C, it is not surprising that GTP-γ-S did not inhibit transport completely. In particular, transport of the first HA molecules that exited from the TGN after release of the cold block may not be affected by the analogue. We, therefore, examined the kinetics of transfer of HA to the cell surface in permeabilized cells incubated with or without GTP-γ-S and compared these to the kinetics observed in intact cells (Fig. 9, *A–C*). This analysis revealed that during the first 30 min after transfer to 35°C, transport proceeded with similar efficiencies in intact and permeabilized cells not treated with GTP-γ-S (Fig. 9 *A*). After this time, transport essentially ceased in the permeabilized cells, whereas in intact cells it continued, so that after 90 min ~90% of the HA molecules had reached the apical surface. It is noteworthy that in both permeabilized and intact cells (see for example lane *b* in Fig. 9 *B*) very little transport took place during the first 15 min after transfer to 35°C (an average of 6%, in three independent experiments with intact cells) and that in permeabilized cells the bulk of the transport took place during the first 15 min after this initial lag phase (Fig. 9, *A* and *C*). When GTP-γ-S was added to cells permeabilized immediately after the cold block, the small amount of transport that takes place in the lag phase was unaffected but subsequent transport was nearly completely inhibited (Fig. 9 *A*). This is consistent with the notion that transport of the few HA molecules that reach the cell surface during the first 15 min (the lag phase) utilizes endogenous GTP, already bound to GTP-binding proteins before the temperature was raised. On the other hand, transport of HA molecules that are delivered to the surface during the following 15 min would utilize GTP-binding proteins charged after the perforation. In fact, the lag phase could represent a restoration period in which charging of GTP binding proteins takes place.

To determine whether GTP and its hydrolysis are required during the lag phase that takes place immediately after transfer to 35°C, or during the delivery phase that follows, we examined the effect of GTP-γ-S on transport when the analogue was added after the lag phase was completed, but before significant delivery of HA to the cell surface had occurred. Thus, cells that were labeled for 15 min with ^{35}S-methionine and incubated for 90 min at 19.5°C, to accumulate the HA molecules in the TGN, were warmed to 35°C and maintained at this temperature for 15 min before perforation and incubation for transport in the presence of GTP or GPT-γ-S (Fig. 10 *A*). It was observed that in this case, in which cells were perforated after the lag phase, a substantially larger fraction (72% in three experiments, Table I) of

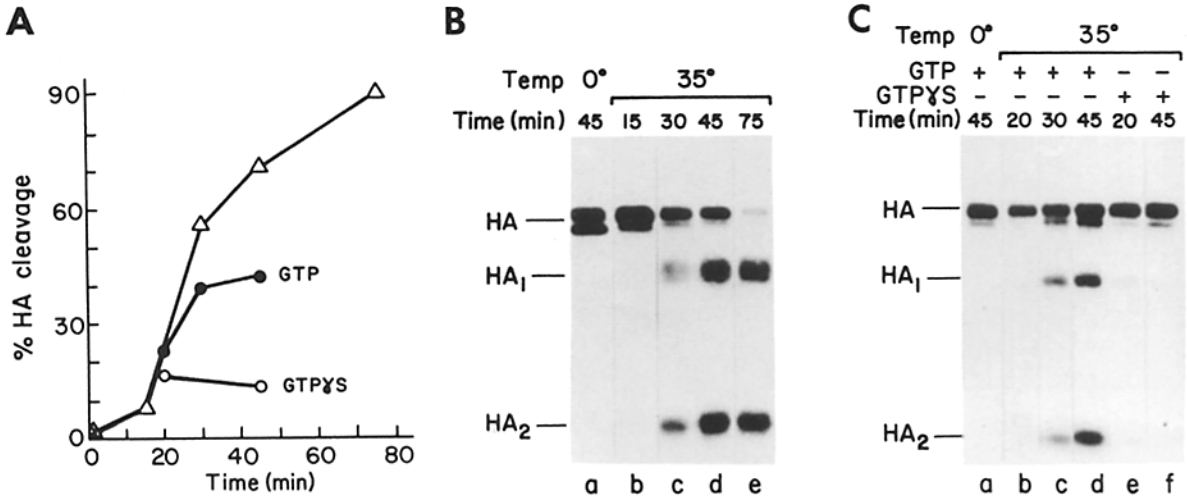

Figure 9. Kinetics of arrival of influenza HA to the apical plasma membrane of MDCK cells after transfer from 19.5°C to 35°C: Transport resumes with a 15-min lag and is impaired when GTP-γ-S is added at the time of the temperature shift. Confluent MDCK cell monolayers grown on Polycarbonate Transwell™ filters infected with Influenza virus, pulse labeled at 37°C and chased at 19.5°C were left intact or permeabilized with SLO from the basolateral surface before raising the temperature to allow transport to resume. The arrival of HA to the apical surface was assessed as described in the legend to Fig. 8. (*A*) The amount of HA on the apical surface at different times after release from the cold-block was measured by densitometric scanning of the fluorographs shown in *B* and *C*. (△) Intact cells which were brought to 0°C to mimic the permeabilization procedure and then incubated at 37°C in serum-free DME. (●) and (○) Cells that were perforated with SLO and incubated for transport with GTP or with GTP-γ-S, respectively (see *C*). (*B*) Intact cells that after the 19.5°C incubation were incubated at 0°C (*a*), or at 37°C (*b–e*) in serum-free DME for the times indicated. (*C*) Monolayers permeabilized with SLO were incubated at 0°C (*a*) or at 35°C (*b–f*) for the times indicated, with complete transport medium (*a–d*) or with medium lacking GTP but containing 100 μM GTP-γ-S (*e* and *f*).

the accumulated HA molecules were transported to the cell surface and that this transport was not inhibited by GTP-γ-S (Fig. 10 *A*, compare lanes *b* and *c* with *e* and *f*; Table I). The observation that GTP-γ-S inhibited transport only when it was added before the lag phase demonstrates that the GTP molecules must be present during this phase for the subsequent delivery of HA to the cell surface to take place. Thus, either GTP hydrolysis is required only during the lag phase, or GTP molecules incorporated into the transport machinery during this phase are hydrolyzed during the delivery phase.

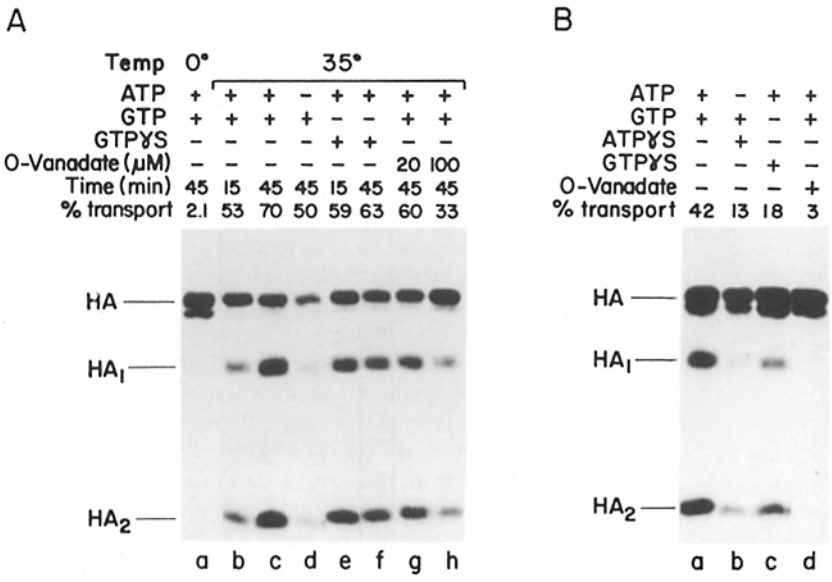

Figure 10. In cells incubated at 37°C for 15 min after release from the cold block, GTP-γ-S has little inhibitory effect on transport whereas ortho-vanadate is still inhibitory. (*A*) Monolayers of MDCK cells that were infected and pulse labeled at 37°C and chased at 19.5°C for 90 min (see legend to Fig. 8) were incubated for 15 min at 37°C in serum-free DME. After this period, the monolayers were cooled again, permeabilized with SLO added to the basolateral surface, and incubated at 0°C (*a*) or at 35°C (*b–h*) for the times indicated with complete transport medium without (*a–c*) or with 20 μM (*g*) or 100 μM (*h*) ortho-vanadate added, or with medium lacking ATP and the ATP-regenerating system (although with 0.2 mM GTP still present) but containing 2-deoxyglucose and antimycin A (*d*). In *e* and *f*, GTP in the transport medium was replaced with 100 μM GTP-γ-S. (*B*) After incubation at 19.5°C, monolayers were permeabilized with SLO added to the basolateral surface and incubated at 35°C for 45 min in complete transport medium without (*a*) or with 100 μM ortho-vanadate added (*d*), or in media in which ATP was replaced by 2 mM ATP-γ-S (*b*), or GTP was replaced by 100 μM GTP-γ-S (*c*).

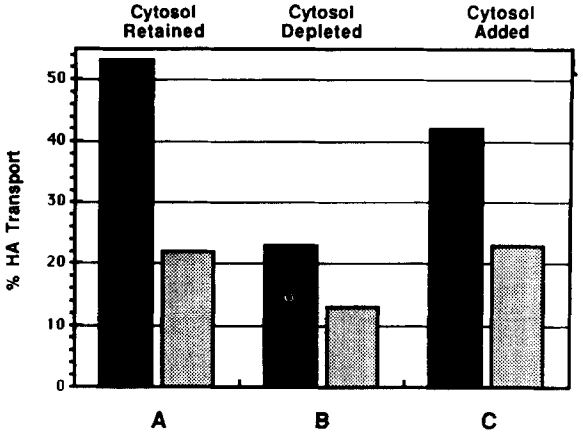

Figure 11. Addition of exogenous cytosol restores the transport capacity of monolayers that, after permeabilization with SLO, were washed to remove endogenous cytosolic components. After a brief (4 min) incubation in transport medium at 35°C to allow pore formation, cold synchronized, virus-infected labeled monolayers that received SLO from the basolateral surface were cooled again to 0°C in buffer A (*B* and *C*) or incubated with transport medium at 35°C for 45 min (*A*). Perforated monolayers that were cooled to 0°C and were washed at 4°C, four times with buffer A to remove endogenous cytosol, were then supplied with transport medium (*B*) or with transport medium supplemented with MDCK cytosol (*C*) (2.82 mg/ml). In all cases transport was carried out with (*light bars*) or without (*dark bars*) addition of GTP-γ-S.

The effect of several other potential inhibitors of transport was examined in this system (Table I). Vanadate, an inhibitor of ATPases (Shimizu, 1981; Gibbons et al., 1978; Kobayashi et al., 1978) and phosphatases (Swarup et al., 1982; Chasteen, 1983), had a marked inhibitory effect (93%) when added immediately after the cold block (Fig. 10 *B*, lane *d*; Table I) and still inhibited significantly (>50%) when added after the lag phase was completed (Fig. 10 *A*, lane *h*; Table I). Similar differential effects were observed when apyrase or antimycin A and 2-deoxyglucose were added to deplete the supply of ATP before or after a 15-min shift to 35°C (Fig. 8 *A*, lane *d*; *B*, lane *d*; Fig. 10 *A*, lane *d*). Okadaic acid (kindly provided by Dr. J. D. Buxbaum, NYU School of Medicine), a relatively specific serine threonine protein phosphatase inhibitor (Bialojan and Takai, 1988; Hescheler et al., 1988), had no effect on transport (Table I). The protein kinase C inhibitors, AMG-C_{16} [1-O-Hexadecyl-2-O-methyl-ras-glycerol (Bachem Bioscience, Inc., Philadelphia, PA) and H-8 {N-[2-(methylamino)-ethyl]-5-iso-quinoline-sulfonamide, diHCl} (Calbiochem Corporation, San Diego, CA) (Hidaka et al., 1984; van Blitterswijk et al., 1987; Kramer et al., 1989), had practically no inhibitory effect on either phase of transport, whereas the dipeptide gly-phe, which has been reported to inhibit secretion (Strous et al., 1988), but not gly-gly, which has no effect on secretion, inhibited transport when added before, but not when added after the lag phase (Table I).

Transfer of HA from the TGN to the Cell Surface Requires Cytosolic Components

The experimental protocol used in this work to study transport in SLO-permeabilized cells assures that the cytosol is retained during the incubation and is present at the maximally feasible concentration, since the perforation step is carried out in a very small volume of transport medium. To determine whether cytosolic components are required for transport, 4 min after the temperature was raised to 35°C to affect permeabilization the cells were cooled once again and washed several times with the transport solution, to remove any released cytosolic components. The permeabilized cells were then rewarmed and incubated for transport with or without the addition of exogenous cytosol. It was observed (Fig. 11) that in the absence of the cytosolic supplement transport to the cell surface was reduced by 60% with respect to controls in which cytosol was not removed. Addition of cytosol from MDCK cells, however, restored transport to 80% of the control value (Fig. 11). Thus, in future experiments it should be possible to use this system to identify specific cytosolic components involved in transport.

Discussion

Efficient and Selective Permeabilization of Apical or Basolateral Plasma Membrane Domains by a Two-Step Procedure Employing SLO

Although cell-free systems employing crude extracts or defined subcellular fractions have provided important information regarding vesicular transport (for review see Balch, 1989), their usefulness for studying interorganellar protein traffic is limited by the disruption of cellular architecture that takes place during homogenization or cell lysis. On the other hand, semi-intact (Beckers and Balch, 1989; De Curtis and Simons, 1988) or permeabilized cell systems (Gomperts and Fernandez, 1985) can, in principle, retain a high degree of subcellular organization, including the integrity of the cytoskeleton, which may be essential for directed vesicular movements (e.g., Rindler et al., 1987; Eilers et al., 1989; Achler et al., 1989). Moreover, such systems may be particularly useful in the analysis of questions related to the mechanisms that regulate vesicular traffic to and from the different plasma membrane domains of polarized epithelial cells.

Several methods have been described (Beckers et al., 1987; Simons and Virta, 1987) to produce permanent plasma membrane discontinuities that involve the mechanical disruption of the cell surface, but these compromise the integrity of cultured cell monolayers. One method, designed to preserve the polarized organization of epithelial cells, involves pressing a nitrocellulose filter to the surface of a monolayer and peeling it off to strip pieces of apical plasma membrane from the cells (Simons and Virta, 1987). This procedure, however, when applied to polarized MDCK cell monolayers on a glass coverslip, disrupts intercellular junctions and exposes to the bathing medium the outside surface of the basolateral plasma membrane domains (Simons and Virta, 1987). Moreover, when it was applied to MDCK cell monolayers grown on a permeable filter support, vesicles emerging from the TGN carrying viral glycoproteins destined to either the apical or basolateral surfaces did not fuse with the plasma membrane and were released into the medium (Bennett et al., 1988). Although it was suggested that this resulted from the loss of cytosolic proteins during perforation, it seems possible that it was also a consequence of the massive damage to the plasma membrane caused by the stripping procedure. We have, therefore, sought to de-

velop a method to selectively permeabilize either surface of MDCK cell monolayers that causes minimal structural damage to the permeabilized surface and allows the preservation in the system of the released cytosolic proteins. For this purpose, we have utilized SLO, a bacterial toxin that selectively binds to cholesterol within the plasma membrane in a temperature-independent manner and, at physiological temperatures, oligomerizes within the plasma membrane to form pores that allow passage of large proteins such as urease, catalase and ferritin (see Bhakdi and Tranum-Jensen, 1987; Buckingham and Duncan, 1983; Smyth and Duncan, 1978).

Although SLO has been used previously to permeabilize mammalian cells (e.g., Cockcroft et al., 1987; Howell and Gomperts, 1987; Churcher and Gomperts, 1990), in those instances the permeabilization was carried out in a single step by incubating the cells with the toxin at 37°C, which in our experience (our unpublished observations) greatly diminishes the capacity of the permeabilized cells to carry out protein synthesis and intracellular transport of membrane proteins, the latter, possibly because of damage to intracellular membranes. The procedure described in this paper permits the selective permeabilization of one or the other plasma membrane domain of polarized cells grown on a permeable support, because the toxin is added to only one surface of the monolayer, where it is allowed to bind at 4°C. Excess toxin is then removed and pore formation is induced by raising the incubation temperature in a medium appropriate for the physiological process being investigated. The fact that, under the conditions we used, only the surface to which the toxin was applied was perforated, was apparent from the failure of antibodies or other impermeant probes, such as fluorescently labeled phalloidin, to enter the cells when applied to the opposite side. Moreover, the high efficiency of the permeabilization procedure was demonstrated by the effectiveness of the same impermeant probes in labeling the cytoplasm of the entire cell population when applied from the same side as the toxin. These experiments, as well as the demonstration that a transepithelial electrical resistance was maintained, proved that the permeabilization treatment did not abolish the barrier between the two plasma membrane domains provided by the tight junctions. With this procedure, we were able to study in epithelial cells the effect of impermeant reagents on the selective transport of a membrane protein from the Golgi apparatus to the apical plasma membrane, a process known to involve sorting steps in the TGN (Rindler et al., 1984; Matlin and Simons, 1984; Misek et al., 1984; Rindler et al., 1985). This would not have been possible with mechanical perforation methods, which invariably damage the apical surface.

Although we used this system primarily to study TGN to plasma membrane transport, its usefulness for studying other physiological processes should be obvious, since the permeabilized cells, when properly supplemented, remained capable of protein synthesis, translocation of newly synthesized proteins into the ER, and transport of proteins from the ER to the Golgi apparatus. In fact, we were able to show, in agreement with the other investigators (Beckers et al., 1989; Melançon et al., 1987), that transport of the VSV G and influenza HA glycoproteins from the ER to the medial region of the Golgi apparatus requires both ATP and GTP.

Involvement of GTP-binding Proteins in the Transport of HA from the TGN to the Apical Cell Surface

To study the delivery of newly synthesized influenza HA glycoprotein molecules from the TGN to the apical surface, labeled HA molecules were allowed to accumulate in the TGN by incubating pulse-labeled, virus-infected cells for 90 min at 19.5°C. As previously reported (Matlin and Simons, 1983), at the end of this period none of the HA protein molecules had yet reached the cell surface, as indicated by their inaccessibility to an apically applied exogenous protease. Upon transfer of the cultures to 35°C, efficient delivery (~50%) of the viral glycoprotein to the intact apical surface took place within the next 30–45 min. This transport was shown to be dependent on the hydrolysis of both ATP and GTP. The dependence on ATP was demonstrated by the nearly complete inhibition that occurs when this nucleoside triphosphate was depleted by the addition of deoxyglucose and antimycin A, or replaced by ATP-γ-S in the presence of GTP. The requirement for GTP and its hydrolysis was evident from the strong inhibitory effect of GTP-γ-S on the arrival of HA to the apical surface. Although studies with yeast mutants (Salminen and Novick, 1987; Goud et al., 1988; Walworth et al., 1989) have implicated GTP-binding proteins in the last stage of protein transport to the cell surface, our findings provide biochemical evidence for this requirement in mammalian cells.

When the kinetics of transport of HA from the TGN to the cell surface was examined, after the cells were warmed to 35°C, it was observed that there was a 15-min lag before the wave of radioactive HA molecules began to arrive at the cell surface. It was striking that GTP-γ-S only inhibited HA transport when it was added before the lag period, but not when the cells were permeabilized 15 min after release from the cold block and GTP-γ-S was then added. This suggests that the incorporation of GTP into the putative binding protein(s) that participate in the transport of HA from the TGN to the cell surface, takes place in the early stages of the transport process, probably during the formation of the carrier vesicles that bring the viral protein to the plasma membrane. Indeed, the lag phase may represent a period of recovery from the cold block during which extensive vesicle formation and GTP binding could take place. The notion that vesicle formation takes place during the first 15 min after release from the cold block is supported by recent experiments (D. Gravotta and E. Ivessa, unpublished observations), in which the effect on transport of brefeldin A, a drug which causes disassembly of the Golgi apparatus, including the TGN, and return of its components to the ER (Lippincott-Schwartz et al., 1989, 1990), was examined in intact cells that received the drug either immediately after the cold block, or after further incubation for 15 min at 35°C. It was observed that bredfeldin A completely blocked transport when added immediately after the cold block, but had little effect when added after the lag phase. This is consistent with the notion that the HA protein leaves the Golgi apparatus during the lag phase so that after that time its delivery cannot be affected by a drug that acts by eliminating the donor organelle.

Our observations on the differential inhibitory effect of GTP-γ-S on TGN to cell surface transport when added at different times after release from the cold block can be inter-

preted in the context of a model for vesicular transport similar to that proposed by Bourne (1988). Thus, our finding that, in permeabilized cells, GTP-γ-S only inhibited transport of HA when added immediately after the release from the cold block, but not 15 min later when the viral glycoprotein was still located inside the cell, is to be expected if charging of the GTP-binding proteins takes place during an early stage in transport, for example, during vesicle formation, and GTP-γ-S cannot displace GTP molecules that were bound to their acceptor proteins during the lag phase. Following this reasoning, the fact that addition of GTP-γ-S 15 min after release from the cold block did not prevent >60% of the HA protein that accumulates within the cell from reaching the cell surface, implies that a single round of transport has the capacity to deliver that amount of HA to the apical membrane, without the need to recycle any essential components.

The notion that GTP-binding proteins function cyclically in vesicular transport has received support from the behavior of an in vitro generated mutation of *sec4* (Walworth et al., 1989) that behaves as a dominant lethal, loss of function, allele. This would be expected for a GTP-binding protein that is permanently in the "active configuration" and, when expressed at high enough levels, blocks access of the wild type sec4 protein to the sites in the acceptor membrane which induce GTP hydrolysis. Further evidence that GTP binding takes place early in vesicular transport but hydrolysis takes place at a late step, has come from the recent demonstration that a synthetic peptide corresponding to a specific region of rab3, a mammalian GTP-binding protein belonging to the sec4/YPT-1-family, was able to inhibit ER to Golgi transport when added to a semi-intact cell system at a time at which transport was no longer sensitive to GTP-γ-S (Plutner et al., 1990). The inhibitory peptide used in that work corresponds to the putative effector domain of the GTP-binding protein, i.e., the domain that interacts with a putative effector protein that activates GTP hydrolysis. The fact that this peptide inhibited at a late step in transport is consistent with the expectation that it inhibits GTP hydrolysis by preventing the interaction of a GTP-binding protein with its effector in the receiving membrane.

A Model for the Role of GTP-binding Proteins in the Segregation of Plasma Membrane Proteins in the TGN and their Vectorial Delivery to the Cell Surface

What specific role(s) could GTP-binding proteins play in the targeted delivery of proteins from the TGN to the apical or basolateral surfaces of epithelial cells? It is generally believed (see Caplan and Matlin, 1989; Rodriguez-Boulan and Nelson, 1989; Simons and Wandinger-Ness, 1990) that, since the apical plasma membrane domain is a specialized structure, sorting of its proteins in the TGN requires signals within the polypeptides that mediate their active segregation into a specific class of vesicles that is targeted to the apical surface. On the other hand, it is possible that not all proteins destined to the basolateral surface, which is equivalent to the nondifferentiated surface of nonpolarized cells, contain specific signals directing their segregation into vesicles that are delivered to that surface. Such signaless proteins could be sorted by a default mechanism, i.e., by exclusion from apically directed vesicles.

We propose that the formation of apically directed vesicles in the TGN, and possibly that of basolaterally directed vesicles as well, occurs by a mechanism similar to that which, in the plasma membrane, leads to the segregation of many receptors and other specific proteins into clathrin-coated endocytic vesicles and, in the TGN, to the incorporation of the mannose-6-phosphate receptor and its bound lysosomal hydrolase ligands into clathrin-coated vesicles directed to developing lysosomes. This mechanism (Pearse, 1988; Glickman et al., 1989; Dahms et al., 1989; Lobel et al., 1989) appears to involve the recognition of sorting signals in the cytoplasmic segments of the proteins to be segregated by macromolecular complexes termed "adaptors" (Pearse, 1988) that link them to the clathrin molecules whose assembly into a cage leads to vesicle formation. By analogy, members of one class of proteins addressed to the apical plasma membrane would contain signals in their cytoplasmic domains that are recognized by putative "apical adaptors" that, by interacting with hypothetical nonclathrin coat subunits, would mediate formation of specific vesicles addressed to the apical surface (Fig. 12). Apically secreted proteins, or transmembrane proteins that lack sorting signals in their cytoplasmic domains but must be delivered to the apical surface, would be sorted in a piggy-back fashion by interacting, via signals in their luminal domains, with the luminal domains of proteins of the first class that, hence, serve as their sorting receptors (Fig. 12). The HA envelope glycoprotein studied in this work appears to be in this category since its sorting information has been localized to its luminal domain (Roth et al., 1987; Compton et al., 1989). The behavior of the sorting receptors would be analogous to that proposed for the mannose-6-phosphate receptor which, in the TGN, would effect the sorting of lysosomal hydrolases bound to its luminal domain by interacting with adaptors through its cytoplasmic tail (Glickman et al., 1989; Lobel et al., 1989).

Adaptors, of course, must function cyclically, being released at some point from the receptor tails to return to the donor membrane for another cycle of vesicle formation and a switching mechanism must ensure that the released adaptors do not rebind to receptors that have already been delivered to the acceptor membrane (Pearse, 1988). Moreover, some type of molecular recognition mechanism must operate to restrict the fusion of the carrier vesicles to the proper acceptor membrane. We suggest that GTP-binding proteins play a key role in the sorting of plasma membrane proteins into carrier vesicles in the TGN that is executed by adaptor proteins, in the regulation of adaptor recycling, and in vesicle targeting, as depicted in Fig. 12. Adaptors, which are multisubunit proteins (see Morris et al., 1989), may contain or become associated with specific GTP-binding proteins that in their GTP-bound state allow the adaptors to recognize the receptor tails (Fig. 12). After vesicle uncoating, the adaptor would remain bound to the receptor tail in the transport vesicle (Patzer et al., 1982) with its associated "active" GTP-binding protein and this complex would constitute the site on the vesicle surface that docks with a specific docking protein in the acceptor membrane (Fig. 12). Indeed, studies in yeast (Salminen and Novick, 1989) have identified a gene product, sec15p, through which the sec4p GTP-binding protein may act in mediating vesicle targeting. Docking of the vesicle on the acceptor membrane would normally trigger GTP hydrolysis, with the docking protein functioning as a GTPase-activating protein (GAP; Trahey and McCormick,

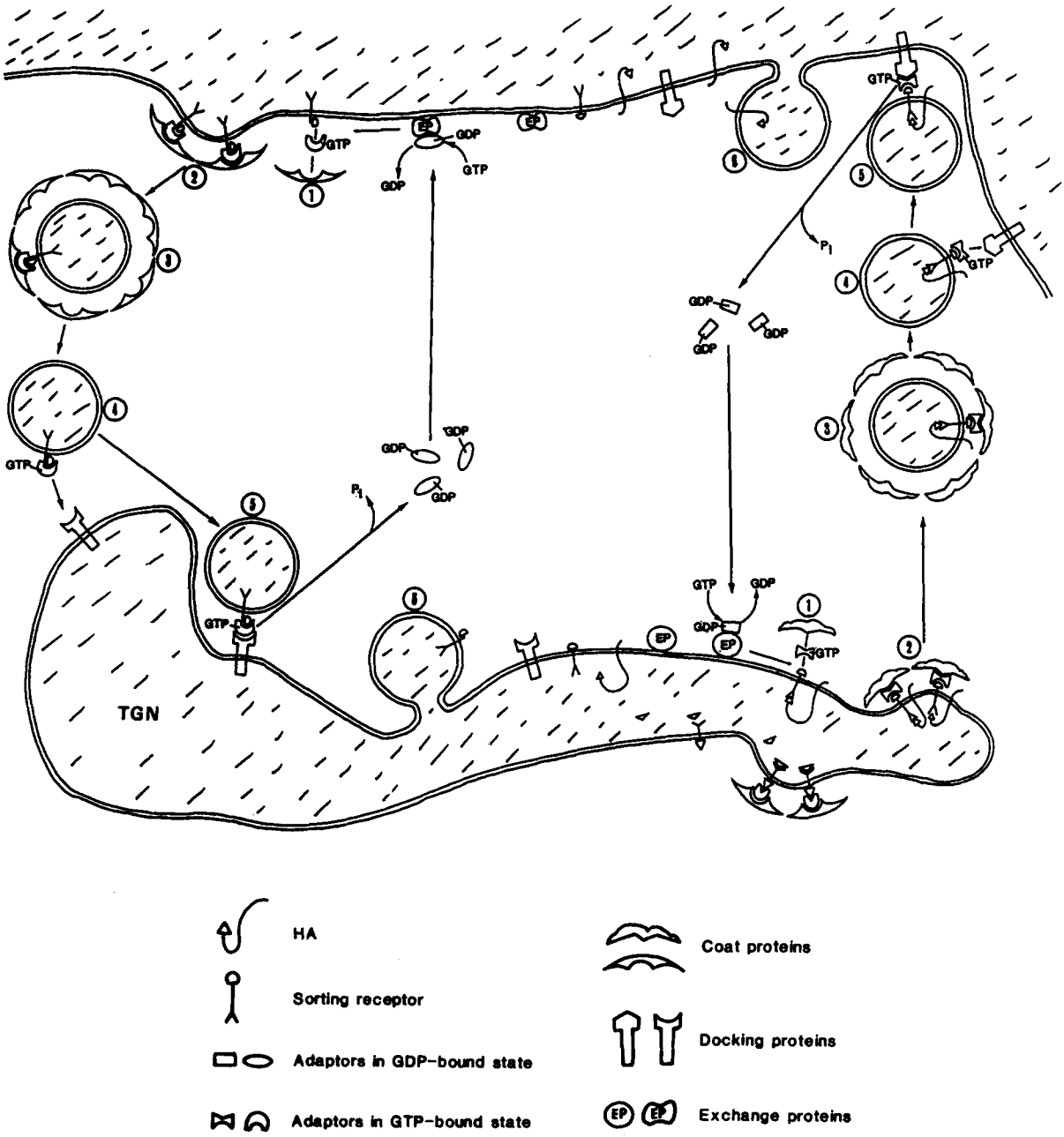

Figure 12. Hypothetical role of adaptor-GTP-binding proteins in the transport of a membrane protein from the TGN to the cell surface. (*right side*) In the TGN, the adaptor-GTP-binding protein complex in its active, GTP-bound configuration binds to the cytoplasmic domain of a sorting receptor that in its luminal portion interacts with HA (*1* and *2*). This leads to the formation of a nonclathrin-coated vesicle in which the viral glycoprotein in segregated (*3*). The vesicle becomes uncoated and this allows docking at the plasma membrane by interaction of the adaptor with a cognate "docking protein" (*4* and *5*). After docking, GTP is hydrolyzed and the adaptor GTP-binding protein complex in its inactive, GDP-bound configuration is released into the cytosol. Vesicle fusion, mediated by other proteins not depicted here, then takes place (*6*). The adaptor GTP-binding protein complex is converted to its "active" GTP-bound form by an exchange protein (*EP*) located in the donor membrane. This ensures that the adaptor functions cyclically, always binding in the TGN, and not in the plasma membrane, to the cytoplasmic domain of the protein that it sorts. (*left side*) A similar sequence of events is depicted, in which a sorting receptor in the plasma membrane is brought back to the TGN in a clathrin-coated vesicle (*1–6*). This is formed by virtue of the interaction of the cytoplasmic domain of the protein with a different type of adaptor that is activated in the plasma membrane and recognizes a different signal in the cytoplasmic domain of the sorting receptor.

1987). The ensuing conformational change would place the docked vesicle in a position to interact with components of the "fusion machine" (Malhotra et al., 1988; Wilson et al., 1989; Clary and Rothman, 1990) that completes vesicles delivery. After GTP hydrolysis, the adaptor, with the GTP-binding protein in the GDP conformation, would be released from the vesicle and its subsequent binding to a receptor tail could only take place in the donor membrane after GTP is exchanged for GDP by a resident exchange protein (Bourne, 1988). Thus, the specificity of TGN to apical plasma mem-

brane transport would rely on the affinities of the adaptor and its associated GTP-binding protein for a specific set of receptor tails and a specific docking protein in the apical membrane, as well as the specificity of the GDP/GTP exchange enzyme in the TGN donor membrane. The retrieval of sorting receptors to the TGN would, of course, require vesicular transport in the opposite direction, and this would rely on a different signal in the cytoplasmic tail of the receptor, as well as its set of cognate adaptor-GTP-binding protein, GDP/GTP exchange enzyme (in the apical plasma membrane), and docking protein (in the TGN) (Fig. 12).

Clearly, mechanisms of the type just proposed could operate to direct vesicular flow between other membrane systems, even when the segregation of specific proteins into a vesicle is not required, such as in the apparent "bulk flow" of proteins from ER to Golgi and through this organelle (Weiland et al., 1987). In this case, of course, the adaptors would still have to recognize some molecular entity exposed on the cytoplasmic surface of the donor membrane. The multiplicity of GTP-binding proteins in the rab gene family, that includes the yeast YPT-1 and Sec-4 gene products, appears to be sufficiently high (Zahraoui et al., 1990; Chavrier et al., 1990) to support the numerous bidirectional transport steps that must take place throughout the endomembrane system. Indeed, several specific GTP-binding proteins within this family have been localized to various portions of this system (Goud et al., 1990; Chavrier et al., 1990).

Energy Requirements for Transport to the Cell Surface

As previously noted, we observed that transport of influenza HA from the ER to the medial region of the Golgi apparatus and from the TGN to the apical plasma membrane was nearly completely abolished when ATP was either depleted from the permeabilized cell system or was replaced with ATP-γ-S. When transport from the TGN to the apical surface was dissected into two stages, by incubating cells after the cold block for 15 min at 35°C before permeabilization, depletion of ATP or addition of ATP-γ-S, only partially inhibited the second step of transport, suggesting that this step can proceed at lower ATP concentrations than the first step, which probably involves vesicle formation.

It has long been known that protein transport from the ER to the Golgi apparatus is an energy-dependent process (Jamieson and Palade, 1968), and recent studies with cultured cells, cell-free and semi-intact cell systems have demonstrated that a requirement for ATP is a general feature of vesicle transport along the secretory pathway (Balch et al., 1984*a*,*b*; Balch et al., 1986; Balch and Keller, 1986; Nowack et al., 1987; Bennett et al., 1988; de Curtis and Simons, 1988). IntraGolgi transport has been studied in the greatest detail and may provide a paradigm for studies on transport between other compartments, such as the TGN and the plasma membrane. At least four distinct steps in intraGolgi transport have been shown to require ATP (Balch et al., 1984*b*; Malhotra et al., 1988). The first of these is a "priming" reaction that precedes vesicle formation in the donor membrane and may correspond to or include the ATP-dependent synthesis of fatty-acyl CoA needed to form the vesicle (Glick and Rothman, 1987; Pfanner et al., 1989). It is noteworthy that this reaction has a greater dependence on ATP than subsequent steps, presumably reflecting the high K_m for ATP of the fatty acyl CoA ligase (Glick and Rothman, 1987). Although the participation of fatty acyl CoA in other vesicle formation events has not been established, our observation of an apparently higher requirement for ATP of the early stage in TGN to surface transport may reflect the need for it in vesicle formation at the *trans*-Golgi donor membrane. That ATP is required for vesicle formation in the TGN has been demonstrated directly in perforated cold-synchronized cells (Bennett et al., 1988), as well as in cell-free extracts (de Curtis and Simons, 1989; Tooze and Huttner, 1990). The apparent contradictory finding that in permeabilized BHK cells transport of the Semliki virus glycoprotein p62 from the *trans*-Golgi to the nonpolarized cell surface was not inhibited significantly by ATP-γ-S (de Curtis and Simons, 1989), whereas vesicle formation from the TGN in a cell-free system from the same cells was ATP dependent (de Curtis and Simons, 1989) may simply be a consequence of the capacity of the perforated cells to produce sufficient ATP to compete with the non-hydrolyzable analogue. Other ATP-dependent steps in intraGolgi vesicular transport that follow vesicle formation take place after attachment of the vesicle to the acceptor membrane. The first of these involves the *N*-ethylmaleimide–sensitive factor (NSF), which seems to play a key role in all membrane fusion events (Malhotra et al., 1988; Beckers et al., 1989; Diaz et al., 1989). It is, therefore, likely that the ATP requirement we observed during the second step in TGN to plasma membrane transport includes at least a similar NSF-dependent step.

The ATP requirement for vesicular transport to the apical surface could also reflect the participation of a microtubule-based motor in this process. It has previously been shown that the polarized delivery of the influenza HA to the apical surface is dependent on the integrity of microtubules (Rindler et al., 1987) and this has been shown to be also the case for the transport of other proteins to the apical surface of other polarized epithelial cells (Eilers et al., 1989; Achler et al., 1989). Delivery of HA from the TGN to the apical surface of MDCK cells would require vesicle transport in the retrograde direction since longitudinal microtubules in these cells are oriented with minus ends towards the apical surface (Bacallao et al., 1989). Transport in this direction is known to involve the participation of dynein, a microtubule-activated cytoplasmic ATPase, and it is noteworthy that the activity of this protein is highly sensitive to vanadate, an agent that we showed was a very potent inhibitor of apical transport, even when added 15 min after release from the cold block. Vanadate, however, has multiple cellular targets, including other ATPases, such as the Na^+,K^+ ATPase, and protein phosphatases. Since okadaic acid, an inhibitor of type 2A, type I, and polycation-modulated phosphatases (Bialojan and Takai, 1988) had not effect on transport, it can be excluded that vanadate acted through the inhibition of such enzymes. The failure of the protein kinase C inhibitors H-8, (Hidaka et al., 1984) and AMG-C_{16} (Kramer et al., 1989) to affect transport also excluded the possibility that the ATP dependence of transport resulted from the participation of that enzyme in this process.

The permeabilized system also allowed us to test effect on TGN to cell surface transport of dipeptides that inhibit metalloendoproteases and have been shown to block vesicular transport in both the exocytic and endocytotic pathways

(Strous et al., 1988). As expected, the dipeptide gly-phe had a strong inhibitory effect on transport. However, the inhibition only occurred when the dipeptide was added before the first stage of transport, which suggests that a metalloendoprotease may be involved in vesicle formation, but not in vesicle fusion.

A major virtue of cell-free systems for studying transport is that they permit the identification and purification of individual macromolecular and small molecule cytosolic components that participate in the process and the subsequent elucidation of their mode of action. We showed that the SLO-permeabilized cells can be rendered dependent on exogenous cytosol for TGN to cell surface transport by introducing a washing step after pore formation. According to the model we proposed, the cytosolic fraction should contain adaptors and GTP-binding proteins with specificity for sorting different proteins into different vesicles and for delivering these vesicles to specific acceptor membranes. We anticipate that, since the cellular architecture of polarized epithelial cells is retained in this system, it should be possible to utilize it in assays designed to identify cytosolic factors specific for transport steps that accomplish protein delivery to one or the other cell surface domains.

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