Cholesterol Is Required for Surface Transport of Influenza Virus Hemagglutinin

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Abstract. Transport from the TGN to the basolateral surface involves a rab/*N*-ethylmaleimide–sensitive fusion protein (NSF)/soluble NSF attachment protein (SNAP)/SNAP receptor (SNARE) mechanism. Apical transport instead is thought to be mediated by detergent-insoluble sphingolipid–cholesterol rafts. By reducing the cholesterol level of living cells by 60–70% with lovastatin and methyl-β-cyclodextrin, we show that the TGN-to-surface transport of the apical marker protein influenza virus hemagglutinin was slowed down, whereas the transport of the basolateral marker vesicular stomatitis virus glycoprotein as well as the ER-to-Golgi transport of both membrane proteins was not affected. Reduction of transport of hemagglutinin was accompanied by increased solubility in the detergent Triton X-100 and by significant missorting of hemagglutinin to the basolateral membrane. In addition, depletion of cellular cholesterol by lovastatin and methyl-β-cyclodextrin led to missorting of the apical secretory glycoprotein gp-80, suggesting that gp-80 uses a raft-dependent mechanism for apical sorting. Our data provide for the first time direct evidence for the functional significance of cholesterol in the sorting of apical membrane proteins as well as of apically secreted glycoproteins.

E PITHELIAL cells have to deliver newly synthesized proteins to the apical and basolateral plasma membrane domains of the polarized cell surface. Most of the studies analyzing how this polarized surface delivery is accomplished have been carried out in MDCK cells. Sorting takes place in the TGN and at least two vesicular carriers exist for apical and basolateral delivery (Lafont et al., 1994; Pimplikar and Simons, 1994; Ikonen et al., 1995). Recent data indicate that also nonpolarized cell lines, such as BHK, CHO, GH3 (a rat anterior pituitary line), or mouse 3T3 fibroblasts use apical and basolateral cognate routes to deliver proteins from the TGN to the cell surface and that the same signals that operate in epithelial sorting probably also function in fibroblasts (Müsch et al., 1996; Yoshimori et al., 1996).

Basolateral sorting signals have a cytoplasmic disposition and can be grouped in two classes, one with signals related to clathrin-coated pit localization motifs, and one with unrelated motifs (Mellman, 1996). Apical sorting signals are of at least two types. Glycosyl-phosphatidylinositol (GPI)[1]-anchored proteins use their GPI anchors as sorting determinants (Brown et al., 1989; Lisanti et al., 1989). Another sorting signal is constituted by the mannose-rich core part of *N*-glycans. This signal is used by apical secretory proteins, and probably by apical transmembrane proteins as well (Scheiffele et al., 1995). Apical sorting information may also reside in the transmembrane domains of some apical proteins (Kundu et al., 1996), favoring partitioning into sphingolipid- and cholesterol-rich domains in a similar manner as GPI anchors do. The machinery decoding these different sorting signals in the TGN has been poorly characterized. The basolateral route uses the rab/*N*-ethylmaleimide–sensitive fusion protein (NSF)/soluble NSF attachment protein (SNAP)/SNAP receptor (SNARE) mechanism for delivery, whereas the apical pathway seems to involve a new mechanism (Ikonen et al., 1995) using sphingolipid–cholesterol rafts, which contain apical cargo and several proteins (VIP21/caveolin-1 [Dupree et al., 1993], VIP36 [Fiedler et al., 1994], VIP17/MAL [Zacchetti et al., 1995], and annexin XIIIb [Fiedler et al., 1995]) with potential function in apical sorting and delivery.

Our working hypothesis for apical targeting postulates the formation of sphingolipid–cholesterol rafts within the exoplasmic leaflet of the Golgi membrane (Simons and van Meer, 1988). According to the model proposed (for review see Simons and Ikonen, 1997) rafts are closely packed domains assembled within the fluid bilayer. They are formed by sphingolipids associating laterally with each other. The carbohydrate head groups occupy larger areas

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1. *Abbreviations used in this paper*: GPI, glycosyl-phosphatidylinositol; HA, hemagglutinin; PLAP, placental alkaline phosphatase; VSV G, vesicular stomatitis virus glycoprotein.

than their mostly saturated hydrocarbon chains, and the voids that would tend to form underneath the bulky head groups are filled with cholesterol molecules. Moreover, cholesterol is also present in the cytoplasmic leaflet (Schroeder et al., 1991), where it too could function as a spacer to fill voids created by the long sphingolipid fatty acid chains interdigitating with the cytoplasmic leaflet (Morrow et al., 1995; but see also Boggs and Koshy, 1994). These sphingolipid–cholesterol rafts, which can be isolated as detergent-insoluble, glycolipid-enriched complexes (DIGs) using Triton X-100 at 4°C, are thought to act in the TGN as sorting platforms for inclusion of protein cargo destined for delivery to the apical membrane. GPI-anchored proteins (Brown and Rose, 1992; Rodgers et al., 1994; Scheiffele et al., 1997), apical transmembrane proteins (Skibbens et al., 1989; Fiedler et al., 1993; Danielsen, 1995; Scheiffele et al., 1997), and also doubly acylated tyrosine kinases of the Src family (Resh, 1994; Rodgers et al., 1994; Casey, 1995; Scheiffele et al., 1997) associate with rafts. Several studies have demonstrated that this association is because of cooperative interactions between sphingolipids, cholesterol, and proteins.

If rafts indeed do represent the units for apical TGN-to-surface transport, then inhibiting the synthesis of or segregating cholesterol or sphingolipids would be expected to affect both detergent insolubility and transport of raft-associated proteins. These predictions, as far as they have been studied, are at least partially fulfilled for GPI-anchored proteins. Blocking the synthesis of cholesterol and deprivation of sphingolipids reduced the amount of detergent-insoluble human placental alkaline phosphatase (PLAP) (Hanada et al., 1995). Extraction of cellular cholesterol with cyclodextrin (Scheiffele et al., 1997) or depletion of cholesterol by saponin (Cerneus et al., 1993) rendered PLAP completely soluble in Triton X-100. Deprivation of cellular cholesterol also inhibited the formation of the prion protein PrPsc (Taraboulos et al., 1995), and it was shown that the GPI anchor directed subcellular trafficking and controlled conversion into the scrapie isoform (Taraboulos et al., 1995; Kaneko et al., 1997). A reduction of the cellular cholesterol level in MDCK cells decreased the detergent-insolubility of gD1-DAF without affecting its steady state cell surface distribution (Hannan and Edidin, 1996). Recently, the association of the apical transmembrane protein influenza virus hemagglutinin (HA) with rafts also has been shown to critically depend on cholesterol, as judged by the increased Triton X-100 solubility of HA after cyclodextrin treatment (Scheiffele et al., 1997).

Methods commonly used to alter cholesterol levels within living cells include lipid-free lipoprotein–mediated cellular lipid efflux (for review see Oram and Yokoyama, 1996). However, they cannot be used to specifically alter cholesterol levels because phospholipids are also removed. β-Cyclodextrins have been shown to selectively and rapidly extract cholesterol from the plasma membrane, in preference to other membrane lipids (Ohtani et al., 1989; Kilsdonk et al., 1995; Neufeld et al., 1996). Another strategy is to inhibit cholesterol biosynthesis. Most of these inhibitors, such as lovastatin (Alberts et al., 1980), compactin (Endo et al., 1976), SR-12813 (Berkhout et al., 1996), 25-hydroxycholesterol (Kandutsch and Chen, 1974), and 7-ketocholesterol (Brown and Goldstein, 1974), are directed against 3-hydroxy-3-methylglutaryl coenzyme A reductase. Alternatively, cells have also been grown in the presence of delipidated serum.

In this paper we have examined the role of cholesterol in the trafficking of apical and basolateral transmembrane proteins as well as of gp-80, the major apically secreted glycoprotein of MDCK cells. Since long-term growth of cells in the absence of low density lipoprotein (LDL) as the principal source of cholesterol may allow them to compensate for reduced cholesterol levels by changes in lipid desaturase activity and membrane lipid composition (Leikin and Brenner, 1988; Lutton, 1991; Muriana et al., 1992), we have tried to remove cholesterol as acutely as possible. As markers for the apical and for the basolateral pathways we have used influenza virus HA and vesicular stomatitis virus glycoprotein (VSV G), respectively. We show that by using lovastatin and methyl-β-cyclodextrin cholesterol levels can be lowered by 60–70% without significantly affecting cell viability. In cells treated this way, TGN-to-apical surface transport of HA was markedly reduced. On the contrary, neither TGN-to-surface transport of VSV G, nor ER-to-Golgi transport of both markers were affected. Moreover, in MDCK cells there was substantial missorting of HA as well as of gp-80 to the basolateral surface. These data provide the first concrete evidence for the functional significance of sphingolipid–cholesterol rafts in membrane trafficking.

Materials and Methods

Materials

Media and reagents for cell culture were purchased from GIBCO BRL (Eggestein, Germany). Lovastatin was provided by R. Luedecke (MSD Sharp & Dohme, Haar, Germany), and was prepared as a 20-mM stock solution as described (Fenton et al., 1992). N-tosyl-L-phenylalanine chloromethyl ketone (TPCK)-treated trypsin and soybean trypsin inhibitor were obtained from Worthington Biochemical Corp. (Freehold, NJ); endoglycosidase H from Boehringer Mannheim GmbH (Mannheim, Germany); proteinase inhibitors, methyl-β-cyclodextrin, mevalonate, and filipin from Sigma Chemical Co. (Deisenhofen, Germany); protein A–Sepharose CL-4B from Pharmacia Biotech Sevrage (Uppsala, Sweden); NP-40 from Fluka AG (Buchs, Switzerland); SDS from BioRad Laboratories, GmbH (München, Germany); delipidated FCS from PAA Laboratories (Linz, Austria); [35S]methionine and [1a, 2a(N)-3H]cholesterol from Amersham Buchler GmbH (Braunschweig, Germany). Antibodies used were an affinity-purified polyclonal antibody raised against the luminal domain of VSV G (Pfeiffer et al., 1985), a polyclonal antibody against gp-80 (Urban et al., 1987), and monoclonal antibodies against a 58-kD basolateral protein (Balcarova-Ständer et al., 1984), and lactate dehydrogenase (Sigma).

Virus Preparation

Stocks of influenza virus N, whose HA is not cleaved by proteases of the host cell, and of phenotypically mixed VSV were prepared as described before (Matlin and Simons, 1983; Bennett et al., 1988).

Cell Culture and Treatment with Lovastatin

BHK 21 cells (1.2×10^5 cells) were cultured in BHK medium (Glasgow MEM supplemented with 5% FCS, 10% phosphate tryptose broth, 10 mM Hepes, pH 7.3, 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin) on 11-mm-diam coverslips in 24-multi-well plates (NUNC, Roskilde, Denmark) for 48 h in the presence or absence of 4 μM lovastatin and 0.25 mM mevalonate (lovastatin/mevalonate).

MDCK strain II cells (1:80 of the cells from one confluent 75-cm² flask per filter) were grown on 12-mm-diam, 0.4-μm pore–size Transwell polycarbonate filters (Costar Corp., Cambridge, MA), as previously described (Pimplikar et al., 1994). 24 h after plating fresh MDCK medium (Earle's MEM supplemented with 10% FCS, 10 mM Hepes, pH 7.3, 2 mM

glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin) was added. 24 h later fresh medium (750 μl apically, 1,500 μl basolaterally) containing or not lovastatin/mevalonate was added, and the cells were allowed to grow for further 48 h in this medium.

Viral Infection and Extraction with Methyl-β-Cyclodextrin

BHK cells were infected for 1 h at 37°C with the viruses in infection medium (Earle's MEM supplemented with 50 mM Hepes, pH 7.3, 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin) in the presence or absence of lovastatin/mevalonate. Then the medium was aspirated, fresh infection medium containing or not lovastatin/mevalonate was added, and the cells were incubated for 2.5 h at 37°C. Thereafter, the cells grown and infected in the presence of lovastatin/mevalonate were treated for 30 min at 37°C with 1 ml 10 mM methyl-β-cyclodextrin in infection medium. The cells then were carefully washed twice with labeling medium (Earle's MEM without methionine and with low bicarbonate [0.35 g/l], supplemented with 50 mM Hepes, pH 7.3, 2 mM glutamine), and subsequently used for the transport assays (see below).

MDCK cells were infected for 1 h at 37°C with the viruses in infection medium in the presence or absence of lovastatin/mevalonate. After aspiration of the medium, fresh infection medium containing or not lovastatin/mevalonate was added, and the cells were incubated for 2 h at 37°C. Thereafter the cells grown and infected in the presence of lovastatin/mevalonate were treated for 60 min at 37°C with 10 mM methyl-β-cyclodextrin in infection medium (750 μl apically, 1,500 μl basolaterally). The cells were washed twice with PBS+ (PBS containing 0.9 mM CaCl$_2$ and 0.5 mM MgCl$_2$), and then were used for the transport assays (see below).

All subsequent steps were done in the absence of lovastatin/mevalonate. Metabolic labeling in cells treated with lovastatin/mevalonate and cyclodextrin was generally decreased by ~10–20%.

Metabolic Labeling and ER-to-Golgi Transport

BHK cells were labeled in a water bath for 5 min at 37°C with 23 μCi [^{35}S]methionine in 200 μl labeling medium. The pulse was terminated by adding chase medium (labeling medium containing 20 μg/ml cycloheximide and 150 μg/ml methionine), and the cells were incubated for ≤40 min at 37°C. They then were lysed in 120 μl of lysis buffer (PBS containing 2% NP-40 and 0.2% SDS) supplemented with a protease inhibitor cocktail (CLAP; 25 μg/ml each of chymostatin, leupeptin, antipain, and pepstatin A), and insoluble material was removed by centrifugation.

MDCK cells were labeled from the basolateral side for 8 min at 37°C with 15 μCi [^{35}S]methionine in 25 μl labeling medium as described (Lafont et al., 1995). The filters then were transferred into new dishes containing chase medium and incubated at 37°C for up to 30 min. The cells were lysed in 120 μl of lysis buffer and insoluble material was removed by centrifugation.

20 μl of the cell lysate then was treated with endoglycosidase H according to the manufacturer's instructions, and then was analyzed by SDS-PAGE on 7.5% polyacrylamide gels (Laemmli, 1970). Radioactivity in the individual bands was determined using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA), and the percentage of HA and VSV G reaching the Golgi was calculated: percent endo H–resistant = endo H–resistant form/[endo H–resistant form + endo H–sensitive form] × 100.

Metabolic Labeling and TGN-to-Surface Transport

BHK cells were labeled for 5 min, chased for 3 min at 37°C, and then further incubated for 75 min at 19.5°C to block the viral glycoproteins in the TGN. The cells were then incubated for up to 30 min at 37°C. To detect surface arrival of HA, trypsin cleavage was used (Matlin and Simons, 1983; Yoshimori et al., 1996). The amount of VSV G transported was determined by surface immunoprecipitation using an affinity-purified polyclonal antibody against the exoplasmic portion of VSV G (Pfeiffer et al., 1985), essentially as previously described for Semliki Forest virus glycoprotein E1 + E2 (Yoshimori et al., 1996).

Virus-infected MDCK cells were labeled for 8 min at 37°C, and incubated for 75 min at 19.5°C in chase medium. The cells were then shifted for up to 30 min to 37°C. Surface arrival of HA and VSV G were detected by trypsin cleavage and surface immunoprecipitation, respectively, as described (Lafont et al., 1995).

Aliquots were analyzed by SDS-PAGE on 10% polyacrylamide gels (Laemmli, 1970), and radioactivity in the individual bands was determined using a PhosphorImager. The amount of HA transported was calculated: transport index (%) = 2 × HA2/[HA + (2 × HA2)] × 100 (Matlin and Simons, 1983). VSV G reaching the surface was calculated: transport index (normalized arbitrary units) = surface-immunoprecipitated VSV G/total VSV G.

To analyze gp-80 secretion, noninfected MDCK cells were labeled for 15 min at 37°C with 83 μCi [^{35}S]methionine, chased for 5 min at 37°C, and further incubated for 75 min at 19.5°C. Then the cells were shifted for up to 40 min to 37°C, and then apically and basolaterally secreted, as well as intracellularly retained gp-80 was recovered by immunoprecipitation and analyzed by SDS-PAGE under nonreducing conditions. The amount of gp-80 secreted apically was calculated: gp-80 apically secreted (%) = gp-80 in apical medium/[gp-80 in apical medium + gp-80 in basolateral medium + intracellular gp-80] × 100. The amount of gp-80 secreted basolaterally was calculated accordingly.

Triton X-100 Insolubility of Influenza Virus HA

Triton X-100 extractions were performed as described (Brown and Rose, 1992; Fiedler et al., 1993). BHK and MDCK cells grown in the presence of lovastatin/mevalonate were infected with influenza N virus, treated with 10 mM methyl-β-cyclodextrin, and pulse labeled as described above. After a chase at 37°C the cells were extracted on ice for 30 min with 1% (wt/vol) Triton X-100 in 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 2 mM DTT, CLAP (BHK cells, 100 μl; MDCK cells, 300 μl), followed by centrifugation for 30 min at 120,000 g. Triton X-100–soluble and –insoluble fractions were separated on 7.5% polyacrylamide gels (Laemmli, 1970), and analyzed using a PhosphorImager. The amount of detergent-insoluble, complex-glycosylated HA was calculated: percent Triton X-100–insoluble HA = detergent-insoluble HA/[detergent-insoluble HA + detergent-soluble HA] × 100.

[^{3}H]Cholesterol Labeling of Cells and Extraction with Methyl-β-Cyclodextrin

BHK cells were plated in BHK medium on coverslips in the presence or absence of lovastatin/mevalonate as described above. 24 h after plating, the cells were washed by dipping twice in PBS+ and transferred into a new 24-multiwell plate with 400 μl cholesterol-labeling medium (Earle's MEM supplemented with 2.5% delipidated FCS, 10 mM Hepes, pH 7.3, 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin) containing 2.5 μCi [^{3}H]cholesterol in the presence or absence of lovastatin/mevalonate. After an incubation for 8 h at 37°C, the cells were washed twice with PBS+, and cholesterol-labeling medium with or without lovastatin/mevalonate was added. Radiolabeled cholesterol was allowed to equilibrate with the cellular cholesterol pools for 20 h at 37°C (Debry et al., 1997). The cells were then treated for 30 min at 37°C with 1 ml 10 mM methyl-β-cyclodextrin in infection medium. The medium was removed, the cells were washed once with PBS+, and then lysed in 200 μl lysis buffer. After a short centrifugation to remove insoluble material, the [^{3}H]cholesterol released into the medium and remaining in the cells was determined by liquid scintillation counting (model LS6000SC; Beckman Instruments, Fullerton, CA).

MDCK cells were grown on filters and treated with lovastatin/mevalonate as described above. 48 h after plating the cells were washed twice with PBS+, and cholesterol-labeling medium (750 μl apically, 1,500 μl basolaterally) containing 3.3 μCi [^{3}H]cholesterol per filter in the presence or absence of lovastatin/mevalonate was added. After an incubation for 8 h at 37°C, the cells were washed twice with PBS+, and incubated in cholesterol labeling medium with or without lovastatin/mevalonate for an additional 20 h. The cells were then treated for 60 min at 37°C with 10 mM methyl-β-cyclodextrin in infection medium (750 μl apically, 1,500 μl basolaterally). [^{3}H]Cholesterol released into the apical and basolateral medium and remaining in the cells was determined as described for BHK cells.

Fluorimetric Cholesterol Determination

Filter-grown MDCK cells were grown and treated with lovastatin/mevalonate and methyl-β-cyclodextrin as described above. Lipids were extracted from the cell lysates using chloroform/methanol (Bligh and Dyer, 1959). Free cholesterol was determined by a cholesterol oxidase/peroxidase based assay (Gamble et al., 1978).

Western Blot Analysis

BHK cells were grown and treated with lovastatin/mevalonate and methyl-β-cyclodextrin as described above. Aliquots of cell lysates were separated on a 10% polyacrylamide gel (Laemmli, 1970), and proteins were transferred to a nitrocellulose membrane. After staining with Ponceau red the blot was probed with antibodies directed against the cytosolic marker lactase dehydrogenase, and visualized using an HRP-conjugated secondary antibody and enhanced chemiluminescence detection kit (Amersham International).

Confocal Immunofluorescence

Filter-grown MDCK cells were grown and treated with lovastatin/mevalonate and methyl-β-cyclodextrin as described above. Immunofluorescence using the monoclonal antibody 6.23.3 against a basolateral 58-kD protein was performed as described (Fiedler et al., 1995), omitting the denaturation step with guanidine. Images were taken with an inverted confocal laser scanning microscope (LSM-410; Carl Zeiss, Oberkochen, Germany).

Filipin Staining of BHK Cells

BHK cells (0.2×10^5 cells) were grown and treated with lovastatin/mevalonate and methyl-β-cyclodextrin as described above. They were fixed for 30 min on ice with 4% paraformaldehyde, and then stained for 15 min at room temperature with 400 μl of a 125 μg/ml filipin solution in PBS. After washing twice with PBS for 15 min each, the cells were mounted in PBS/50% glycerol. Digital images were taken using a Zeiss Axioskop microscope equipped with a 3-chip color camera (Photonic Sciences, Millham, UK).

Results

Our aim was to specifically disrupt rafts in living cells by depleting cholesterol. We were looking for methods that would rapidly and efficiently remove cholesterol without affecting cell polarity and ER-to-Golgi transport. For this purpose, we have followed two strategies. First, we inhibited de novo synthesis of cholesterol in the ER by lovastatin in the presence of small amounts of mevalonate to allow the synthesis of nonsterol products from mevalonate (Brown and Goldstein, 1980). Second, we rapidly extracted cholesterol from the plasma membrane using methyl-β-cyclodextrin, a compound shown to selectively extract cholesterol from the plasma membrane of a number of cells (Ohtani et al., 1989; Kilsdonk et al., 1995; Neufeld et al., 1996).

Depletion of Cholesterol

The concentration of lovastatin and methyl-β-cyclodextrin used was critical. Treatment for 48 h with 4 μM lovastatin and 0.25 mM mevalonate (subsequently referred to as lovastatin/mevalonate) in medium containing FCS proved to be optimal. Under these conditions the cells showed a normal growth rate. Extraction with 10 mM methyl-β-cyclodextrin was done immediately before pulse labeling. In samples treated this way there was no detachment of BHK cells from the glass coverslips. BHK and MDCK cells treated for 30 and 60 min, respectively, displayed more dramatic effects than shorter treatments (data not shown). Since there is a substantial amount of cholesterol in intracellular membranes and because cholesterol is continuously cycling between the plasma membrane and the ER, this suggests that at least a partial depletion of the intracellular stores was important.

Although treatment with lovastatin for 48 h alone reduced cholesterol levels by only 10%, this treatment significantly increased the effect of methyl-β-cyclodextrin on TGN-to-surface transport of HA (see below).

We have tried to further reduce cellular cholesterol levels by growing cells in the presence of delipidated serum or in hormone-supplemented, serum-free medium (Taub et al., 1979). However, BHK cells treated for 2 d with lovastatin/mevalonate in the presence of delipidated serum were growing more slowly and were easily detached by methyl-β-cyclodextrin. Moreover, there was a general reduction seen in biosynthetic trafficking (although to a lower extent) even in control cells not treated with methyl-β-cyclodextrin, suggesting that delipidation had removed essential components from the serum.

Thus a combination of a treatment with lovastatin/mevalonate for 48 h before infection and an extraction with methyl-β-cyclodextrin immediately before pulse labeling proved to be optimal. All the results presented below were obtained with cells treated in this way.

Methyl-β-Cyclodextrin Efficiently Removes Cholesterol from the Plasma Membrane

We have monitored the extent of cholesterol extraction in two ways. First, we determined the amount of cholesterol being removed from the cells. In preliminary experiments, we have used a cholesterol oxidase/peroxidase-based assay (Gamble et al., 1978), which would allow a direct measurement of the quantity of cholesterol being extracted. However, methyl-β-cyclodextrin interfered with the fluorimetric determination, even when the lipid extraction protocol was changed (Kilsdonk et al., 1995). Therefore we have used a more indirect way by preloading the cells with [³H]cholesterol. After an equilibration with the nonradioactive cellular cholesterol pool, cholesterol extraction was followed by the release of [³H]cholesterol into the medium. BHK cells treated with lovastatin/mevalonate and extracted for 30 min with 10 mM methyl-β-cyclodextrin lost ∼60% of their [³H]cholesterol, whereas cells incubated in medium lost essentially no cholesterol (Fig. 1 *A*). Filter-grown MDCK cells treated for 60 min lost 70% of their [³H]cholesterol (Fig. 1 *B*), a fourth of it being recovered in the apical medium (data not shown). Although shorter extraction times removed similar amounts of cholesterol (Rietveld, A., and K. Simons, unpublished results), effects on TGN-to-surface transport of influenza virus HA (see below) were more pronounced after a more extensive treatment, suggesting that a partial depletion of cholesterol from intracellular membranes was important.

Second, we used filipin, a fluorescent polyene antibiotic that forms complexes with cholesterol (Miller, 1984; Yeagle, 1985; see also Neufeld et al., 1996). When BHK cells were fixed and incubated with filipin, prominent labeling of the plasma membrane and of vesicular structures concentrated in the perinuclear region was observed (Fig. 2 *A*). After treatment with lovastatin/mevalonate and extraction for 30 min with methyl-β-cyclodextrin, followed by an incubation for 75 min at 19.5°C, plasma membrane staining was essentially gone (Fig. 2 *B*). The intracellular staining was less affected, although the signal also became weaker (note that the image in Fig. 2 *B* was acquired using 20 integration frames, whereas for Fig. 2 *A* only 5 integration frames were used). Treatment with lovastatin/mevalonate alone had no effect on filipin staining (data not shown).

Depletion of Cholesterol Leaves the Cells Intact

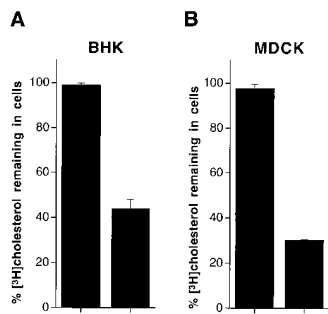Treatment of erythrocytes with β-cyclodextrin not only

Figure 1. Treatment of BHK and MDCK cells with methyl-β-cyclodextrin leads to efficient removal of cellular cholesterol. BHK cells (*A*) and filter-grown MDCK cells (*B*) were labeled with [³H]cholesterol in the absence (−) or presence (+) of lovastatin/mevalonate, and radiolabeled cholesterol was allowed to equilibrate with the nonradioactive pool of cholesterol. The cells then were treated for 30 min (*BHK*) or 60 min (*MDCK*) at 37°C with 10 mM methyl-β-cyclodextrin in infection medium (+) or with infection medium only (−). Radioactivity released into the medium and remaining in the cells was determined. Data were obtained from two independent experiments with samples done in duplicate.

led to an efficient removal of cholesterol, but also to a significant extraction of proteins and eventually to hemolysis (Ohtani et al., 1989). Using the conditions described above we first verified that cells treated this way were left intact and that MDCK cells grown on filters were still properly polarized.

BHK cells were treated with lovastatin/mevalonate and methyl-β-cyclodextrin, lysed, and then analyzed by SDS-PAGE and Western blotting. The protein pattern and the total amount of protein did not change as compared with untreated control cells. Moreover there was no difference in the amount of lactate dehydrogenase, a cytosolic marker protein, indicating that no cell lysis had occurred (data not shown).

Filter-grown MDCK cells were treated with lovastatin/mevalonate and methyl-β-cyclodextrin or were left untreated, and the transepithelial resistance was measured. Although there was an overall variation of the transepithelial resistance between individual experiments, no significant differences between cholesterol-depleted and control cells were detected. In a representative experiment the transepithelial resistances measured were 83.7 ± 11.5 Vcm² and 82.2 ± 10.7 Vcm², respectively (three filters for each condition). In addition, by using immunofluorescence we have looked at the distribution of a 58-kD basolateral protein (Balcarova-Ständer et al., 1984) in cholesterol-depleted, filter-grown MDCK cells. Fig. 2 clearly shows that the lateral distribution of the 58-kD protein did not change after cholesterol extraction (Fig. 2 *D*) as compared with the control cells (Fig. 2 *C*). Our conclusion that cholesterol extraction did not lead to opening up of the junctional complexes was further strengthened by the observation that trypsin added basolaterally did not leak to the apical side (see below).

Removal of Cholesterol Does Not Affect ER-to-Golgi Transport

As association of HA with rafts occurs only in the Golgi complex (Skibbens et al., 1989; Kurzchalia et al., 1992; Fiedler et al., 1993), no effect on ER-to-Golgi transport of HA was expected to occur after cholesterol depletion. Consistent with this hypothesis, ER-to-Golgi transport of
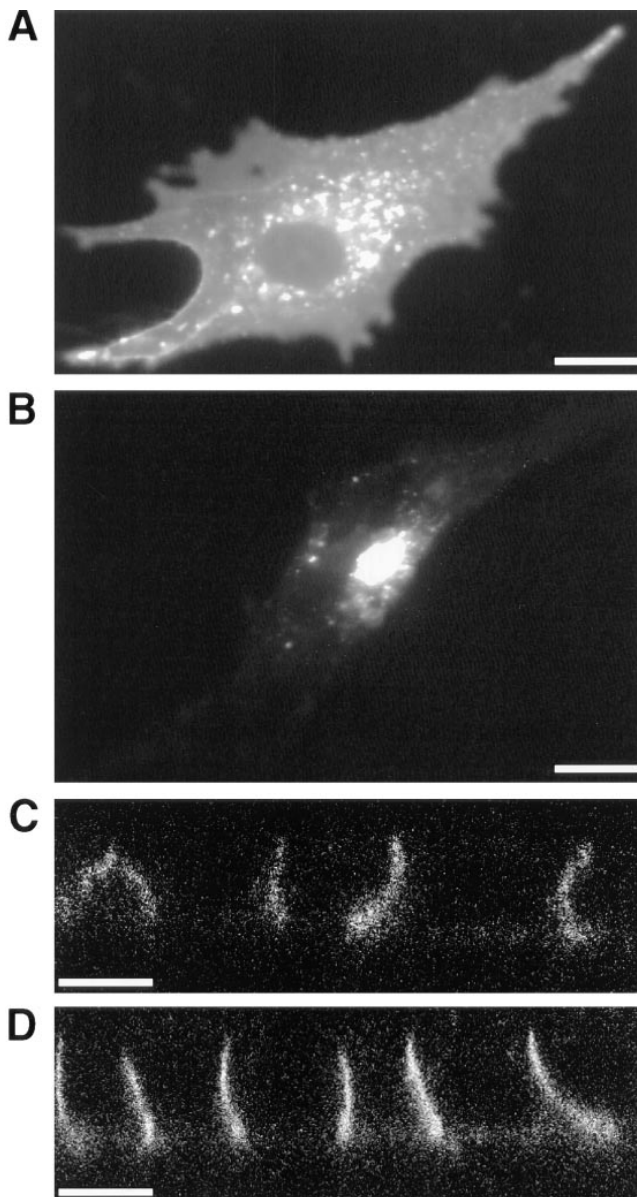

Figure 2. Treatment of BHK cells and of filter-grown MDCK cells with methyl-β-cyclodextrin. BHK cells were left untreated (*A*), or were grown in the presence of lovastatin/mevalonate and then treated for 30 min at 37°C with 10 mM methyl-β-cyclodextrin (*B*) as detailed in Materials and Methods, and then were stained with filipin. Digital images were acquired using 5 (*A*) and 20 (*B*) integration frames, respectively. Filter-grown MDCK cells were left untreated (*C*), or were grown in the presence of lovastatin/mevalonate and then treated for 60 min at 37°C with 10 mM methyl-β-cyclodextrin (*D*) as detailed in Materials and Methods. X–Z confocal views of cells labeled with a monoclonal antibody directed against a basolateral 58-kD protein. Bars, 10 μm.

HA, as well as of the basolateral marker VSV G was not affected by cholesterol extraction, as monitored by the acquirement of resistance to endoglycosidase H digestion. This was the case for BHK (Fig. 3, *A* and *B*) and for filter-grown MDCK (Fig. 3, *C* and *D*) cells.

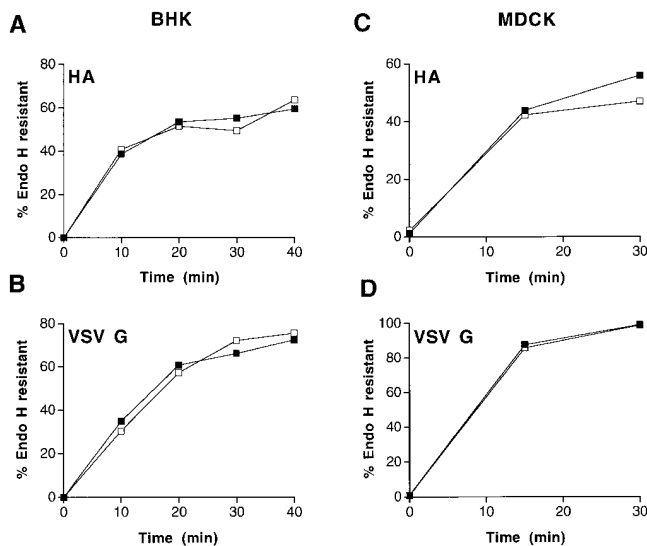Thus although cholesterol continuously cycles between the plasma membrane and the ER and although cholesterol

Figure 3. Removal of cholesterol does not affect ER-to-Golgi transport. BHK cells (*A* and *B*) and filter-grown MDCK cells (*C* and *D*) were treated with lovastatin/mevalonate and methyl-β-cyclo-dextrin as detailed in Materials and Methods. After pulse labeling with [^{35}S]methionine, viral proteins (*A* and *C*, *HA*; *B* and *D*, *VSV G*) were chased for different times at 37°C. Arrival in the Golgi complex was monitored by acquisition of resistance to endoglycosidase H digestion. ■, untreated control cells; □, cells treated with lovastatin/mevalonate and methyl-β-cyclodextrin.

biosynthesis takes place in the ER, from where it probably uses the same vesicular carriers as HA and VSV G to reach the Golgi complex (for review see Liscum and Underwood, 1995), removal of 60–70% of total cellular cholesterol did not interfere with ER-to-Golgi transport of either marker.

Removal of Cholesterol Affects TGN-to-Surface Transport of Influenza Virus HA, but Not of VSV G

Next we looked at the TGN-to-surface transport of HA and VSV G. We (Yoshimori et al., 1996) and others (Müsch et al., 1996) have recently shown that nonpolarized cells, such as BHK cells, also use apical and basolateral cognate routes to deliver proteins from the TGN to the plasma membrane. After treatment of BHK cells with lovastatin/mevalonate and methyl-β-cyclodextrin, there was a dramatic decrease in surface arrival of HA, whereas arrival of VSV G was not affected at all. Representative examples for TGN-to-surface transport assays of HA and VSV G are shown in Fig. 4, *A* and *B*, respectively. 15 min after release from the TGN block, the surface arrival of HA was reduced by ∼60% as compared with untreated control cells, and there was little variation from experiment to experiment (Fig. 4 *C*; mean of four experiments). After 30 min of transport however, there was less overall inhibition of transport and the variability became much larger. Under the same conditions VSV G transport was not affected at any time (Fig. 4 *D*).

Subsequently, we have looked at the TGN-to-surface transport in fully polarized MDCK cells. As in BHK cells, cholesterol depletion led to a marked decrease in arrival of HA on the apical plasma membrane (Fig. 5 *A*), whereas transport of VSV G to the basolateral surface was barely
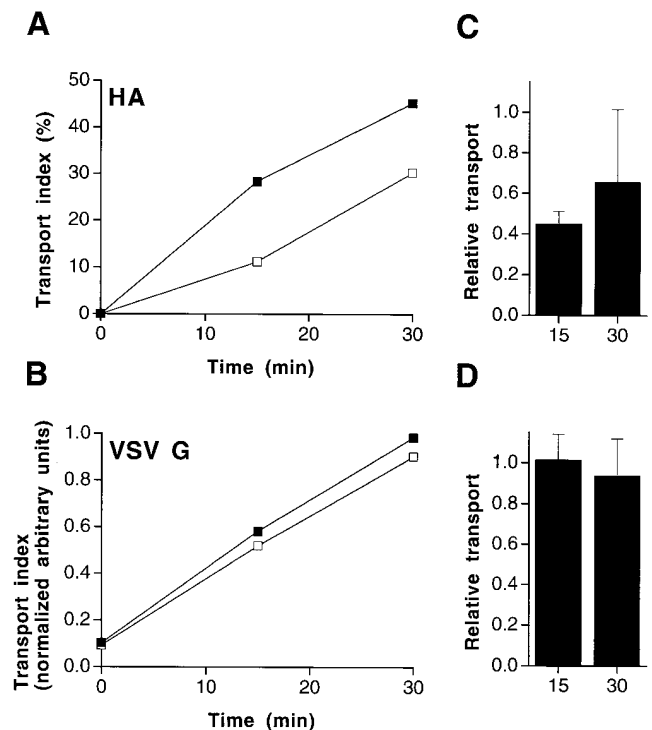

Figure 4. Removal of cholesterol from BHK cells affects TGN-to-surface transport of influenza virus HA, but not of VSV G. (*A* and *B*) Representative example of a TGN-to-surface transport. BHK cells were treated with lovastatin/mevalonate and methyl-β-cyclodextrin as detailed in Materials and Methods. After pulse labeling with [^{35}S]methionine and a TGN block at 19.5°C, viral proteins (*A*, *HA*; *B*, *VSV G*) were chased to the cell surface for different times at 37°C. Arrival on the surface was detected by trypsin treatment (*HA*) or by surface immunoprecipitation (*VSV G*), respectively. ■, untreated control cells; □, cells treated with lovastatin/mevalonate and methyl-β-cyclodextrin. (*C* and *D*) Averaged data of four independent experiments. Relative transport indices for HA (*C*) and VSV G (*D*) after 15 and 30 min of incubation at 37°C were calculated from four experiments. Transport in cells treated with lovastatin/mevalonate and methyl-β-cyclodextrin was expressed relative to the transport in untreated control cells at the respective time.

affected (Fig. 5, *B* and *D*). HA transport was inhibited by ∼60% after 15 min, and 40% after 30 min of transport as compared with untreated control cells (Fig. 5 *C*; mean of five experiments). Remarkably, the experimental variation for 30 min of transport was much smaller than was observed in BHK cells (Fig. 4 *C*).

Attempts to replete cholesterol by adding it back as a cholesterol–cyclodextrin complex was not interpretable because also control cells to which no cholesterol was added corrected their apical HA delivery over time (data not shown).

Removal of Cholesterol from MDCK Cells Leads to Partial Missorting of Influenza Virus HA

As there was clearly a reduction of transport of HA to the apical surface, we wondered whether the remaining HA had been retained in the Golgi, or possibly had been missorted to the basolateral side. Therefore we have looked at the appearance of HA on the basolateral membrane of filter-grown MDCK cells. As expected (Rodriguez-Boulan
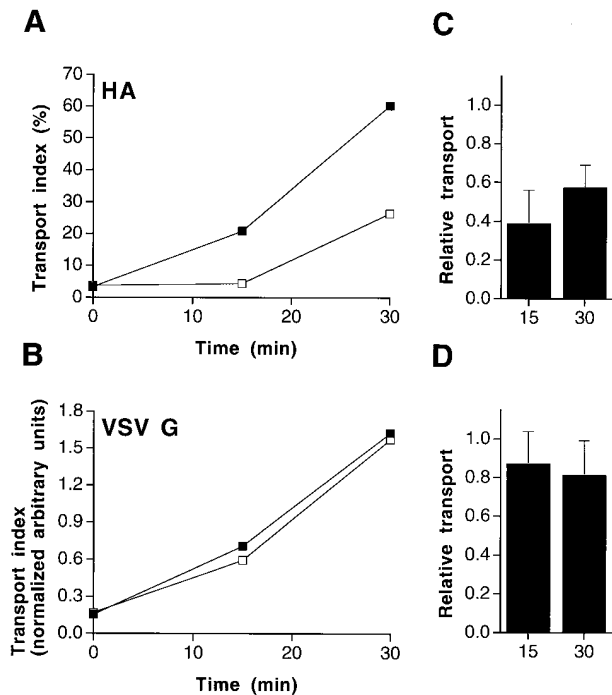
Figure 5. Removal of cholesterol from MDCK cells affects TGN–apical surface transport of influenza virus HA, but not basolateral VSV G transport. (*A* and *B*) Representative example of a TGN-to-surface transport. Filter-grown MDCK cells were treated with lovastatin/mevalonate and methyl-β-cyclodextrin as detailed in Materials and Methods. After pulse labeling with [³⁵S]methionine and a TGN block at 19.5°C, viral proteins (*A*, *HA*; *B*, *VSV G*) were chased to the cell surface for different times at 37°C. Arrival of HA on the apical surface was detected by trypsin treatment. Basolateral arrival of VSV G was detected by surface immunoprecipitation. ■, untreated control cells; □, cells treated with lovastatin/mevalonate and methyl-β-cyclodextrin. (*C* and *D*) Averaged data of five independent experiments (see Fig. 4).

Figure 6. Removal of cholesterol from MDCK cells leads to partial missorting of influenza virus HA. Filter-grown MDCK cells were treated with lovastatin/mevalonate and methyl-β-cyclodextrin as detailed in Materials and Methods. After pulse labeling with [³⁵S]methionine and a TGN block at 19.5°C, HA was chased to the cell surface for different times at 37°C. Arrival on the apical or basolateral membrane, respectively, was detected by trypsin treatment. (*A*) untreated control cells; (*B*) cells treated with lovastatin/mevalonate and methyl-β-cyclodextrin. HA1 and HA2 are the products generated from HA by trypsin cleavage. The duration of transport (in minutes) is indicated above the lanes.

and Pendergast, 1980; Fuller et al., 1985), barely any HA was cleaved into HA1 and HA2 when trypsin was added to the basolateral medium of untreated control cells (Fig. 6 *A*). In cholesterol-depleted cells there was little HA detectable on the basolateral side after 15 min of release from the TGN block. However, after 30 min of transport a significant amount of HA had appeared on the basolateral membrane (Fig. 6 *B*).

The detection of HA on the basolateral surface could be artifactual, being caused by trypsin added basolaterally leaking to the apical side. However, this is unlikely since cyclodextrin treatment did not lead to an apparent change in transepithelial resistance (see above). Moreover, soybean trypsin inhibitor was always present on the apical side. Nevertheless we did a control experiment where, after 30 min of transport, trypsin was added to the apical side only, or to the basolateral side only, or to both sides at the same time. In lovastatin/mevalonate and methyl-β-cyclodextrin treated cells, 31% of the total amount of HA was cleaved when trypsin was added apically, 21% were cleaved when trypsin was added basolaterally, and 50% were cleaved when trypsin was added from both sides. In un-
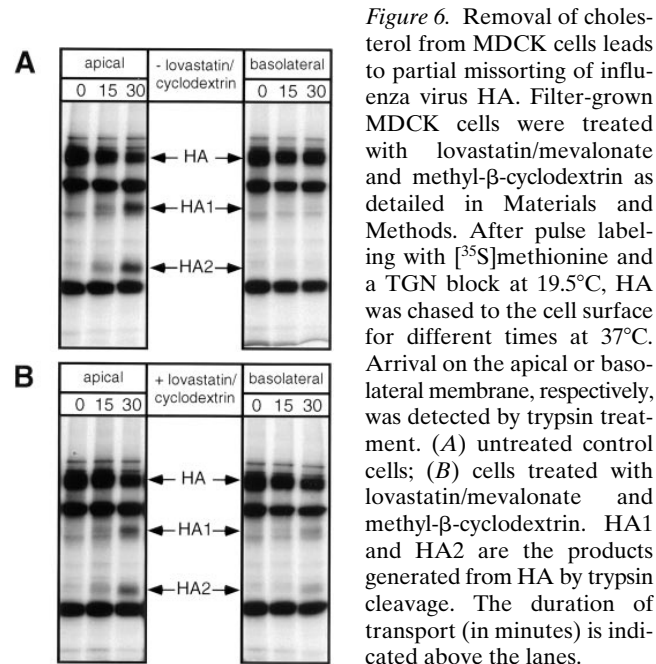
treated control cells 63% of the total amount of HA was cleaved from the apical side whereas only ~1% was accessible to trypsin on the basolateral side. These data clearly show that the junctional complexes were left intact, still functioning as a fence for molecules of at least the size of trypsin. In addition they show that whereas overall surface arrival of HA was only slightly reduced (53 vs. 64%) in cholesterol-depleted cells, 40% of surface HA (or 21% of total cellular HA) was missorted to the basolateral membrane after 30 min of transport.

Removal of Cholesterol Renders Influenza Virus HA Triton X-100 Soluble

Would a reduction of apical transport by depletion of cholesterol be accompanied by a dissociation of HA from sphingolipid–cholesterol rafts? Triton X-100 insolubility at 4°C has been extensively used to monitor the association of GPI-anchored proteins and of some transmembrane proteins with rafts (for review see Simons and Ikonen, 1997), and it has been shown that partitioning of newly synthesized HA into detergent-insoluble, glycolipid-enriched complexes (DIGs) occurs only after delivery to the late Golgi complex (Skibbens et al., 1989; Kurzchalia et al., 1992; Fiedler et al., 1993). Like BHK cells transiently transfected with HA (Scheiffele et al., 1997), treatment of virally infected BHK as well as MDCK cells with lovastatin/mevalonate and methyl-β-cyclodextrin rendered complex glycosylated HA dramatically less Triton X-100 insoluble (Fig. 7). Thus, as judged by the increased Triton X-100 solubility of HA, there was indeed a disruption of the interaction of HA with rafts.

Removal of Cholesterol Leads to Missorting of gp-80

Finally, we wondered whether depletion of cholesterol would also affect the polarized secretion of the apical secretory glycoprotein gp-80. As inhibition of *N*-glycosyla-
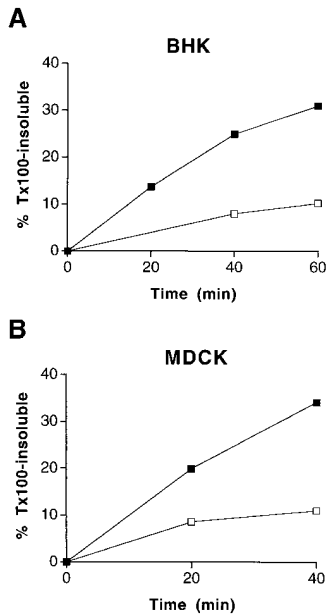
A

BHK

B

MDCK

Figure 7. Treatment of BHK and MDCK cells with methyl-β-cyclodextrin leads to increased Triton X-100 solubility of influenza virus HA. BHK cells (*A*) and filter-grown MDCK cells (*B*) were treated with lovastatin/mevalonate and methyl-β-cyclodextrin as detailed in Materials and Methods. The cells then were extracted on ice with 1% Triton X-100, followed by centrifugation at 120,000 *g* to obtain detergent-soluble and -insoluble fractions. ■, untreated control cells; □, cells treated with lovastatin/mevalonate and methyl-β-cyclodextrin.

A

- cyclodextrin

B

+ cyclodextrin

Figure 8. Removal of cholesterol from MDCK cells leads to missorting of gp-80. Filter-grown MDCK cells were treated with lovastatin/mevalonate and methyl-β-cyclodextrin as detailed in Materials and Methods. After pulse labeling for 15 min with [^{35}S]methionine and a TGN block at 19.5°C, gp-80 was allowed to be secreted for 20 and 40 min at 37°C, and was recovered from apical and basolateral media, as well as from the cells by immunoprecipitation. The immunoprecipitates were analyzed by nonreducing SDS-PAGE, followed by quantitation with a PhosphorImager. (*A*) Untreated control cells. (*B*) Cells treated with lovastatin/mevalonate and methyl-β-cyclodextrin. ■, gp-80 secreted apically; □, gp-80, secreted basolaterally.

tion by tunicamycin leads to randomized secretion from MDCK cells (Urban et al., 1987), the carbohydrate moiety may be recognized by a lectin-like sorting receptor, possibly raft-associated VIP36 (Fiedler et al., 1994), which would concentrate the protein in apical carrier vesicles.

In untreated control cells, newly synthesized gp-80 was preferentially secreted apically (Fig. 8 *A*). In MDCK cells treated with lovastatin/mevalonate and methyl-β-cyclodextrin gp-80 secretion into the apical medium decreased and basolateral secretion increased, thus inverting the polarity of secretion (Fig. 8 *B*).

Importantly, the observed missorting cannot be due to diffusion of gp-80 from the apical into the basolateral medium caused by the opening up of the junctional complexes, since there was no change in transepithelial resistance after cholesterol depletion (see above). Moreover we have shown that the comparatively small molecule trypsin did not leak into the other compartment under these conditions (see above).

Taken together, these data indicate that removal of 60–70% of total cellular cholesterol leads to a disruption of the interaction of HA with rafts and causes a dramatic reduction of transport of HA to the apical surface in MDCK cells. Furthermore there is a substantial amount of HA as well as of gp-80 being missorted to the basolateral surface. The basolateral marker protein VSV G is not affected under the same conditions.

Discussion

Our data provide direct evidence for the functional significance of cholesterol as a component of sphingolipid–cholesterol rafts in membrane trafficking. They clearly demonstrate that reducing the cholesterol level of living cells leads to a slowing down of TGN-to-surface transport of influenza virus HA, whereas transport of the basolateral marker VSV G is not affected. Reduction of transport of HA is accompanied by an increased solubility in the detergent Triton X-100. Cholesterol depletion specifically af-
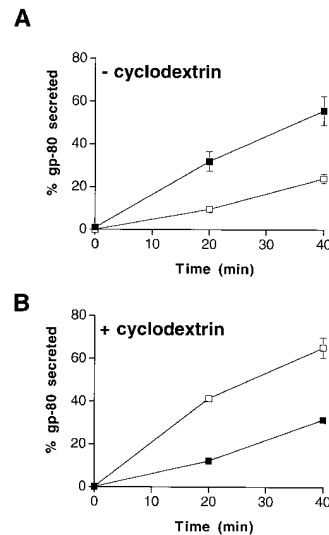
fects HA-containing TGN-derived transport vesicles, but has no effect on ER-to-Golgi transport of HA, demonstrating that rafts become important only in the Golgi complex. Moreover, reduction of the cellular cholesterol level also leads to basolateral secretion of the apical secretory glycoprotein gp-80.

To find optimal conditions to remove cholesterol from cells, we performed an extensive series of pharmacological studies. The difficulty was to establish the window in which enough cholesterol had been depleted to see the resulting effects without affecting the viability of the cells. If too much cholesterol was removed from the cells the cellular machinery responsible for biosynthetic transport stopped functioning. Therefore we had to leave 30–40% of the cellular cholesterol, and this level of cholesterol allowed some apical transport to occur. We combined two different ways to rapidly and efficiently remove cholesterol, i.e., inhibiting the new synthesis of cholesterol by lovastatin and extraction of cholesterol from the plasma membrane with methyl-β-cyclodextrin. Extraction of mouse L cells and Fu5AH cells with 100 mM 2-hydroxypropyl-β-cyclodextrin revealed a rapidly extractable ($t_{1/2} = 19$–20 s) and a slowly extractable pool ($t_{1/2} = 15$–28 min) of cellular cholesterol, both of which are thought to reside in the plasma membrane (Yancey et al., 1996). Cholesterol continuously cycles between the plasma membrane and the ER, such that there is always a substantial amount of cholesterol in intracellular membranes (for review see Liscum and Underwood, 1995). Since treatment with methyl-β-cyclodextrin for longer times displayed more dramatic effects on TGN-to-surface transport of HA, and since filipin staining suggested that at least a partial removal of intracellular cholesterol had occurred, it seems likely that surface as well as intracellular cholesterol pools needed to be depleted to get maximal effects.

Lovastatin treatment alone reduced cellular cholesterol levels by only 10%. Nevertheless, lovastatin treatment for 48 h significantly increased the effect of methyl-β-cyclo-

dextrin on TGN-to-surface transport of HA. It's possible that lovastatin had altered the intracellular distribution of cholesterol, possibly reducing the amount in the Golgi complex. This would be in agreement with data showing that cholesterol deprivation affected the fluorescence properties of C_6-NBD-ceramide in the Golgi apparatus of living cells (Martin et al., 1993). The overall Golgi structure however seemed to be unchanged after treatment with lovastatin/mevalonate and methyl-β-cyclodextrin, as judged by the fluorescence pattern obtained from green fluorescent protein fused to VSV G (Keller, P., and J. White, unpublished results).

Depletion of cholesterol not only leads to reduced TGN-to-surface transport of HA but also to a substantial amount of missorting in MDCK cells. Similarly, expression of HA at very high levels often leads to the appearance of HA on the basolateral side (Matlin and Simons, 1984). Is this caused by mistargeting of apical transport vesicles to the basolateral side or rather by HA molecules being incorporated into basolateral vesicles? There is no direct answer to this question, however, considering how clathrin-coated pits exclude or include proteins may be helpful.

GPI-anchored Thy-1 and HA are associated with rafts and are efficiently excluded from coated pits (Bretscher et al., 1980; Roth et al., 1986). Based on these observations, it was suggested that coated pits would act as molecular filters, selecting some proteins for entry and excluding others (Bretscher et al., 1980). Several HA mutants with cytoplasmic domains into which internalization signals have been introduced are known to be endocytosed (Roth et al., 1986; Lazarovits and Roth, 1988; Zwart et al., 1996). Recently a HA mutant has been described in which four amino acids at the exoplasmic boundary of the transmembrane domain had been changed to alanines. Although this mutant did not contain any cytoplasmic internalization signal, it was internalized at a rate close to the constitutive uptake of membrane lipid by coated pits in fibroblasts (Lazarovits et al., 1996). Interestingly, this mutant was also no longer raft associated since it was fully soluble in Triton X-100 at 4°C (Scheiffele et al., 1997). Thus, dissociation from rafts allowed this molecule to enter clathrin-coated pits even in the absence of a distinct coated pit localization signal. It is therefore possible that HA similarly has the potential to enter basolateral transport vesicles once it is no longer associated with rafts, i.e., after depletion of cholesterol. It would thus travel as a "stowaway" in basolateral vesicles to the cell surface.

Another explanation for how missorting could occur comes from the finding that missorting after cholesterol depletion is also observed for the apical secretory glycoprotein gp-80. Since gp-80 cannot directly interact with raft lipids, we believe that the association of gp-80 with rafts is mediated by a lectin-like sorting receptor, possibly VIP36 (Fiedler et al., 1994). Cholesterol depletion probably would break the interactions of this receptor with rafts, and therefore would indirectly also affect sorting of gp-80. Our observations are in contrast to a recent report suggesting that sorting of gp-80 was a raft-independent process (Graichen et al., 1996). This conclusion was drawn from the fact that gp-80 could not be detected in the detergent-insoluble pellet after extraction with Triton X-100 at

pH 7.5. Data from our laboratory however indicate that gp-80 is found in the CHAPS-insoluble complex after extraction at pH 6.2, approximately the pH of the TGN (Scheiffele, P., and K. Simons, unpublished results).

Thus the observed missorting of HA and gp-80 could be caused by cholesterol depletion leading to missorting of the apical sorting receptor, which then would direct apical glycoproteins basolaterally.

Depletion of cholesterol from MDCK cells did not cause missorting of the GPI-anchored protein gD1-DAF (Hannan and Edidin, 1996). In this study, however, cholesterol levels were reduced by only 25% after 3 d of low density lipoprotein (LDL) deprivation, as compared with 60–70% using the short-term treatment with methyl-β-cyclodextrin as described in our report. We have attempted to look at the transport of PLAP in MDCK cells. Although proper quantitation of TGN-to-surface transport in pulse-chase experiments was not possible because of a low signal-to-noise ratio, there was clear missorting of PLAP to the basolateral surface after 40 min of transport (data not shown). From these studies it is clear that only extensive depletion of cholesterol allows normally raft-associated proteins to be distributed basolaterally.

The association of proteins to rafts is due to cooperative interactions between proteins, cholesterol and sphingolipids. Therefore several groups have attempted to study the role of sphingolipids in vesicular transport. Inhibition of sphingolipid synthesis by the fungal metabolite fumonisin caused partial missorting of the GPI-linked protein GP-2 in MDCK cells (Mays et al., 1995). Since ceramide can also act as a second messenger (Hannun, 1994), it is difficult to differentiate the effects of inhibition of sphingolipid synthesis on raft function and on signaling. Interestingly, another inhibitor of sphingolipid synthesis, 1-phenyl-2-(decanoyl-amino)-3-morpholino-1-propanol (PDMP), caused a reduction of transport of VSV G throughout the whole biosynthetic pathway (Rosenwald et al., 1992). PDMP retarded transport immediately after addition and therefore cannot represent effects caused by changes in the mass of cellular sphingolipids. PDMP however is known to increase levels of N,N-dimethylsphingosine (Felding-Habermann et al., 1990; Igarashi et al., 1990), an inhibitor of protein kinase C (Hakomori, 1990), and therefore may exert its effect on vesicular transport by altering protein kinase C metabolism. Indeed it has recently been shown that N,N-dimethylsphingosine potently inhibited the release of post-Golgi vesicles from purified Golgi fractions of VSV G–infected MDCK cells (Simon et al., 1996). We also have attempted to deplete sphingolipids by using either CHO-SPB1 cells, a temperature-sensitive mutant of sphingolipid biosynthesis (Hanada et al., 1990), or by treatment of BHK cells with fumonisin. The data we have obtained for transport of HA and VSV G were more consistent with an effect of sphingolipid depletion on second messengers (Keller, P., and K. Simons, unpublished results). Thus it is likely that these means to inhibit sphingolipid synthesis exert their effects by both altering raft function and second messenger pathways.

In this paper we have provided concrete evidence for the functional significance of rafts in membrane trafficking. We postulate that one of the main roles of cholesterol in mammalian cells is to function as a co-organizer of sphin-

golipid–cholesterol domains. A challenge for the future will now be to integrate the lipid organization of membranes into the rapidly progressing research on protein involvement in membrane structure and function.

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