

Hierarchy of Mechanisms Involved in Generating Na/K-ATPase Polarity in MDCK Epithelial Cells

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Abstract. We have studied mechanisms involved in generating a polarized distribution of Na/K-ATPase in the basal-lateral membrane of two clones of MDCK II cells. Both clones exhibit polarized distributions of marker proteins of the apical and basal-lateral membranes, including Na/K-ATPase, at steady state. Newly synthesized Na/K-ATPase, however, is delivered from the Golgi complex to both apical and basal-lateral membranes of one clone (II/J), and to the basal-lateral membrane of the other clone (II/G); Na/K-ATPase is selectively retained in the basal-lateral membrane resulting in the generation of complete cell surface polarity in both clones. Another basal-lateral membrane protein, E-cadherin, is sorted to the basal-lateral membrane in both MDCK clones, demonstrating that there is not a general sorting defect for basal-lateral membrane proteins in clone II/J cells. A glycosyl-phosphatidylinositol (GPI)-anchored protein (GP-2) and a glycosphingolipid (glucosylceramide, GlcCer) are preferentially transported to the apical membrane in clone II/G cells, but, in clone II/J cells, GP-2 and

GlcCer are delivered equally to both apical and basal-lateral membranes, similar to Na/K-ATPase. To examine this apparent inter-relationship between sorting of GlcCer, GP-2 and Na/K-ATPase, sphingolipid synthesis was inhibited in clone II/G cells with the fungal metabolite, Fumonisin B₁ (FB₁). In the presence of FB₁, GP-2 and Na/K-ATPase are delivered to both apical and basal-lateral membranes, similar to clone II/J cells; FB₁ had no effect on sorting of E-cadherin to the basal-lateral membrane of II/G cells. Addition of exogenous ceramide, to circumvent the FB₁ block, restored GP-2 and Na/K-ATPase sorting to the apical and basal-lateral membranes, respectively. These results show that the generation of complete cell surface polarity of Na/K-ATPase involves a hierarchy of sorting mechanisms in the Golgi complex and plasma membrane, and that Na/K-ATPase sorting in the Golgi complex of MDCK cells may be regulated by exclusion from an apical pathway(s). These results also provide new insights into sorting pathways for other apical and basal-lateral membrane proteins.

POLARIZED epithelial cells form boundaries between different biological compartments and regulate the ionic composition of those compartments by vectorial transport of ions and solutes (Rodriguez-Boulant and Nelson, 1989). Restriction of ion pump and transporter protein distributions to specific plasma membrane domains, termed apical and basal-lateral, which face these different compartments, is critical for these vectorial transport functions (Almers and Stirling, 1984).

Recent studies have sought to define mechanisms involved in sorting proteins to these different membrane do-

main. In MDCK cells, sorting of one class of apical protein in the TGN may be mediated by a glycosyl-phosphatidylinositol (GPI)¹ anchor (Brown et al., 1989; Lisanti et al., 1989). Since GPI-anchored proteins do not contain cytoplasmic sorting information, they may be sorted to the apical membrane through an association with clusters or rafts of glycosphingolipids (GSLs) (Simons and Wandinger-Ness, 1990), which are enriched in the apical membrane of polarized epithelial cells (Simons and van Meer, 1988). GSLs cluster through intermolecular hydrogen bonding, which segregates GSLs from glycerolipids in lipid bilayers

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1. *Abbreviations used in this paper:* FB₁, Fumonisin B₁; GPI, glycosyl-phosphatidylinositol; GSLs, glycosphingolipids; GlcCer, glucosylceramide; PI-PLC, phosphatidylinositol-specific phospholipase C; TER, transepithelial electrical resistance; C6-NBD-ceramide, N-6[7-nitro-2,1,3-benzoxadiazol-4-yl] aminocaproyl sphingosine galactoside; NBD-SM, N-6[7-nitro-2,1,3-benzoxadiazol-4-yl] aminocaproyl sphingomyelin.

(Thompson and Tillack, 1985; Rock et al., 1990), and may be sufficient to segregate GPI-anchored proteins from basal-lateral proteins in the TGN (Simons and van Meer, 1988; Simons and Wandinger-Ness, 1990). This notion is supported by the isolation of membrane complexes enriched in GSLs and GPI-anchored proteins from polarized epithelial cells (Brown and Rose, 1992; Garcia et al., 1993; Sargiacomo et al., 1993) and the detection of an immobile fraction of GPI-anchored proteins upon their arrival at the apical membrane (Hannan et al., 1993). For non-GPI-anchored apical proteins, sorting information in the extracellular domain may be recognized by an apical sorting receptor that interacts with GSLs (Simons and Wandinger-Ness, 1990).

Sorting signals for some basal-lateral membrane proteins are comprised of a specific, short cytoplasmic amino acid sequence located close to the transmembrane domain (reviewed in Rodriguez-Boulant and Powell, 1992). These signals were found to be colinear with the signal for endocytosis in the LDL and Fc receptors and IgG120 (Hunziker et al., 1991). Further analysis showed that the basal-lateral signal functionally overlaps the signal for endocytosis in some proteins (Tyr⁵⁴³ mutation of hemagglutinin [Brewer and Roth, 1991], lysosomal acid phosphatase [Prill et al., 1993], NGF receptor [Le Bivic et al., 1991], and Fc receptor [Matter et al., 1994], but not in others (poly IgA receptor [Casanova et al., 1991], LDL receptor [Matter et al., 1992]). Detailed analysis of LDL receptor showed that this basal-lateral sorting signal contains a Tyr residue and a cluster of three negatively charged amino acids, COOH-terminal to the critical Tyr (Matter et al., 1993; Matter et al., 1994). Removal of a basal-lateral sorting signal often results in transport of the protein to the apical membrane, indicating a hierarchy of sorting determinants (Mellman et al., 1993; Matter et al., 1994). At present, however, little is understood about the cellular machinery that decodes these signals.

In contrast to the direct sorting of proteins containing single membrane-spanning domains, the regulation of Na/K-ATPase localization to the basal-lateral membrane appears to be more complex. Previous studies demonstrated that in one clone of MDCK cells the polarized distribution of Na/K-ATPase was generated by differences in the rates of protein turnover at the apical and basal-lateral membranes (Hammerton et al., 1991; Siemers et al., 1993). Studies with another clone of MDCK cells indicated that newly synthesized Na/K-ATPase is delivered predominantly to the basal-lateral membrane (Gottardi and Caplan, 1993a).

To more completely address these differences in Na/K-ATPase sorting, and to better understand the mechanisms involved in sorting of proteins and GSLs, we compared the delivery of newly synthesized proteins and GSLs to the apical and basal-lateral domains in two independently isolated clones of MDCK cells (clone II/G and II/J). We find that in clone II/G cells, Na/K-ATPase is sorted to the basal-lateral membrane, and GlcCer and a GPI-anchored protein are sorted to the apical membrane. However, in clone II/J cells, we find that GlcCer, GP-2, and Na/K-ATPase are delivered to both apical and basal-lateral membranes. By inhibiting sphingolipid synthesis in clone II/G cells, we show that there is a direct inter-relationship

between sorting of GSLs, a GPI-anchored protein, and Na/K-ATPase. These results provide new insights into mechanisms that regulate Na/K-ATPase sorting and distribution, and indicate how sorting of other apical and basal-lateral proteins may be regulated in the Golgi complex of MDCK cells.

Materials and Methods

Cells and Tissue Culture Methodology

Madin-Darby canine kidney clone II/J cells were isolated in the Nelson laboratory from a single cell clone from the stock of MDCK cells obtained from The American Type Culture Collection (Gaithersburg, MD; Nelson and Veshnock, 1986); clone II/G cells were isolated independently (Gaush et al., 1966; Louvard, 1980) and have been grown and characterized in the van Meer laboratory. Based upon the criteria of ultrastructural organization, transepithelial electrical resistance (200–300 $\Omega \cdot \text{cm}^2$), and localization of Na/K-ATPase, ankyrin, fodrin, E-cadherin, and GP-135 by immunofluorescence, clone II/G and II/J cells are indistinguishable (Mays et al., 1995).

Cells were maintained in DME supplemented with penicillin, streptomycin, and kanamycin and 10% FBS. For experiments with FB₁ (25 $\mu\text{g}/\text{ml}$ for 48–72 h; Division of Food Sciences and Technology, CSIR, Pretoria, South Africa), the FBS concentration was lowered to 5%. Cells were routinely grown at low density on plastic dishes before generating confluent monolayers on Costar Transwell™ 0.45- μm filters coated with type I collagen (Hammerton et al., 1991; Siemers et al., 1993). Cells grown on filters for extended times were refed with the appropriate media every other day. Low passage replicates of the clones were propagated for 4–6 wk and then discarded.

To generate a confluent monolayer of “contact-naive” cells (Nelson and Veshnock, 1987), cells were grown at low density (2×10^6 cells/150-cm-diam dish) for 2 d. After light trypsinization, cells were centrifuged at 1,000 g for 5 min and resuspended in DME containing 5 μM Ca^{2+} supplemented with 10% FBS that had been exhaustively dialyzed against PBS (without Ca^{2+}). Approximately 2.5×10^6 cells in 1.5 ml of medium were added to a 2.4 cm² Costar Transwell™ filter coated with type I collagen; 2.5 ml of medium were added to the outside (basal-lateral) compartment of the filter. After 2–6 h at 37°C, by which time >95% of cells had attached to the filter, medium was replaced with DME/FBS containing 1.8 mM Ca^{2+} to induce synchronous cell-cell contacts across the monolayer (Hammerton et al., 1991; Siemers et al., 1993); this modification from a previous protocol (Nelson and Veshnock, 1987) minimizes the time that cells are exposed to low [Ca^{2+}] in the growth medium.

For metabolic labeling, cells were preincubated in DME/FBS in the absence of methionine (DME/FBS-met) for 15 min., and then in DME/FBS-met containing 250–500 μCi ³⁵S-methionine/cysteine (Dupont) for 1–3 h at 37°C (pulse period). Cells were rinsed twice in prewarmed DME/FBS containing a 10,000-fold excess of unlabeled methionine, and then incubated in that medium for different periods (chase period). After the chase period, cells were rinsed four times in Tris-saline at 4°C before processing for protein extraction (see below).

Stable Transfection of MDCK II/J and II/G Cells with GP-2 cDNA

A cDNA for rat GP-2 was obtained by screening a rat pancreas $\lambda\text{gt}11$ library (Clontech, Palo Alto, CA) with a monoclonal anti-GP-2 antibody and subcloned into the eukaryotic expression vector, pRC-CMV (Invitrogen, San Diego, CA). The resulting expression vector contained the GP-2 gene preceded by the CMV promoter and a neomycin resistance gene. The pRC-CMV (GP-2) vector was transfected into clone II/J and II/G MDCK cells using the Lipofectin Reagent (GIBCO BRL, Gaithersburg, MD) and stable transformants were selected with G418 (GIBCO BRL) at a concentration of 0.4 mg/ml. G418 resistant cells were isolated by limiting dilution and screened by immunoprecipitation of GP-2 from ³⁵S-methionine/cysteine labeled cells (for example see Fig. 1).

Antibodies

The polyclonal antibody raised against β subunit of canine renal Na/K-ATPase was previously generated and characterized (Marrs et al., 1993). A rabbit polyclonal antibody was raised against the H5 cytoplasmic loop

of sheep α subunit Na/K-ATPase expressed as a glutathione-S-transferase fusion protein (a generous gift of Dr. Robert Farley, University of Southern California; for details see Piepenhagen et al., 1995). An antibody raised against the cytoplasmic domain of E-cadherin has been described previously (Marrs et al., 1993). A murine anti-rat GP-2 monoclonal antibody, 4A9 (IgG1 subclass), was generated as previously described (Lowe et al., 1994).

Figure 1 shows the specificity of antibodies used in the analysis of protein trafficking in this study. Cells were metabolically labeled with ^{35}S -methionine/cysteine and proteins were immunoprecipitated with either specific antibody (Ab), or the corresponding preimmune serum (Pi), or a non-specific monoclonal antibody (NS). The antibody to E-cadherin immunoprecipitates E-cadherin (*; ~120 kD), and coimmunoprecipitates β -catenin (94 kD); the antibody to α subunit Na/K-ATPase immunoprecipitates α subunit Na/K-ATPase (*; 100 kD); the antibody to β subunit Na/K-ATPase immunoprecipitates several proteins in the range of 35–50 kD (*) that represent different stages of glycosylation of β subunit Na/K-ATPase; the antibody to GP-2 immunoprecipitates a major ~85-kD protein (*) and an incompletely glycosylated 70-kD GP-2 that can be chased to the 85-kD form in nonradioactive medium (Fritz, B. A., and A. W. Lowe, manuscript submitted for publication).

Domain Specific Biotinylation of the Plasma Membrane of MDCK Cells Growing on Filters

Proteins on the cell surface of MDCK cells were biotinylated as described previously (Hammerton et al., 1991; Siemers et al., 1993). Briefly, confluent monolayers of MDCK cells on Costar Transwell™ filters were washed twice in ice-cold Ringer's saline containing 2 mM Ca^{2+} . Either 300 $\mu\text{g}/\text{ml}$ NHS-SS-Biotin (Pierce Chemical Co., Rockford, IL) or 200 $\mu\text{g}/\text{ml}$ sulfo-NHS-biotin (Pierce), prepared immediately before use in Ringer's saline containing 2 mM Ca^{2+} , was added to either the apical (400 μl) or basal-lateral (800 μl) compartment of the filter, and the cells were incubated for 30 min at 4°C with constant rocking; Ringer's saline containing 2 mM Ca^{2+} was added to the compartment that did not contain the biotinylating reagent. The biotinylation reaction was quenched by washing cells in four changes of Tris-saline at 4°C. The presence of functional tight junctions across the monolayer was assessed by measuring the diffusion of ^3H -inulin (DuPont NEN, Boston, MA) from the apical to the basal-lateral compart-

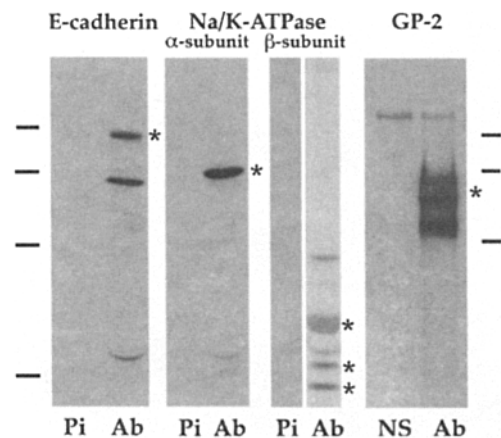


Figure 1. Characterization of antibody specificity by protein immunoprecipitation. MDCK cells were metabolically labeled, extracted, and proteins were immunoprecipitated with either specific antibody (Ab), the corresponding preimmune serum (Pi), or a nonspecific monoclonal antibody (NS); the proteins were separated by SDS-PAGE and processed for fluorography (see Materials and Methods). Antibodies specific for E-cadherin, α or β subunits of Na/K-ATPase, or GP-2 were analyzed. All antibodies are highly specific and immunoprecipitate the corresponding antigen (*); see text for details. Molecular weight standards are shown on the left for immunoprecipitates of E-cadherin, and α and β subunit Na/K-ATPase, and on the right for the GP-2 immunoprecipitate: β -galactosidase (116 kD); phosphorylase b (97 kD); bovine serum albumin (68 kD); left only, ovalbumin (45 kD).

ment during the incubation with biotinylated cross-linking reagents. Only filters which inhibited diffusion of >99.5% of tracer were used further.

Immunoprecipitation

Cells were extracted sequentially in an isotonic buffer containing 0.5% Triton X-100 at 4°C, followed by a buffer containing 1% SDS at 100°C, as described in detail previously (Hammerton et al., 1991; Siemers et al., 1993). Cell extracts (250–500 μl) were precleared with 10 μl of preimmune serum and 60 μl *Staphylococcus aureus* cells (Pansorbin; Calbiochem Novabiochem, La Jolla, CA). Primary antibody (10–20 μl) was added to the precleared extracts, and 60 min later immune complexes were precipitated with 50 μl of a suspension of Protein A-sepharose beads (Pharmacia LKB Nuclear, Gaithersburg, MD). Immunoprecipitates were washed under stringent conditions, as described previously (Pasdar and Nelson, 1989). Antibody-antigen complexes were dissociated and biotinylated proteins were re-precipitated with avidin-agarose (Pierce) and washed under stringent conditions, as described previously (Hammerton et al., 1991; Siemers et al., 1993). For immunoprecipitation of GP-2, cells were extracted in buffer containing 1% Triton X-114 as described in detail previously (Lisanti et al., 1988), and aqueous and detergent phases were formed by incubating the extract at 37°C for 30 min. After phase separation by centrifugation at 1,200 g for 5 min, GPI-linked proteins were released into the aqueous phase by incubation with 0.5 U/ml phosphatidylinositol-specific phospholipase C (PI-PLC; Oxford Glycosystems, Abingdon Oxon, UK). After a second phase separation, GP-2 was immunoprecipitated from the aqueous phase with GP-2 monoclonal antibody, as described above, and proteins were separated in SDS–7.5% polyacrylamide gels as described previously (Hammerton et al., 1991; Siemers et al., 1993). Amounts of labeled protein in the gels were determined directly using a Fujitas 2000 phosphorimager.

Analysis of Lipid Sorting

The lipid sorting assay using NBD-sphingolipids (van Meer et al., 1987; van't Hof and van Meer, 1990) was performed with the following modification. 5 μM C6-NBD-ceramide in liposomes was added to the apical surface in Hanks' buffered salt solution containing 1% BSA, while the basal-lateral surface was bathed in the same solution without NBD-ceramide. After 1 h at 37°C, the media were collected and both surfaces were washed with Hanks' buffered salt solution containing 1% BSA for 30 min at 10°C. Lipids were extracted from the collected apical and basal-lateral media, and separated by TLC. The amount of fluorescence in the various spots was quantitated as before (van Meer et al., 1987). The polarity of NBD-glucosylceramide (NBD-Cer) and NBD-sphingomyelin (NBD-SM) delivered to each membrane domain after synthesis in the Golgi complex was calculated by dividing the relative fluorescence of extracted lipids at the apical membrane by the relative fluorescence of the same lipid species at the basal-lateral membrane. Relative polarity was determined as the ratio of NBD-Cer polarity divided by the polarity of NBD-SM (van't Hof and van Meer, 1990).

To circumvent the FB_1 -induced block in sphingolipid synthesis, exogenous ceramide was added to cells during the incubation with FB_1 (see above). Liposomes containing 65 nmol/ml NBD-ceramide and 435 nmol/ml egg phosphatidylcholine (Avanti Polar Lipids Alabaster, AL) were prepared as follows. 3 ml 10 \times stock ceramide (650 nmol/ml) and phosphatidylcholine (4350 nmol/ml) in chloroform were dried under nitrogen. Lipids were resuspended in 3 ml DME and sonicated at 4°C for 5 min using the micro-tip of a Branson Sonifier at setting no. 4. Liposomes were diluted into 27 ml DME containing 5% FBS and 25 $\mu\text{g}/\text{ml}$ FB_1 and added to cells.

Metabolic Labeling of Glycolipids

Confluent monolayers of cells were labeled for 20 h in glucose-free media containing 10 $\mu\text{Ci}/\text{ml}$ ^3H -galactose (Dupont). At the end of the labeling period, cells were washed three times in PBS and then extracted twice in chloroform/methanol (2:1). Aqueous and solvent phases were partitioned by the addition of equal volumes of chloroform and 20 mM acetate. After 10 min at 22°C, the organic phase was removed and dried under nitrogen. Lipids are dissolved in two drops of chloroform/methanol and spotted on silica thin layer chromatography plates (Whatman Inc., Clifton, NJ). Lipids were separated in a solvent containing chloroform/methanol/0.2% CaCl_2 (60:35:8). The TLC plate was sprayed with Enhance (DuPont), and then exposed to x-ray film. Lipid standards (Avanti Polar Lipids) were separated under identical conditions as lipids extracted from MDCK cells,

and visualized on the TLC plate as previously described (van't Hof and van Meer, 1990). R_f values were determined by measuring the distance migrated by a lipid species relative to the distance between the solvent front and the origin.

Results

Na/K-ATPase Is Restricted to the Basal-Lateral Membrane Domain of Clone II/J and Clone II/G MDCK Cells Despite Differences in Pathways of Newly Synthesized Protein Delivery from the Golgi Complex to the Cell Surface

The plasma membrane distribution of α - (data not shown) and β subunit Na/K-ATPase in different clones of MDCK cells was quantitated by cell surface biotinylation (Fig. 2); results are similar to the time course of development of cell surface polarity of α subunit Na/K-ATPase (see also Hammerton et al., 1991). Four hours after cell-cell adhesion, Na/K-ATPase is present in apical and basal-lateral membranes of both clones of MDCK cells. After 24 h, the amount of Na/K-ATPase in the basal-lateral membrane of clone II/J cells is slightly greater than that in the apical membrane domain, whereas in clone II/G cells >80% of Na/K-ATPase is restricted to the basal-lateral membrane. Complete cell surface polarity of Na/K-ATPase in both clones requires >48 h, at which time the protein is present predominantly (>95%) in the basal-lateral membrane domain (Fig. 2); note that most Na/K-ATPase is solubilized under the stringent extraction conditions used here. The time course of Na/K-ATPase localization to the (basal-) lateral membrane determined by cell surface biotinylation

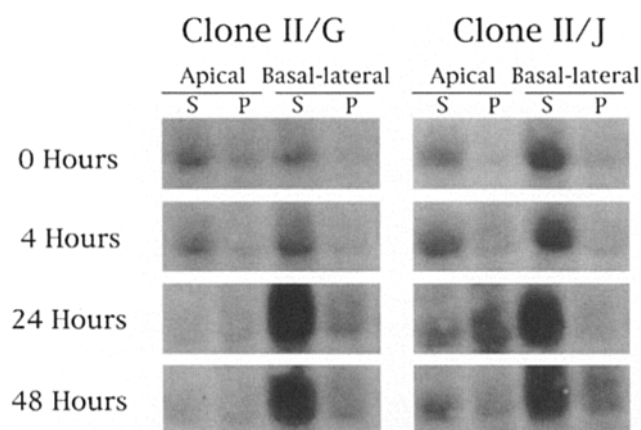


Figure 2. Steady state distribution of Na/K-ATPase in the apical and basal-lateral membranes of clone II/G and clone II/J MDCK cells at different times after induction of cell-cell contact. Confluent cultures of clone II/G and clone II/J MDCK cells were initially established on collagen-coated Costar Transwell™ filters in medium containing 5 μ M Ca^{2+} , and cell-cell contacts were induced subsequently by addition of 1.8 mM Ca^{2+} . At the times indicated (0, 4, 24, and 48 h), duplicate filters from each clone were labeled in parallel at either the apical or basal-lateral membranes with sulfo-NHS biotin. Cells were extracted (S, Triton X-100 fraction; P, Triton X-100 insoluble fraction), and β subunit Na/K-ATPase was immunoprecipitated. Precipitated protein was separated by SDS-PAGE, proteins were electrophoretically transferred to nitrocellulose membranes, and biotinylated protein was visualized by ECL. Results are shown from a representative experiment of greater than six trials.

compliments observations made by immunofluorescence microscopy (Mays et al., 1995).

We next examined the contribution of direct delivery of newly synthesized Na/K-ATPase to the basal-lateral membrane as a mechanism for generating the polarized distribution of the protein in the (basal-) lateral membrane (Gottardi and Caplan, 1993b). Cells were pulse labeled for 1 h with ^{35}S -methionine/cysteine, and the first wave of newly synthesized protein to arrive at the cell surface was detected by biotinylation (see Materials and Methods). Results show that similar amounts of newly synthesized Na/K-ATPase are delivered to both apical and basal-lateral membranes of clone II/J cells at all times analyzed (Fig. 3), even at times (e.g., 48 h) when the steady state distribution of Na/K-ATPase is restricted to the (basal-) lateral membrane (see Fig. 2). A parallel analysis of Na/K-ATPase sorting in clone II/G cells reveals that, after 6 h, newly synthesized Na/K-ATPase is also delivered to both apical and basal-lateral membrane domains, similar to that in clone II/J cells. However, after 48 h, >85% of newly synthesized Na/K-ATPase is delivered directly to the basal-lateral membrane (Fig. 3). In studies similar to Hammerton et al. (1991), we found that Na/K-ATPase delivered to the apical membrane has a shorter half-life than that delivered to the basal-lateral membrane in both clones (data not shown).

E-Cadherin Is Restricted to the Basal-Lateral Membrane Domain, and is Directly Delivered from the Golgi Complex to the Basal-Lateral Membrane in Clone II/G and II/J MDCK Cells

To determine if Na/K-ATPase trafficking to both membrane domains in clone II/J cells represented a general

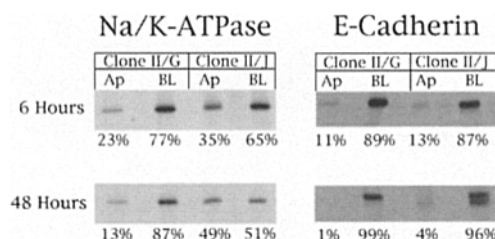


Figure 3. Delivery of newly synthesized Na/K-ATPase and E-cadherin to different plasma membrane domains of clone II/G and clone II/J MDCK after induction of cell-cell contact. Confluent cultures of clone II/G and clone II/J MDCK cells were initially established on collagen-coated Costar Transwell™ filters in medium containing 5 μ M Ca^{2+} , and cell-cell contacts were induced subsequently by addition of 1.8 mM Ca^{2+} . At the times indicated (6 and 48 h), duplicate filters from each clone were metabolically labeled in parallel with ^{35}S -methionine/cysteine for 1 h at 37°C, and then duplicate filters were labeled at either the apical (Ap) or basal-lateral (BL) membrane with NHS-SS-biotin. Cells were extracted in buffer containing 1% SDS. Half of the sample was used for immunoprecipitation of α subunit Na/K-ATPase, and the other half for E-cadherin. Biotinylated proteins were isolated from the immunoprecipitates with avidin-agarose, separated by SDS-PAGE. Amounts of labeled protein in the gels were determined directly using a Fujibias 2000 phosphorimager. Results are shown from a representative experiment of greater than four trials in which the ratio of α subunit Na/K-ATPase delivered to different membrane domains varied by <10%.

trend for basal-lateral membrane proteins in this clone of MDCK cells, we examined the sorting of another basal-lateral marker protein, E-cadherin. For direct comparison, we used half of the protein sample for analysis of Na/K-ATPase and the remainder for E-cadherin (Fig. 3 is representative of this parallel analysis).

Cell surface biotinylation showed that at steady state E-cadherin is rapidly restricted to the basal-lateral membrane domain after induction of cell adhesion (see Wollner et al., 1992). The time course of E-cadherin localization to the (basal-) lateral membrane determined by cell surface biotinylation compliments observations made by immunofluorescence microscopy (Mays et al., 1995). Cell surface biotinylation after metabolic labeling shows that 6 h after induction of cell-cell contact, ~90% of newly synthesized E-cadherin is delivered to the basal-lateral membranes of both clone II/G and II/J MDCK cells. After 48 h, >95% of newly synthesized E-cadherin is delivered directly to the basal-lateral membrane in both clones of MDCK cells (Fig. 3). The reason for the appearance of E-cadherin as a doublet (Fig. 3; *Clone II/G, 48 Hours, BL*) is unknown, but may be due to partial protein degradation during sample preparation.

Since cell surface biotinylation and protein extraction were performed on the same cells for analysis of Na/K-ATPase and E-cadherin, we can rule out the possibility that variations in cell culture, protein labeling, or extraction techniques are responsible for differences in the sorting patterns of these proteins in clone II/J cells. We conclude that clone II/J cells do not have a basic sorting defect for basal-lateral membrane proteins (see also Wollner et al., 1992).

Differences in Sorting of a GPI-anchored Protein, GP-2, and Glycosphingolipids between Clone II/J and Clone II/G MDCK Cells

We examined whether there were differences in the patterns of sorting of components of the apical membrane between clone II/J and II/G cells. We compared the sorting of a GPI-anchored protein, GP-2 (Fukouka et al., 1991; Hoops and Rindler, 1991). In clone II/G MDCK cells, >90% of newly synthesized GP-2 is sorted directly to the apical membrane, and <10% of GP-2 is sorted to the basal-lateral membrane (Fig. 4). In direct contrast, similar amounts of GP-2 are sorted to the apical and basal-lateral membranes of clone II/J MDCK cells (Fig. 4). Note that GP-2 was isolated in the Triton X-114 detergent phase and was sensitive to cleavage by PI-PLC, demonstrating that the protein had been delivered to both membrane domains anchored to the membrane by a GPI moiety. The electrophoretic mobility of GP-2 biotinylated in the basal-lateral membrane is slightly slower than that of GP-2 that had been biotinylated in the apical membrane (Fig. 4); the reason for this difference appears to be due to differential proteolytic processing (B. A. Fritz and A. W. Lowe, unpublished results).

We next examined the delivery of newly synthesized GSLs to the cell surface to determine whether differences in GP-2 sorting between the two MDCK clones reflected the pattern of GSL sorting (Table I). GSL sorting was determined using the fluorescent ceramide analog C6-NBD-ceramide. NBD-ceramide is incorporated into the plasma

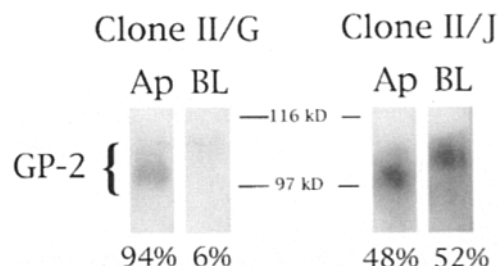


Figure 4. Differences in delivery of newly synthesized GPI-anchored protein, GP-2, to membrane domains of clone II/G and clone II/J MDCK cells. GP-2, a GPI-anchored protein expressed in pancreatic acinar cells, was stably expressed in clone II/G and clone II/J MDCK cells. Confluent monolayers of cells were grown for 72 h, and metabolically labeled as described in the legend to Fig. 3. Proteins were biotinylated in either the apical (Ap) or basal-lateral (BL) membrane with NHS-SS-biotin to capture the first wave of newly synthesized proteins targeted to each cell surface. Cells were extracted with Triton X-114. After separation and isolation of the detergent phase, GPI-linked proteins were released into the aqueous phase by incubation with PI-PLC, and biotinylated GP-2 was isolated by sequential precipitation with a monoclonal antibody and then avidin-agarose. Immunoprecipitates were separated by SDS-PAGE, and the amount of labeled GP-2 in the gels were determined directly using a Fujibass 2000 phosphorimager. Results shown are representative of three independent trials in which the ratios of GP-2 delivery to different membrane domains varied by <12%.

membrane, internalized and delivered to the Golgi complex (Lipsky and Pagano, 1985). In the Golgi complex, NBD-ceramide is used as a precursor in the synthesis of NBD-glucosylceramide and -sphingomyelin, which are subsequently resorted back to the cell surface (Lipsky and Pagano, 1985). The absolute amounts of NBD-glucosylceramide and NBD-sphingomyelin delivered to the cell sur-

Table I. Comparison of Sorting Pathways of NBD-Glucosylceramide and NBD-Sphingomyelin in Clone II/G and II/J Cells at Different Times After Induction of Cell-Cell Contacts

Cell type	Polarity		Relative polarity
	NBD-GluCer	NBD-SM	
Clone II/G			
6 Hours	1.60 ± 0.07	1.05 ± 0.07	1.52 ± 0.04
48 Hours	1.55 ± 0.05	0.76 ± 0.05	2.05 ± 0.05
72 Hours	1.47 ± 0.20	0.64 ± 0.10	2.33 ± 0.41
Clone II/J			
6 Hours	1.18 ± 0.02	0.98 ± 0.01	1.25 ± 0.05
48 Hours	0.94 ± 0.05	0.74 ± 0.01	1.27 ± 0.05
72 Hours	0.93 ± 0.15	0.66 ± 0.09	1.41 ± 0.11

Confluent monolayers of clone II/G and clone II/J MDCK cells were grown in parallel as described in Materials and Methods. At the indicated times after induction of cell-cell contacts, cells were incubated for 1 h at 37°C with liposomes containing NBD-ceramide. After incorporation into the membrane and delivery to the Golgi complex, NBD-ceramide is converted to either NBD-sphingomyelin or NBD-glucosylceramide, which are then delivered to the apical and basal-lateral membrane. NBD-sphingomyelin and NBD-glucosylceramide were extracted from the apical and basal-lateral membrane separately. The polarity of NBD-glucosylceramide (NBD-GluCer) and NBD-sphingomyelin (NBD-SM) delivered to each membrane domain following synthesis in the Golgi complex was calculated by dividing the relative fluorescence of extracted lipids at the apical membrane by the relative fluorescence of the same lipid species at the basal-lateral membrane. Relative polarity was determined as the ratio of NBD-Cer polarity divided by the polarity of NBD-SM (van't Hof and van Meer, 1990). Results are from four to nine independent experiments and are presented as the mean ± standard error.

face in the two clones of cells were similar (raw data used to compile Table I).

Table I shows that although NBD-glucosylceramide is extracted from both apical and basal-lateral membranes of clone II/G MDCK cells, the apical/basal-lateral membrane ratio of NBD-glucosylceramide is 1.47 ± 1.6 in clone II/G cells. Preferential sorting of NBD-glucosylceramide to the apical membrane developed rapidly after induction of cell-cell adhesion (Table I). In clone II/J, however, the apical/basal-lateral membrane ratio of NBD-glucosylceramide is ~ 1 , demonstrating that equal amounts of NBD-glucosylceramide were delivered to both apical and basal-lateral membranes at all times examined. Apart from this measurement of absolute polarity of NBD-glucosylceramide delivered, sorting of NBD-glucosylceramide can be measured as preferential delivery of GlcCer to the apical membrane when compared to the delivery of NBD-sphingomyelin (van Meer et al., 1987; van't Hof and van Meer, 1990). The extent of this sorting is expressed as the ratio of the polarities of delivery of the two delivered lipids and is termed "relative polarity" (Table I). This analysis shows that after 72 hours, the relative polarity of NBD-glucosylceramide reached a maximum of 2.33 ± 0.41 in clone II/G cells, and 1.41 ± 0.11 in clone II/J cells. We conclude that the relative amounts of NBD-glucosylceramide and NBD-sphingomyelin delivered to the cell surface were similar in both clones, but NBD-glucosylceramide is preferentially delivered to the apical membrane of clone II/G cells, and randomly delivered to both membrane domains in clone II/J cells.

Inhibition of Sphingolipid Synthesis by the Fungal Metabolite Fumonisin B₁ Disrupts Sorting of Na/K-ATPase and GP-2 in Clone II/G MDCK Cells

The results described above indicate an interrelationship between the pathways of sorting of GlcCer and GP-2, and GlcCer and Na/K-ATPase. In clone II/J MDCK cells, GP-2 and GlcCer are delivered to both apical and basal-lateral membranes, whereas in clone II/G cells both are sorted preferentially to the apical membrane. In clone II/G cells, Na/K-ATPase is sorted predominantly to the basal-lateral membrane, whereas GlcCer (and GP-2) are sorted to the apical membrane; in clone II/J cells, Na/K-ATPase, GP-2 and GlcCer are all delivered approximately equally to both the apical and basal-lateral membranes.

To examine this inter-relationship directly, sphingolipid synthesis was inhibited in clone II/G MDCK cells and the effects on sorting of Na/K-ATPase, GP-2 and E-cadherin were examined. Sphingolipid synthesis was inhibited by FB₁, a toxin from the fungus *Fusarium moniliforme*, which specifically competes with sphingosine as a substrate of ceramide synthase (Fig. 5 A). Although it was reported that epithelial cell growth is sensitive to high doses of FB₁ (Shier et al., 1991), we found that cell viability was not affected at doses that inhibited sphingolipid synthesis when cells were grown on filters, rather than on plastic, and in reduced concentrations of FBS (R. W. Mays, unpublished results). We titrated the concentration of FB₁ that affected cell growth and sphingolipid synthesis and found that 25 μ g/ml FB₁ was not toxic to confluent monolayers of cells

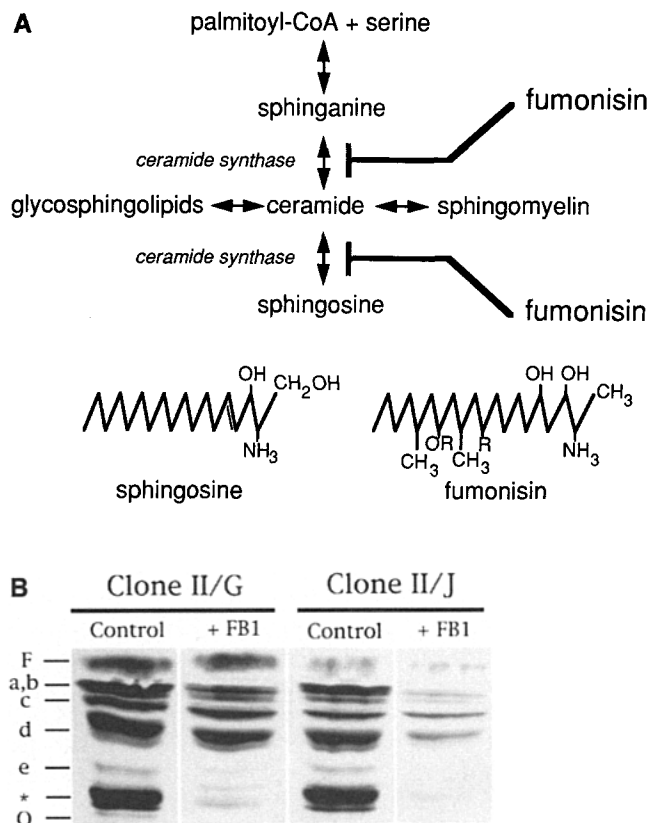


Figure 5. FB₁ inhibits glycolipid synthesis in clone II/G and clone II/J MDCK cells. (A) Schematic representation of the structure of FB₁ compared with that of sphingosine. FB₁ inhibits ceramide synthase activity by competing with sphingosine, resulting in an inhibition of sphingolipid synthesis. Note that addition of exogenous ceramide circumvents the FB₁ block in sphingolipid synthesis. (B) Duplicate cultures of clone II/G and clone II/J MDCK cells were grown for 72 h, and labeled with ³H-galactose for 20 h in the absence or presence of FB₁. Cellular lipids were extracted in chloroform/methanol and separated by thin layer chromatography. Radiolabeled lipids were visualized by fluorography, and identified by comparison to the migration of purified lipid standards. F, solvent front; a, glucosylceramide; b, galactosylceramide; c, dihexosesphingolipid; d, trihexosesphingolipid and galactosylceramide-sulfate; e, globoside; *, Forssman antigen and GM₃ (NeuAc-Gal-Glc-Cer); O, origin. Results are representative of two independent experiments.

on filters. Note that FB₁-treated cells exhibited control levels of ³⁵S-methionine-cysteine incorporation into proteins (see Figs. 6–8), sorted E-cadherin to the basal-lateral membrane (see Fig. 6), and maintained tight junction integrity and overall ultrastructural organization (R. W. Mays, unpublished results).

To determine the effects of FB₁ on glycolipid synthesis, confluent monolayers of each clone were established in the absence or presence of FB₁, and incubated with ³H-galactose. Lipids were extracted in chloroform-methanol, and separated on TLC plates (Fig. 5 B). Both clone II/J and clone II/G cells have similar profiles and amounts of ³H-galactose-labeled glycolipids, including a significant amount of Forssman antigen. Addition of FB₁ blocked synthesis of Forssman's antigen and globoside, significantly decreased the synthesis of glucosyl- and galactosyl-ceramide, and

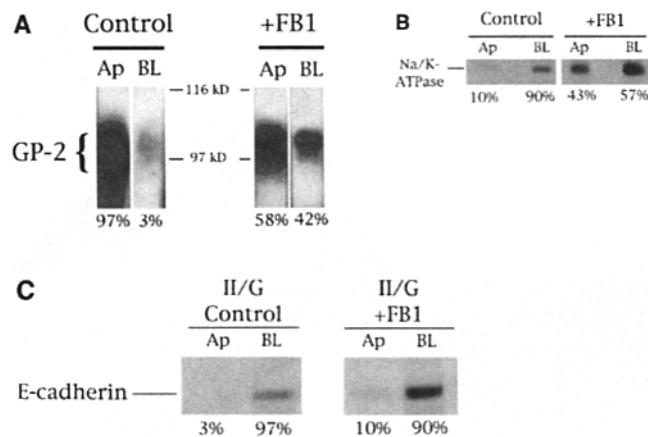


Figure 6. FB_1 inhibits polarized sorting of Na/K-ATPase and GP-2, but not E-cadherin, in clone II/G MDCK cells. Confluent cultures of clone II/G MDCK cells, and clone II/G cells expressing GP-2, were grown for 72 hours after the induction of cell-cell contact in either the absence (*Control*) or presence of FB_1 ($+\text{FB}_1$), as described in Materials and Methods. Cells were metabolically labeled with ^{35}S -methionine/cysteine, biotinylated at either the apical (*Ap*) or basal-lateral (*BL*) membrane with NHS-SS-biotin. Cells were extracted in buffer containing either Triton X-114 (*A*) or Triton X-100 (*B* and *C*). Biotinylated GP-2 (*A*), α subunit Na/K-ATPase (*B*), or E-cadherin (*C*) were isolated from cell extracts, and separated by SDS-PAGE. Amounts of labeled protein in the gels were determined directly using a Fujibas 2000 phosphorimager. The results are representative of four independent trials, and the ratios of protein in each membrane varied by $<10\%$.

partially affected synthesis of dihexosesphingolipid, trihexosesphingolipid, and galactosylceramide-sulfate in both clones. Clone II/J cells appear to be slightly more sensitive to the effects of FB_1 on glycolipid synthesis than clone II/G cells (Fig. 5 *B*); the reason for this difference is unknown.

To determine if protein sorting in clone II/G cells is affected by inhibition of sphingolipid synthesis, the delivery of newly synthesized GP-2, Na/K-ATPase and E-cadherin were analyzed in the presence and absence of FB_1 (Fig. 6). In the presence of FB_1 , sorting of GP-2 (Fig. 6 *A*) and Na/K-ATPase (Fig. 6 *B*) is disrupted. In control cells, 97% of GP-2 and 10% of Na/K-ATPase are delivered to the apical membrane (also, see Figs. 3 and 4, respectively), whereas in the presence of FB_1 , the percentage of GP-2 and Na/K-ATPase delivered to the apical membrane became 58% and 43%, respectively. Note that the absolute amounts of Na/K-ATPase and GP-2 delivered to the cell surface were similar to those in control cells, indicating that decreased amounts of GSLs did not affect the overall trafficking of these proteins to the cell surface. Significantly, the fidelity of sorting of E-cadherin, another basal-lateral membrane protein, was only slightly reduced in FB_1 -treated clone II/G cells (Fig. 6 *C*). We also analyzed the steady state cell surface distribution of Na/K-ATPase and E-cadherin in cells treated with FB_1 . By immunofluorescence microscopy, we found that both proteins were restricted to the lateral membranes of cells in a distribution indistinguishable from those in control cells (R. W. Mays, unpublished result). Thus, similar to clone II/J cells, selective retention of Na/K-ATPase in the (basal-) lateral membrane of clone II/G

cells is sufficient to generate cell surface polarity of this protein.

Two important controls were performed to assess the effects of FB_1 on protein sorting in clone II/G cells. First, clone II/G cells were incubated in the presence of FB_1 and liposomes containing exogenous ceramide to circumvent the FB_1 -induced block in ceramide synthesis (Harel and Futerman, 1993). Under this condition, $>75\%$ of newly synthesized Na/K-ATPase is sorted to the basal-lateral membrane (Fig. 7 *A*). A similar analysis sorting showed that 70% of GP-2 was delivered to the apical membrane domain in the presence of both FB_1 and ceramide (R. W. Mays, unpublished result). Note that E-cadherin sorting is not significantly affected by addition of exogenous ceramide (Fig. 7 *B*). In a second control experiment, FB_1 was removed from the culture medium, and the cells were allowed to recover for 48 h before protein sorting was examined. We found that polarized sorting of GP-2 and Na/K-ATPase is restored to the apical and basal-lateral membranes, respectively (R. W. Mays, unpublished results).

Delivery of a Secreted Protein, GP-84, to the Apical Membrane Is Not Affected by the Addition of FB_1

To examine whether delivery of proteins other than GP-2 to the apical membrane is affected by FB_1 , we analyzed the cell surface targeting of the glycoprotein, GP-84. GP-84 is the predominant protein secreted into the apical medium from polarized MDCK cells (Kondor-Koch et al., 1985; Gottlieb et al., 1986; Urban et al., 1987). GP-84 is detected simply by analysis of culture medium by SDS-PAGE without reduction of disulfide bonds (see Wollner et al., 1992). Fig. 8 shows that in both control clone II/G cells and in cells treated with FB_1 , newly synthesized GP-84 is secreted almost exclusively into the apical medium. In a previous

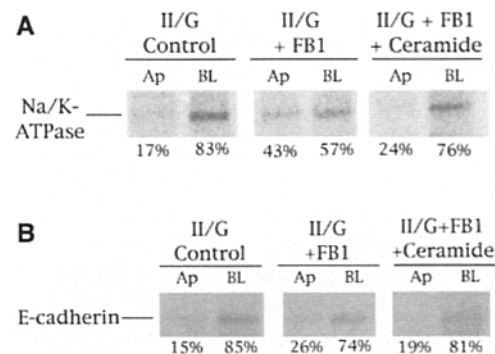


Figure 7. Na/K-ATPase and E-cadherin sorting in the presence of both FB_1 and exogenous ceramide in clone II/G MDCK cells. Confluent cultures of clone II/G MDCK cells were grown for 72 h after the induction of cell-cell contact in either the absence (*Control*), or presence of FB_1 (FB_1), or presence of FB_1 and ceramide ($+\text{FB}_1$ + ceramide), as described in Materials and Methods. Cells were metabolically labeled with ^{35}S -methionine/cysteine, biotinylated at either the apical (*Ap*) or basal-lateral (*BL*) membrane with NHS-SS-biotin. Cells were extracted, and biotinylated α subunit Na/K-ATPase (*A*) or E-cadherin (*B*) were isolated from cell extracts, separated by SDS-PAGE. The amount of labeled protein in the gels was determined directly using a Fujibas 2000 phosphorimager. The results are representative of three independent trials, and the ratios of protein in each membrane varied by $<15\%$.

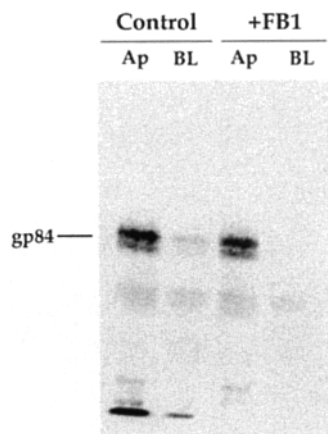


Figure 8. Characterization of the secretory pathway of GP-84 in clone II/G MDCK cells. Confluent monolayers of cells were established in the absence (*Control*) and presence of FB_1 (*+FB1*) and metabolically labeled as described in Materials and Methods. After 1 h, 50- μl aliquots of the apical (*Ap*) and basal-lateral (*BL*) media were removed, adjusted to a final concentration of 1% SDS (without reducing agent), and separated by SDS-PAGE. The amount of

labeled GP-84-2 in the gels was determined directly using a Fujibas 2000 phosphorimager. GP-84 is the predominant secreted protein in the growth medium; a longer exposure of the gel reveals additional proteins. The result is representative of two independent experiments.

study, we showed that GP-84 is also secreted into the apical medium from clone II/J cells (Wollner et al., 1992).

Discussion

Defining mechanisms involved in restricting protein distributions to different membrane domains is important for understanding vectorial transport of ions and solutes in epithelia, synaptic transmission in neurons, and cell activation in the immune system (Rodriguez-Boulant and Powell, 1992). Studies with polarized epithelial cells *in vitro* have provided important insights into the mechanisms involved. Although sorting signals for some classes of apical and basal-lateral membrane proteins have been identified (see Introduction), mechanisms involved in sorting of proteins that span the membrane multiple times, such as most ion channels and transporters, are poorly understood. In the present study, we have exploited the finding that newly synthesized Na/K-ATPase is sorted differently in the Golgi complex of the two MDCK cell clones, although, in both clones, steady state polarity of Na/K-ATPase is achieved in the basal-lateral membrane. Our search for a basis for this difference in sorting has uncovered evidence that sorting of Na/K-ATPase may be indirect, and that complete cell surface polarity is generated by a hierarchy of mechanisms in the Golgi complex and at the plasma membrane. In addition, our results have uncovered several pathways involved in sorting other proteins to either the apical or basal-lateral membrane.

Mechanisms for Sorting Na/K-ATPase in the Golgi Complex

We found that Na/K-ATPase is delivered equally to both apical and basal-lateral membranes in clone II/J cells, whereas >85% is sorted to the basal-lateral membrane of clone II/G cells (Fig. 3). We can rule out several trivial explanations for the difference in Na/K-ATPase sorting between clone II/G and II/J cells. First, the experimental protocol for detecting the arrival of newly synthesized pro-

tein(s) at the cell surface excludes the possibility that proteins were first delivered to the basal-lateral membrane and then redirected to the apical membrane. Second, the analysis of each clone of cells was performed simultaneously, with the same reagents, and the same procedures for cell surface biotinylation, protein extraction and immunoprecipitation (Fig. 3). Therefore, the pattern of Na/K-ATPase sorting in clone II/J cells is not due to an experimental artifact, since clone II/G cells would have been subjected to the same artifact. Third, we directly compared the sorting of E-cadherin and Na/K-ATPase from the same cell extracts, and showed that nearly 100% of newly synthesized E-cadherin was sorted to the basal-lateral membrane in both clones of MDCK cells (Fig. 3). Therefore, there is not a general sorting defect for basal-lateral membrane proteins in clone II/J cells.

It is important to note that Na/K-ATPase sorting differences between clone II/J and II/G cells do not necessarily imply abnormality, since Na/K-ATPase is localized to the apical and lateral membranes during normal development of renal epithelial cells (Avner et al., 1992). Since the original population of MDCK cells was obtained by enzymatic digestion of canine kidney cortex (Gaush et al., 1966), which contains many cell types, clone II/J cells may represent an immature cell, or a mature cell type that normally sorts Na/K-ATPase in this manner.

The polarized targeting of newly synthesized Na/K-ATPase to the basal-lateral membrane correlates strongly with targeting of GlcCer and GP-2 to the apical membrane (Fig. 9). This correlation raises the intriguing question as to whether GSL sorting in the Golgi complex influences both GP-2 and Na/K-ATPase sorting. Therefore, we examined the inter-relationship between GSL and Na/K-ATPase sorting in clone II/G cells with the fungal metabolite FB_1 . FB_1 , a competitive inhibitor of ceramide synthase (see Fig. 4 A; Shier et al., 1991; Wang et al., 1991; Norred et al., 1992; Yoo et al., 1992), blocks ceramide and glycosphingolipid synthesis (Fig. 5 B). Note, however, that incorporation of ^{35}S -methionine/cysteine into proteins (see Figs. 6 and 7), sorting of E-cadherin to the basal-lateral membrane (Figs. 6 C and 7 B), the functional integrity of tight junctions, and overall structural organization of cells are unaffected (data not shown), showing that FB_1 is not toxic under the conditions used here.

In the presence of FB_1 , newly synthesized GP-2 and Na/K-ATPase are delivered to both apical and basal-lateral membranes in clone II/G cells, in contrast to the polarized sorting of these proteins in control cells (Fig. 9). When the FB_1 -induced block in sphingolipid synthesis is circumvented by addition of exogenous ceramide (Fig. 5 A), GP-2 and Na/K-ATPase are again preferentially sorted to the apical and basal-lateral membranes, respectively (Fig. 7, see also Fig. 9). At present, we do not know if ceramide acts directly on Na/K-ATPase and GP-2 sorting at the level of the Golgi complex, or as a second messenger (Hunnun, 1994). Nevertheless, taken together these results demonstrate that exogenous ceramide circumvents the FB_1 -induced block in sorting of Na/K-ATPase and GP-2 to the basal-lateral and apical membranes, respectively. It is noteworthy that the sorting patterns of Na/K-ATPase, GP-2 and E-cadherin in clone II/G cells treated with FB_1 are similar to those in clone II/J control cells (Fig.

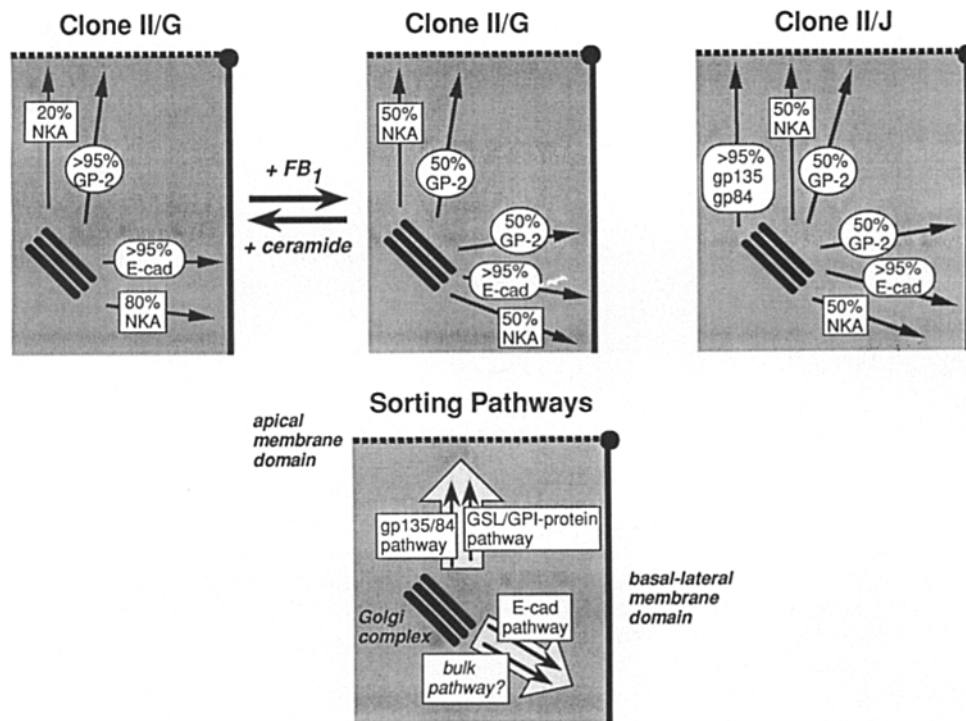


Figure 9. Schematic representations of the sorting pathways of proteins in clone II/G and II/J MDCK cells. Sorting pathways, and percentage of protein in each pathway, from the Golgi complex to either the apical or basal-lateral membrane are represented by solid arrows for GP-2, Na/K-ATPase and E-cadherin in clone II/G cells (*top, left*), clone II/G cells incubated with FB₁ (*top, middle*), or clone II/J (*top, right*) cells. Generalized sorting pathways (*bottom*) for proteins in MDCK cells; Large, gray background arrows represent bulk pathways, onto which is superimposed specialized pathways (*solid arrows*). For discussion, see text.

9), indicating that a difference between the clones is at the level of GSL sorting.

To explain the variable sorting behavior of Na/K-ATPase, we suggest that it utilizes bulk pathways (Pfeffer and Rothman, 1987) for delivery to either cell surface domain (Fig. 9). We suggest that in polarized epithelial cells specialized sorting mechanisms are superimposed on these bulk pathways to regulate sorting of specific proteins to either the apical or basal-lateral membranes (Fig. 9). For example, specialization of the apical bulk pathway may be mediated by GSL clustering in the Golgi complex. We suggest that Na/K-ATPase is physically excluded from GSL clusters and, therefore, the GSL-controlled pathway to the apical membrane (clone II/G cells, see above). Elimination of this specialization of the apical pathway (i.e., when GSLs are not sorted (clone II/J cells) or not synthesized (clone II/G cells + FB₁)) uncovers the (constitutive) bulk pathway that allows Na/K-ATPase to be delivered to the apical membrane (Fig. 9). Since some Na/K-ATPase leaks into the apical pathway when GSL sorting occurs (10–20% in clone II/G cells; see Figs. 3, 7, and 9), we suggest that exclusion of Na/K-ATPase is not complete, perhaps because specialization of the apical bulk pathway by GSL sorting is not absolute (i.e., not all transport vesicles contain sufficient amounts of GSLs to exclude Na/K-ATPase). Note that addition of exogenous ceramide does not entirely rescue the exclusion of Na/K-ATPase from the apical pathway, indicating that the amount of GSLs and/or degree of GSL clustering is critical for this process (see also Rock et al., 1990).

How then is Na/K-ATPase sorted (directly) to the basal-lateral membrane? Since Na/K-ATPase can be delivered to both apical and basal-lateral membranes in both clones of MDCK cells, Na/K-ATPase may not have a strong basal-lateral sorting signal compared, for example, to

E-cadherin; note that E-cadherin is efficiently sorted to the basal-lateral membrane in both clones of cells, even under experimental conditions that alter Na/K-ATPase sorting (see Fig. 9). If Na/K-ATPase had a basal-lateral sorting signal as strong as that of E-cadherin, it should not enter the apical pathway, but rather be sorted directly to the basal-lateral membrane. This is clearly not the case (Fig. 9). It is possible, however, that Na/K-ATPase contains some basal-lateral sorting information, as suggested by others (Gottardi and Caplan, 1993a, b), and enters the specialized basal-lateral pathway(s), together with E-cadherin, as directed by this signal. Alternatively, Na/K-ATPase may be sorted to the basal-lateral membrane because a constitutive basal-lateral bulk pathway exists, or the specialized basal-lateral pathway is not exclusionary (Fig. 9). In this context, it is interesting to note that an apical membrane protein, GP-2, can also leak into the basal-lateral pathway in II/G cells treated with FB₁. This result supports the notion that either the basal-lateral pathway does not exclude proteins that do not have a strong basal-lateral sorting signal, or that a separate basal-lateral bulk pathway exists.

Specialized Sorting Pathways for Apical and Basal-Lateral Proteins in the Golgi Complex

Our results support the hypothesis (Brown et al., 1989; Lisanti et al., 1989; Simons and Wandinger-Ness, 1990) that there is a relationship between sorting of a GPI-anchored protein, GP-2, and GSLs in MDCK cells (Fig. 9). Since GP-2 is delivered to both apical and basal-lateral membranes in the absence of GSLs (clone II/G + FB₁) or absence of GSL sorting (clone II/J cells), GSLs appear to be critical for its delivery to the apical membrane. Thus, GP-2 may not contain a strong apical sorting signal that is

independent of GSL sorting. GSL-mediated sorting of some GPI-anchored proteins to the apical membrane may not be as critical as it is for GP-2. For example, when the GPI-anchor is removed from placental alkaline phosphatase (Casanova et al., 1991) or Thy-1 (Powell et al., 1991), the secreted proteins are still delivered to the apical membrane. Whether or not these proteins are sorted by a receptor that clusters with GSLs, or by a GSL-independent pathway is unclear.

Although GSLs appear to play a role in sorting of GPI-anchored proteins, there is strong evidence that sorting of some apical membrane proteins is independent of both GSL synthesis and sorting. The membrane glycoprotein GP-135 and secreted glycoprotein GP-84 (see also Fig. 8) are delivered directly to the apical membrane in clone II/J cells (Wollner et al., 1992), although neither GP-2 nor GSLs are sorted in these cells (Fig. 9). Therefore, GP-135 and GP-84 appear to be representative of a class of proteins that is sorted into a specialized apical pathway that is independent of GSL sorting (Fig. 9). In support of this, we showed directly that the fidelity of GP-84 secretion from the apical membrane of clone II/G cells was identical in the presence or absence of FB₁ (Fig. 8). In the context of different sorting pathways for apical membrane proteins, Nicolas et al. (1994) identified two populations of apical transport vesicles that contained either GPI-anchored proteins or other proteins.

E-cadherin is almost exclusively sorted to the basal-lateral membrane in both clones of MDCK cells (Fig. 9). We suggest that sorting of E-cadherin into the basal-lateral pathway is an active process that does not depend upon exclusion from the apical pathway. At present, we do not know the mechanism involved in sorting E-cadherin to the basal-lateral membrane. Specific sorting of basal-lateral membrane proteins in the Golgi complex is thought to be mediated by cytosolic adaptor proteins that recognize signals in the cytoplasmic domain of basal-lateral membrane proteins (Rodriguez-Boulant and Powell, 1992; Mellman et al., 1993). Comparison of the amino acid sequences of the cytoplasmic domains of E-cadherin and LDL receptor, in which basal-lateral sorting signals have been well characterized (Matter et al., 1994), demonstrates that some homologies are present, including several tyrosine residues that are close to runs of three acidic amino acids (see also discussion in Matter et al., 1994). It will be interesting to determine the relevance of these motifs to the sorting of E-cadherin to the basal-lateral membrane.

Mechanism for Generating Complete Cell Surface Polarity

Our observations on the sorting of Na/K-ATPase in the Golgi complex and the fate of protein delivered to different membrane domains indicate that there is a two-stage hierarchy of mechanisms involved in restricting Na/K-ATPase to the (basal-) lateral membrane in these MDCK cells. Some degree of protein sorting may occur in the Golgi complex, after which the final cell surface distribution of proteins is determined by differences in protein turnover at the apical and basal-lateral membranes (see also Hammerton et al., 1991). Na/K-ATPase retention in the basal-lateral membrane may be determined by linkage

to the spectrin-based membrane-cytoskeleton (Nelson et al., 1990; Hammerton et al., 1991).

A similar hierarchy of mechanisms appears to regulate the initial generation of polarity of other basal-lateral membrane proteins in MDCK cells (Wollner et al., 1992). An extreme example of this process is found in hepatocytes (Bartles et al., 1987). In these cells, all proteins appear to be delivered to the basal-lateral (sinusoidal) membrane, where resident basal-lateral membrane proteins are retained and apical membrane proteins are internalized and resorted to the apical (bile canalicular) membrane. Thus, polarized epithelial cells appear to have a hierarchy of stages in protein sorting that "proof-read" the status of cell surface polarity to ensure that proteins accumulate in the correct membrane domain.

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