RESEARCH COMMUNICATION

Cholesterol depletion reduces apical transport capacity in epithelial Madin–Darby canine kidney cells

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Reduction of the cholesterol level in membranes of epithelial Madin–Darby canine kidney (MDCK) cells reverses the apical-to-basolateral transport ratio of the apical membrane marker protein influenza virus haemagglutinin and the secreted glycoprotein gp80. At the same time, basolateral transport of the vesicular stomatitis virus G protein is unaffected [Keller and Simons (1998) *J. Cell Biol.* **140**, 1357–1367]. To investigate whether cholesterol depletion influences apical sorting mechanisms specifically, or apical transport capacity more generally, we studied the effect of cholesterol depletion on the secretion of three different classes of molecules from the apical and basolateral surfaces of MDCK cell layers: glycoprotein

gp80, sulphated proteoglycans and proteins, and non-glycosylated rat growth hormone. In each case, cholesterol depletion reduced the fraction secreted to the apical medium and increased the fraction secreted basolaterally. The fact that this was observed for all sulphated proteins and proteoglycans and for the non-glycosylated rat growth hormone, which is randomly secreted in untreated cells, indicates that cholesterol depletion reduces the apical transport capacity, rather than interfering with specific recognition and sorting processes.

Key words: apical secretion, basolateral secretion, epithelial MDCK cells, lipid rafts.

INTRODUCTION

Correct sorting and transport of newly synthesized proteins to specialized plasma membrane domains in epithelial and other polarized cell types is a fundamental requirement for the generation and maintenance of cellular polarity. In Madin–Darby canine kidney (MDCK) cells, sorting signals in basolateral membrane proteins have been localized to their cytoplasmic tails. Apical sorting information has been localized to N-glycans [1–4], O-glycans [5], chondroitin sulphate chains [6] and proteinaceous domains [7,8]. A model for sorting and delivery of proteins from the *trans*-Golgi network (TGN) to the apical plasma membrane is based on the formation of membrane lipid microdomains rich in glycosphingolipids and cholesterol, which have been given the popular name ‘rafts’. According to this model, apical proteins partition into rafts by means of direct protein–lipid interactions [9], or indirectly by protein–protein or protein–carbohydrate interactions, and become enriched in raft-based apical transport containers as these are formed and pinch off from the TGN [10,11]. Important proteins that could participate in the apical sorting machinery are VIP17/MAL [12], VIP21/caveolin-1 [13–15] and annexin XIIIb [16].

One way to study the role of rafts in apical transport is to deplete the cells of one of the major raft components, cholesterol. Such treatment has been shown to reverse the transport polarity from predominantly apical to basolateral of both the apical membrane marker influenza virus, haemagglutinin, and the secreted glycoprotein, gp80 [17]. The resulting effect of cholesterol depletion on apical transport of these proteins could either result from a reduction in the efficiency of apical sorting mechanisms or, alternatively, an inhibition of the general apical transport

capacity. To distinguish between these possibilities, we investigated the effect of cholesterol depletion on secretion of gp80, a glycoprotein which contains apical sorting information [1], on sulphated proteins and proteoglycans (PGs), and on the randomly secreted non-glycosylated protein, rat growth hormone (rGH) [2,18]. Our results indicate that cholesterol depletion changes the apical-to-basolateral secretion ratio of both sorted and non-sorted proteins by reducing the apical secretion capacity.

MATERIALS AND METHODS

Materials

Media and reagents for cell culture were purchased from Gibco BRL and BioWhittaker (Verviers, Belgium). Lovastatin was provided by R. Lueddecke (Merck Sharp and Dohme, Haar, Germany) or purchased from Calbiochem, and was prepared as a 20 mM stock solution, as described in [19]. Methyl- β -cyclodextrin and mevalonate were obtained from Sigma, and Protein A–Sephacrose CL-4B, [³⁵S]sulphate and [³⁵S]cysteine/methionine were from Amersham Pharmacia Biotech. Rabbit polyclonal antibodies against gp80 [1] and rGH (Biogenesis, Poole, Dorset, U.K.) were used. SDS/PAGE gels were either made with SDS from Bio-Rad Laboratories, bis/polyacrylamide solution from Saveen biotech AB, Malmö and other reagents from Sigma, or purchased as gradient gels from Bio-Rad.

Cell culture, cholesterol depletion and metabolic labelling

MDCK II cells transfected with cDNA for wild-type rGH, previously characterized in [2], were used. These cells secrete

Abbreviations used: GPI, glycosylphosphatidylinositol; rGH, rat growth hormone; PG, proteoglycan; TGN, *trans*-Golgi network; MDCK, Madin–Darby canine kidney; gp80, glycoprotein 80.

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the non-glycosylated form of rGH to both the apical and the basolateral medium. Cells were grown and seeded on to filters, as previously described for MDCK II cells [20,21]. Cholesterol depletion was carried out essentially as described in [17]. All incubations were at 37 °C in a CO₂ incubator. MDCK II cells were seeded on to Costar polycarbonate (0.4 µm pore size) Transwell filters (4.7 cm²) in minimal essential medium supplemented with 10% fetal calf serum, 2 mM glutamine, 100 units of penicillin, and 100 µg/ml streptomycin at a density of 1:6 of a 75 cm² flask per filter. After 24 h, new medium was added (1 ml apically and 2 ml basolaterally), in the presence or absence of 4 µM lovastatin and 0.25 mM mevalonate. After 48 h, the cells on filters were washed twice by dipping in pre-warmed PBS and extracted by 10 mM methyl-β-cyclodextrin for 60 min in metabolic labelling medium (medium without sulphate or cysteine/methionine and with dialysed serum) in the presence of the metabolic marker substance (0.3 mCi/ml of either [³⁵S]sulphate or [³⁵S]cysteine/methionine). The metabolic labelling was carried out for another 60, 90 or 120 min in the presence of [³⁵S]sulphate or [³⁵S]cysteine/methionine, but all filters were now without cholesterol depletion, since a longer extraction period would stop cellular transport functions. (The cellular cholesterol level does not return to normal during the 120 min chase.) The apical and basolateral media were subjected to centrifugation to remove loose cells and analysed with respect to their content of secreted molecules.

Analysis of secretion of sulphate-labelled macromolecules

Medium aliquots (1 ml) were chromatographed on 4 ml Sephadex G-50 fine (Amersham Pharmacia Biotech) columns. Only ³⁵S-macromolecules were recovered in the 1.5 ml eluted with 0.05 M Tris/HCl (pH 8.0) with 0.15 M NaCl. The total activity in the fractions was determined by scintillation counting of 30 µl of the 1.5 ml total, and aliquots representing equal volumes were loaded on SDS/PAGE 4–12% gradient mini-gels.

Immune precipitation of gp80 and rGH

Medium samples were collected at the end of the 3 h methyl-β-cyclodextrin chase period, where the cells were incubated with 0.3 mCi/ml of [³⁵S]cysteine/methionine. The medium was centrifuged to pellet eventual loose cells. Cysteine and methionine (up to 5 mM) were added to the supernatants (1 ml) and incubated overnight in the cold with 2 µl of antiserum against either gp80 or rGH. To the incubations were added 50 µl of a 1:1 slurry of Protein A–Sepharose CL-4B (Amersham Pharmacia Biotech), and washed and processed for SDS/PAGE as previously described [17,22]. The extent of protein secretion was visualized by autoradiography. rGH secretion was quantified from autoradiographs by densitometric scanning in a Molecular Dynamics Personal Densitometer SI (Molecular Dynamics, Sunnyvale, CA, U.S.A.).

RESULTS AND DISCUSSION

The effect of cholesterol depletion on apical secretion of sorted and non-sorted proteins was studied by following three different markers. First, we repeated the experiments performed earlier [17] to confirm that cholesterol depletion reverses the polarity of secretion of gp80, which is secreted predominantly at the apical membrane of MDCK cells. Also, in the MDCK II cell line expressing rGH cDNA, cholesterol depletion reduced the

Figure 1 Effect of cholesterol depletion on apical and basolateral secretion of gp80

MDCK II-rGH cells were grown to confluency on polycarbonate filters for 4 days. From day 2 the cells were subjected to cholesterol depletion treatment and metabolic labelling with [³⁵S]sulphate on day 4, as described in the Materials and methods section. Secretion of gp80 was quantified by immunoprecipitation. A representative SDS/PAGE pattern of gp80 secreted apically (**A**) and basolaterally (**B**) from MDCK II-rGH filter-grown monolayers is shown (three similar experiments were performed). The apical and basolateral media were harvested 60, 90 and 120 min (as indicated) after the end of the 60 min in the absence (control) or presence (depleted) of methyl-β-cyclodextrin.

apical secretion of gp80 and increased its basolateral secretion (Figure 1).

We next studied the effect of cholesterol depletion on secretion of sulphated proteins and PGs secreted to the apical and basolateral media. Sulphation of proteins and PGs takes place exclusively in the Golgi apparatus on tyrosine residues in the polypeptide chain or on sugar moieties. Thus only macromolecules that exit from the Golgi apparatus are metabolically labelled with [³⁵S]sulphate. Following the effect of cholesterol depletion on secretion of ³⁵S-sulphated macromolecules to the apical and basolateral media would thus indicate how secretion from the TGN is affected by this treatment. As shown in Figure 2, cholesterol depletion reduced the apical secretion, and an increase in the basolateral secretion of all sulphated macromolecules visible by SDS/PAGE. This may indicate that cholesterol depletion has a general, and opposite, effect on secretion to the apical and basolateral surfaces of MDCK cells. There was, on average, a 56% reduction in apical secretion and a 21% increase in basolateral secretion of sulphated macromolecules, as determined by scintillation counting after purification by G-50 fine gel filtration. A slight delay in the overall secretion led to some accumulation of sulphated macromolecules in the cells depleted of cholesterol. However, since subsets of sulphated molecules may be sorted in MDCK cells [6], for instance among the different PGs in the upper region of the SDS/PAGE gel, we wanted to investigate the effect of cholesterol depletion on the secretion of a single non-sorted, non-glycosylated protein, rGH. This protein is secreted to the same extent from both sides of epithelial MDCK cell layers and is thus not subject to any sorting in the TGN [2,18].

We found that upon cholesterol depletion there was a marked shift towards more basolateral secretion of rGH (Figure 3). Thus cholesterol depletion influences the apical-to-basolateral secretion ratio of molecules, both with and without apical sorting information. It has previously been shown in another epithelial cell line [23], that the apical sorting capacity of two secretory proteins may be exceeded by increasing their expression level. In the present paper the expression level was kept constant, whereas the apical targeting capacity was reduced.

Figure 2 Effect of cholesterol depletion on apical and basolateral secretion of sulphated macromolecules

MDCK II-rGH cells were grown to confluency on polycarbonate filters for 4 days. The cells were subjected to cholesterol depletion treatment from day 2 and metabolic labelling with [35 S]sulphate on day 4, as described in the Materials and methods section. The apical and basolateral media were harvested 60, 90 and 120 min (as indicated) after the end of the 60 min in the absence (control) or presence (depleted) of methyl- β -cyclodextrin. Secretion of 35 S-sulphated macromolecules apically (**A**) and basolaterally (**B**) from MDCK II-rGH filter-grown monolayers was visualized by SDS/PAGE after removal of free [35 S]sulphate by Sephadex G-50 chromatography. (**A**, **B**) Apical and basolateral media from one representative experiment is shown (three similar experiments were performed).

Ordered lipid domains play important roles in cellular processes [10]. Lipid microdomains (or 'rafts'), containing glycosylphosphatidylinositol (GPI)-anchored proteins, cholesterol and glycosphingolipids, have been shown by direct methods to exist in the plasma membrane [24–26], and by indirect methods to exist in endosomes [27] and the Golgi apparatus [10,17,28] of mammalian cells and also in the endoplasmic reticulum of yeast cells [29]. Plasma membrane rafts are disrupted by cholesterol depletion [24,25], a treatment that also affects the putative functions of rafts in endosomes [27,30] and the TGN [17,28].

Figure 3 Effect of cholesterol depletion on apical and basolateral secretion of rGH

MDCK II-rGH cells were grown to confluency on polycarbonate filters for 4 days. From day 2 the cells were subjected to cholesterol depletion treatment, and metabolic labelling with [35 S]cysteine/methionine on day 4, as described in the Materials and methods section. rGH was quantified by immunoprecipitation. SDS/PAGE patterns of rGH secreted apically (**A**) and basolaterally (**B**) from MDCK II-rGH filter-grown monolayers in one representative experiment is shown (three similar experiments were performed). The apical and basolateral media were harvested 60, 90 and 120 min (as indicated) after the end of the 60 min in the absence (control) or presence (depleted) of methyl- β -cyclodextrin. (**C**) Densitometric quantification of rGH secretion from MDCK II-rGH monolayers 60, 90 and 120 min after the 60 min in the absence (control) or presence (depleted) of methyl- β -cyclodextrin. Quantification from X-ray films of apical and basolateral secretion of rGH is shown. Secretion is expressed in arbitrary units as a function of incubation time, where 1 represents secretion from control cells 60 min after the end of the 60 min in the absence (control) or presence (depleted) of methyl- β -cyclodextrin (see the Materials and methods section). Error bars show the range for the experimental values from three different experiments. (x) Secretion from control cells, (■) secretion from cholesterol-depleted cells.

At least two constitutive exocytic routes exit from the TGN towards the plasma membrane in eukaryotic cells [31]. These may be raft-dependent or raft-independent [10,31]: in epithelial

MDCK cells and enterocytes, the basolateral route is raft-independent, whereas the apical route is raft-dependent [17,28]. GPI-linked proteins are mainly transported to the apical membrane in MDCK cells [32] and fulfil the biochemical criteria for classification as raft proteins, by their resistance to solubilization by Triton X-100 at 4 °C [33]. It has been assumed that the GPI anchor itself acts as an apical determinant by its interaction with rafts. The role of GPI anchors in apical sorting has been questioned, however, by the fact that the protein part alone of such proteins is often secreted apically [32,34,35], although not always [36], and that the concentration of GPIs without attached protein is similar in the apical and the basolateral membrane [37]. It has been shown that expressing non-glycosylated rGH with a GPI anchor in MDCK cells resulted in transport to both the apical and basolateral sides of MDCK cell layers, whereas insertion of N-glycan sites into the protein gave predominantly apical transport [3]. This is in agreement with what has been observed for the secreted variant of rGH [2,18], and for other proteins with N-linked [1,4] and O-linked sugars [5,38].

A postulated mechanism by which glycoproteins could utilize rafts to accumulate in apical transport carriers is via lectin receptors [10]. Certain lectins would be recruited to rafts via their transmembrane domains, as has been shown for other proteins [9,39], or via acyl anchors [40]. This would again lead to enrichment of the glycanated lectin ligands in apical carriers that are derived from rafts in the Golgi apparatus. Disruption of rafts by cholesterol depletion would randomize the distribution of lectins and their glycoprotein ligands over the whole *trans*-Golgi membrane, leading to reduced concentration of apical proteins in apical carriers and an increase in basolateral carriers. Such an effect of cholesterol depletion on lectin-based sorting should, however, not alter the apical-to-basolateral transport ratio of the non-glycosylated rGH.

Raft-like domains seem to form early in the secretory pathway [29], and either raft-association or glycanation is of importance for anterograde transport of several proteins through the Golgi apparatus to the cell surface [3]. Both the apical and the basolateral membranes of MDCK cells contain raft lipids, but these are enriched in the apical membrane [41]. Our results indicate that cholesterol depletion reduces the overall transport capacity in the direction of the apical membrane, either by a reduction in carrier size or number.

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