Transcytosis in MDCK Cells: Identification of Glycoproteins Transported Bidirectionally between Both Plasma Membrane Domains

André W. Brändli, Robert G. Parton, and Kai Simons

European Molecular Biology Laboratory, Postfach 10.2209, 6900 Heidelberg, Federal Republic of Germany

Abstract. MDCK cells display fluid-phase transcytosis in both directions across the cell. Transcytosis of cell surface molecules was estimated by electron microscopic analysis of streptavidin-gold–labeled frozen sections of biotinylated cells. Within 3 h, ∼10% of the surface molecules, biotinylated on the starting membrane domain, were detected on the opposite surface domain irrespective of the direction of transcytosis. This suggests that the transcytosis rates for surface molecules are equal in both directions across the cell as shown previously for fluid-phase markers.

A biochemical assay was established to identify transcytosing glycoproteins in MDCKII-RCA^r cells, a ricin-resistant mutant of MDCK. Due to a galactosylation defect, surface glycoproteins of these cells can be labeled efficiently with [³H]galactose. Transcytosis of [³H]galactose-labeled glycoproteins to the opposite membrane domain was detected by surface biotinylation. Detergent-solubilized glycoproteins derivatized with biotin were adsorbed onto streptavidin-agarose and separated by SDS-PAGE. A subset of the cell surface glycoproteins was shown to undergo transcytosis. Transport of these glycoproteins across the cell was time and temperature dependent. By comparative two-dimensional gel analysis, three classes of glycoproteins were defined. Two groups of glycoproteins were found to be transported unidirectionally by transcytosis, one from the apical to the basolateral surface and another from the basolateral to the apical surface. A third group of glycoproteins which has not been described previously, was found to be transported bidirectionally across the cell.

CELL surface proteins internalized by receptor-mediated endocytosis are clustered selectively into coated pits, endocytosed, and transported to endosomes. Three different fates have been described for proteins delivered to endosomes. Receptors can be transported further to the lysosomes for degradation (e.g., EGF receptor), recycle directly back to the cell surface (e.g., low density lipoprotein [LDL] receptor), or be targeted to the *trans*-Golgi network before reappearing on the cell surface (e.g., mannose 6-phosphate receptor) (for review, Steinman et al., 1983; Hubbard, 1989; Van Deurs et al., 1989). In polarized epithelial cells, a fourth pathway is known. Some proteins can be transported across the cell to the opposite surface by transcytosis (Mostov and Simister, 1985).

Transcytosis has been described in various epithelial cell types. The best-characterized examples involve transport of Ig across epithelia. The polymeric Ig receptor transports IgA and IgM unidirectionally from the basolateral to the apical surface in glandular epithelial cells and hepatocytes (Mostov and Simister, 1985). The IgG receptor mediates transcytosis of monomeric IgG in the opposite direction in epithelial cells of the small intestine of neonatal rats (Rodewald and Kraehenbuhl, 1984; Parham, 1989). Also, nerve growth factor (Siminoski et al., 1986), EGF (St. Hilaire et al., 1983; Maratos-Flier et al., 1987), and thyroglobulin (Herzog, 1983) undergo transcytosis in some epithelial cell types. Endo-

thelial cells transport LDL (Vasile et al., 1983), albumin (Ghitescu et al., 1986), and insulin (King and Johnson, 1985) across the cell. It seems like transcytosis is a general process in polarized cell layers.

MDCK cells are epithelial cells that form polarized sheets with functional tight junctions in culture. They have been widely used in studies of cell polarity and membrane traffic (Simons and Fuller, 1985; Rodriguez-Boulan and Nelson, 1989). They are able to transport by transcytosis an implanted viral glycoprotein to the basolateral plasma membrane (Matlin et al., 1983; Pesonen and Simons, 1983). Ricin and fluid phase markers such as horseradish peroxidase are transported in both directions across the cell (von Bonsdorf et al., 1985; Bomsel et al., 1989; Van Deurs et al., 1990). The polymeric Ig receptor, expressed in MDCK cells, functions as observed in vivo, appearing first on the basolateral membrane, and then on the apical surface, where it is cleaved to secretory component and released into the apical medium (Mostov and Deitcher, 1986). Expression of FC receptors in MDCK cells results in apical-to-basolateral transcytosis of immunoglobulins (Hunziker and Mellman, 1989). These results indicate that these cells possess transcytotic routes for both fluid-phase markers and membrane proteins in both directions across the cell. The transcytosis of endogenous glycoproteins, however, has not yet been demonstrated in MDCK cells.

Transcytosing molecules are, by definition, present on both plasma membrane domains and exchange between the two compartments. We have used a mutant of MDCK cells, MDCKII-RCA[r1] (Meiss et al., 1982; Brändli et al., 1988), to study transcytosis. First, we applied an electron microscopic method requiring biotinylation of cell surface molecules to estimate by streptavidin-gold labeling the amount of transcytotic traffic in both directions across MDCKII-RCA[r] cells. Secondly, we established a biochemical assay in order to identify endogenous glycoproteins that are transported by transcytosis across the cell. Transcytosis of cell surface molecules was found to occur with approximately equal rates in both directions, and a new class of glycoproteins was identified which transcytose bidirectionally across the cells.

Materials and Methods

Materials

Media and reagents for cell culture were purchased from Gibco BRL and Seromed (West Berlin, Federal Republic of Germany). UDP-D-[6-^{3}H]galactose (20 Ci/mmol) was from Amersham Buchler GmBH (Braunschweig, FRG). Galactosyltransferase (from bovine milk), methyl [5-(2-thienylcarbonyl)-1H-benzimidazol-2-yl]-carbamate (nocodazole), monensin, chloroquine, streptavidin, and streptavidin-agarose was obtained from Sigma Chemical GmBH (Munich FRG). Entensify and Protosol tissue solubilizer were purchased from Du Pont-New England Nuclear Products (Wilmington, DE). Sulfosuccinimidyl 6-(biotinamido) hexonate (NHS-LC-biotin), and sulfosuccinimidyl 2-(biotinamido) ethyl-1,3-dithiopropionate (NHS-SS-biotin) were from Pierce. Biotin-ε-aminocaproyl hydrazide (BACH) was from Calbiochem. Protein A-Sepharose was purchased from Pharmacia (Uppsala, Sweden). β-Galactosidase from Diplococcus pneumoniae was obtained from Seikagaku Kogyo, Tokyo, Japan.

Cell Culture

Growth media compositions and cell culture protocols for MDCKII-RCA[r] cells have been described previously (Brändli et al., 1988). For filter cultures, premounted polycarbonate Transwell filters (0.4-μm pore size, 24 mm diam) (Costar, Cambridge, MA) were used (a gift from Hank Lane of Costar).

Exogalactosylation

Exogalactosylation was performed according to previously described procedures (Brändli et al., 1988; Brändli and Simons, 1989) modified as follows. 3-d-old filter cultures of MDCKII-RCA[r] cells were washed twice with ice-cold PBS containing 0.9 mM Ca^{2+} and 0.5 mM Mg^{2+} (PBS+), and once with E buffer (10 mM Hepes, pH 7.3, 150 mM NaCl, 0.1% (wt/vol) BSA). The reaction mixture contained E buffer supplemented with 10 mM MnCl$_2$, 0.25 U/ml galactosyltransferase, and 80 μCi/ml UDP-D-[6-^{3}H]galactose. For apical exogalactosylation, 100 μl of the reaction mixture were added to the apical side of the filter. The filter was transferred into a six-well dish filled with 1.5 ml PBS+, and incubated on a rocking platform. For basolateral exogalactosylation, 1 ml of PBS was added apically and the filter chambers were placed on a 100-μl drop of the reaction mixture on Parafilm. After a 45-60 min incubation at 4°C, the reaction was stopped by removing the reaction mixture and washing the filters with PBS+ (three times).

Transcytosis Assay

Prewarmed growth media (1.5 ml apically and 2 ml basolaterally) was added to the exogalactosylated MDCKII-RCA[r] cells. Cells were usually incubated at 37°C in the cell culture incubator. Incubations at temperatures ranging from 10 to 30°C were done in a water bath. In this case the growth

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1. Abbreviations used in this paper: BACH, biotin-ε-aminocaproyl hydrazide; NHS-LC-biotin, sulfosuccinimidyl 6-(biotinamido) hexonate; NHS-SS-biotin, sulfosuccinimidyl 2-(biotinamido) ethyl-1,3-dithiopropionate; MDCKII-RCA[r], ricin-resistant cell line of MDCK strain II.

media contained 0.6 g/liter sodium bicarbonate and 10 mM Hepes, pH 7.3. For nocodazole treatment a stock solution of 33 mM nocodazole in DMSO (stored at −20°C) was prepared. Just before use the stock solution was diluted with prewarmed growth medium to 33 μM. Nocodazole was present in the apical as well as basolateral chambers during the entire incubation period. For some experiments, cells were also incubated with nocodazole-containing medium before exogalactosylation.

Plasma Membrane Domain-restricted Biotinylation

Monolayers of MDCKII-RCA[r] cells were selectively biotinylated with NHS-LC-biotin (Lisanti et al., 1988; Sargiacomo et al., 1989), NHS-SS-biotin (Busch et al., 1989; Graeve et al., 1989; Le Bivic et al., 1989) or BACH (O'Shanessy et al., 1987; Lisanti et al., 1989) as described below. Labeled and chased cells were washed three times with cold PBS+ before biotinylation of cell surface proteins. Time-course and concentration-dependence experiments indicated that optimal labeling was achieved with 1-1.5 mg/ml NHS-LC-biotin for 30 min at 4°C. Immediately before use, NHS-LC-biotin was dissolved in cold PBS+. For apical biotinylation, 250 μl of NHS-LC-biotin solution was added to the apical side of the filter. The filter was transferred into a six-well dish filled with 1.5 ml PBS+, and incubated on a rocking platform. For basolateral biotinylation, 1 ml of PBS+ was added apically and the filter chamber was placed on a 250-μl drop of NHS-LC-biotin solution on Parafilm. After 30 min at 4°C, filter chambers were washed with PBS+ (three times). Periodate-induced biotinylation was carried out as follows. Oxidation of glycoconjugates was initiated by addition of 1.5 ml of 10 mM NaIO$_4$ in PBS+ to one compartment. The opposite compartment received 1.5 ml of PBS+ alone. After 30 min at 4°C, the periodate solution was removed and the cells were washed extensively with PBS+. BACH was dissolved at 1.5 mg/ml in PBS+. The reaction was performed as indicated for NHS-LC-biotin.

β-Galactosidase Treatment

Treatment of exogalactosylated and biotinylated cells with β-galactosidase from Diplococcus pneumoniae was carried out as follows. A stock solution of β-galactosidase was prepared by reconstitution in PBS+ to 1 U/ml and stored at −20°C. The enzyme was diluted to 0.25 U/ml in PBS+, and 200 μl of the β-galactosidase solution was added either apically or basolaterally. The opposite compartment received 1.5 ml of PBS+. After a 90-min incubation at 4°C, the filter chambers were washed with PBS+. Binding of free streptavidin to the biotinylated cell surface proteins was performed similarly using a solution of streptavidin (0.25 mg/ml) in PBS+, and incubating for 90 min on ice.

Isolation of Biotinylated Proteins

Biotinylated proteins were isolated by streptavidin precipitation (Hare and Lee, 1989; Lisanti et al., 1989; Matter et al., 1990) as described below. Cells derivatized with biotin were scraped from the filters into PBS+ using a rubber policeman, transferred into 1.5 ml Eppendorf tubes, and pelleted by centrifugation (13,000 rpm; 5 min). Cell pellets were lysed in 500 μl of lysis buffer A (PBS containing 2% (wt/vol) NP-40, 0.2% (wt/vol) SDS, 35 μg/ml PMSF, 10 μg/ml each of the following protease inhibitors: leupeptin, antipain, chymostatin, and pepstatin) for 30 min at 4°C rotating end over end. Lysates were cleared by centrifugation in a microfuge (13,000 rpm; 10 min). Streptavidin-agarose was washed three times with wash buffer A (PBS with 1% NP-40). 30-40 μl of a 50% (vol/vol) solution of streptavidin-agarose was added to the cleared lysates. Lysates were rotated end over end for at least 2 h (or overnight) at 4°C. The streptavidin-agarose was pelleted by centrifugation in a microfuge, washed three times with wash buffer A, twice with wash buffer B (PBS with 0.1% NP-40 and NaCl to 0.5 M), and once with 50 mM Tris-HCl, pH 7.5. Adsorbed proteins were solubilized in 100 μl of 2× SDS-PAGE sample buffer (100 mM Tris-HCl, pH 6.8; 5 mM EDTA; 4% (wt/vol) SDS; 10% (vol/vol) glycerol; 0.02% (wt/vol) bromophenol blue; 5% (vol/vol) 2-mercaptoethanol) and heated to 95°C for 10 min. Proteins solubilized with SDS sample buffer were run on one-dimensional 9% (wt/vol) acrylamide and 0.24% (wt/vol) bis-acrylamide electrophoresis gels. After electrophoresis gels were fixed (45% (vol/vol) methanol, 7% (vol/vol) acetic acid), treated with Entensify, dried, and exposed for 1-6 wk at −70°C to Kodak XAR-5 film. Fluorograms were quantified by eluting the silver grains from the developed film with 1 M NaOH and measuring the absorbance of the eluate in a spectrophotometer as described by Suissa (1983).

Typically, 20% of the detergent-soluble [^{3}H]galactose-labeled material

of basolaterally exogalactosylated and biotinylated control cells can be recovered on streptavidin-agarose. For apical-to-basolateral transcytosis, 1.2–1.8% of the total detergent-soluble [³H]galactose-labeled material is bound to streptavidin-agarose at the 3-h time point. Therefore, 6–9% of the [³H]galactose-labeled material is transcytosed to the basolateral membrane within 3 h at 37°C.

Immunoprecipitation Protocols

To detect the surface polarity of plasma membrane glycoproteins in MDCKII-RCA^r cells, filter cultures were exogalactosylated as described. ³H-labeled cells were scraped, transferred into Eppendorf tubes, and pelleted by centrifugation. Cell pellets were solubilized in 500 μl of lysis buffer C (1% [wt/vol] NP-40; 50 mM Tris-HCl, pH 7.5; 2 mM EDTA; 150 mM NaCl; 35 μg/ml PMSF; 10 μg/ml each of the following protease inhibitors: leupeptin, antipain, chymostatin, and pepstatin) for 30 min at 4°C on a rotating shaker. The cell lysate was cleared from insoluble material by centrifugation in a microfuge (13,000 rpm, 10 min) at 4°C. 5–10 μl of monoclonal anti-114-kD antibody (ascites fluid) were added to the 500 μl supernatants, and incubated on a rotating shaker for 2 h. Protein A-Sepharose was washed three times with lysis buffer C, incubated with rabbit anti-mouse immunoglobulin (Cappel Laboratories, Malvern, PA) for 2 h at 4°C, and washed with lysis buffer C. The antigen–antibody complex was precipitated with 40 μl of a 50% (vol/vol) suspension of coated protein A-Sepharose for 2 h at 4°C. The precipitate was washed four times with lysis buffer C, twice with lysis buffer C (containing NaCl to 0.5 M), and once with 50 mM Tris-HCl, pH 7.5. The immune complexes were released from the protein A-Sepharose with 100 μl of 2× SDS-PAGE sample buffer or 75 μl of two-dimensional gel sample buffer and processed for electrophoresis.

Labeling of immunoprecipitated glycoproteins from MDCKII-RCA^r cell lysates with [³H]galactose was done as follows. Proteins bound to the protein A immunoadsorbant were washed twice with E buffer. The reaction mixture contained E buffer supplemented with 10 mM MnCl₂, 0.25 U/ml galactosyltransferase, and 80 μCi/ml UDP-D-[6-³H]galactose. 50 μl of the reaction mixture was added to the packed protein A-Sepharose beads. The incubation was for 45 min at 37°C with occasional shaking. The labeled precipitate was washed three times with wash buffer A, twice with wash buffer B, and once with 50 mM Tris-HCl, pH 7.5. Samples were processed for one- or two-dimensional gel electrophoresis.

To determine the fraction of an immunoprecipitated glycoprotein derivatized with biotin the following protocol was applied. The antigen was eluted from the protein A-beads by addition of 100 μl of elution buffer (0.1 M glycine-HCl, pH 2.5; 0.1 M NaCl; 0.1% [wt/vol] BSA), and incubated 10 min at 4°C on a shaker. The protein A-Sepharose beads were pelleted by centrifugation. The supernatant was recovered and brought to neutral pH by addition of 35 μl of 1 M Tris-HCl, pH 7.5. The immunoadsorbant was treated with a second 100 μl aliquot of elution buffer, washed finally with 250 μl of 2% (wt/vol) of NP-40 in PBS. All the eluates and washes were pooled. The eluate was subjected to streptavidin-agarose precipitation and analyzed by one-dimensional SDS-PAGE.

Two-dimensional Gel Analysis

For analysis of transcytosing glycoproteins by two-dimensional IEF/SDS-

PAGE, the cleavable NHS-SS-biotin was used for biotinylation as described for NHS-LC-biotin. Derivatized proteins adsorbed to streptavidin-agarose were released with 75 μl of two-dimensional gel sample buffer (4% [wt/vol] NP-40; 2% [vol/vol] ampholines pH 7-9 [LKB Instruments, Inc., Gaithersburg, MD]; 9.8 M urea; 100 mM DTT) by incubation for 15–30 min at 30°C. Analysis of the lysates by two-dimensional IEF/SDS-PAGE was done as described by Bravo (1984). The tube gels used for the first IEF dimension were 25 cm long and had an internal diameter of 2.5 mm. IEF gels were run at 1,200 V for 17–20 h. The pH gradient ranged from 4.57 to 8.03 and was linear between pH 4.6 and 7.2. The second-dimension resolving gels were 15% (wt/vol) acrylamide and 0.075% (wt/vol) N,N'-bis-acrylamide. Gels were fixed and treated for fluorography.

Electron Microscopy

Preparation of streptavidin-gold was carried out as follows. 9 nm gold was prepared by the tannic acid method (Slot and Geuze, 1985). 12 μg of streptavidin was added to 1 ml of the gold solution adjusted to pH 6.6. The streptavidin-gold was washed three times with PBS containing 0.2% (wt/vol) BSA (PBS-BSA) by centrifugation (32,000 rpm, 30 min) to remove free streptavidin, and finally for 5 min at 13,000 rpm to remove any aggregates. For sectioning, MDCKII-RCA^r cells grown on filters were fixed in 8% (wt/vol) paraformaldehyde in 250 mM Hepes, pH 7.4. Frozen sections of gelatin-embedded filters were cut perpendicular to the plane of the filter as described previously (Parton et al., 1989). The thawed sections were incubated on drops of PBS-BSA for 30 min, and then on 10 μl drops of streptavidin-gold (OD₅₂₀ = 0.2) in the same buffer for 90 min at room temperature. After washing with PBS-BSA (six times, 10-min each), the sections were embedded in a methylcellulose-uranyl acetate mixture as described previously (Griffiths et al., 1983). Control grids were treated as above, but were incubated with 0.5 mg/ml unconjugated streptavidin for 15 min before incubation on streptavidin-gold containing 0.5 mg/ml streptavidin.

Quantification of Electron Microscopic Data

The results shown are from one set of experiments. Quantitatively similar results were obtained in two separate experiments. Sections were taken from two different areas of each filter. Sections of fairly uniform thickness (~100 nm as judged by electron density) were photographed randomly at a magnification of 12,500. The density of gold particles (gold particles/length of membrane profile) on the apical or basolateral surface was measured on negatives (magnified a further 4×) by relating the number of gold particles to the length of membrane profile measured by intersection counting. 100–200 intersections, equivalent to 110–220 μm² of membrane surface, were counted per point. Specific gold particles per μm of surface area were determined by subtracting the values (gold/μm) found under control conditions (binding of streptavidin-gold to sections in the absence of biotinylation or after biotinylation and subsequent quenching with free streptavidin at 0.5 mg/ml) from the measured gold density. The background values were 0.3 ± 0.1 gold/μm for the apical surface, and 0.04 ± 0.02 gold/μm for the basolateral surface. To relate the gold densities obtained for the two plasma membrane domains with each other in terms of traffic across the cell, the different contributions of the two surface domains to the

Table I. Quantitation of Transcytosis in MDCKII-RCA^r Cells by Electron Microscopy

Direction of transcytosis	Incubation time at 37°C	Surface distribution of gold particles			
		Apical membrane		Basolateral membrane	
		Specific gold*	Total gold	Specific gold*	Total gold
	h		%		%
A → B‡	0	21.4 ± 4.8	100	0 ± 0.1	0
	1	19.0 ± 4.2	89	0.7 ± 0.3	3
	3	12.4 ± 3.0	58	2.4 ± 0.7	11
B → A§	0	0 ± 0.1	0	19.8 ± 6.2	100
	1	0.8 ± 0.5	4	20.3 ± 6.0	103
	3	1.5 ± 0.3	8	8.7 ± 1.9	44

* Absolute number of gold particles detected per section associated with the indicated plasma membrane domain per cell in arbitrary units (see Materials and Methods for details).
‡ Transcytosis from the apical to the basolateral plasma membrane.
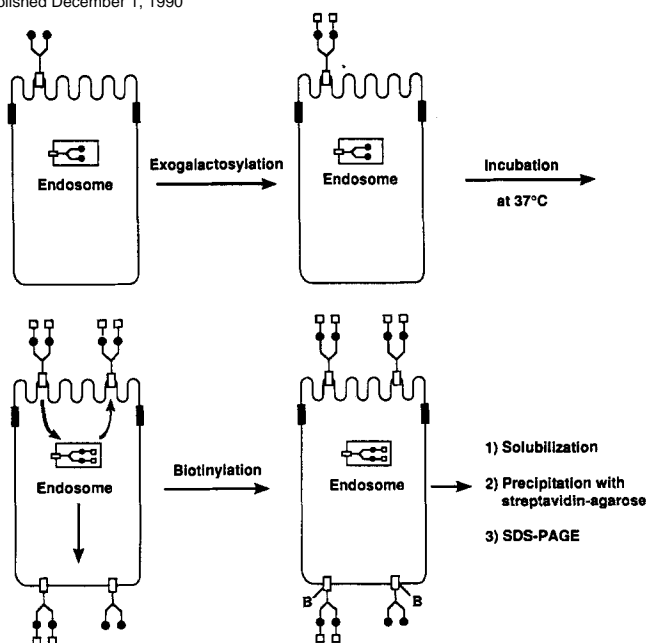§ Transcytosis from the basolateral to the apical plasma membrane.
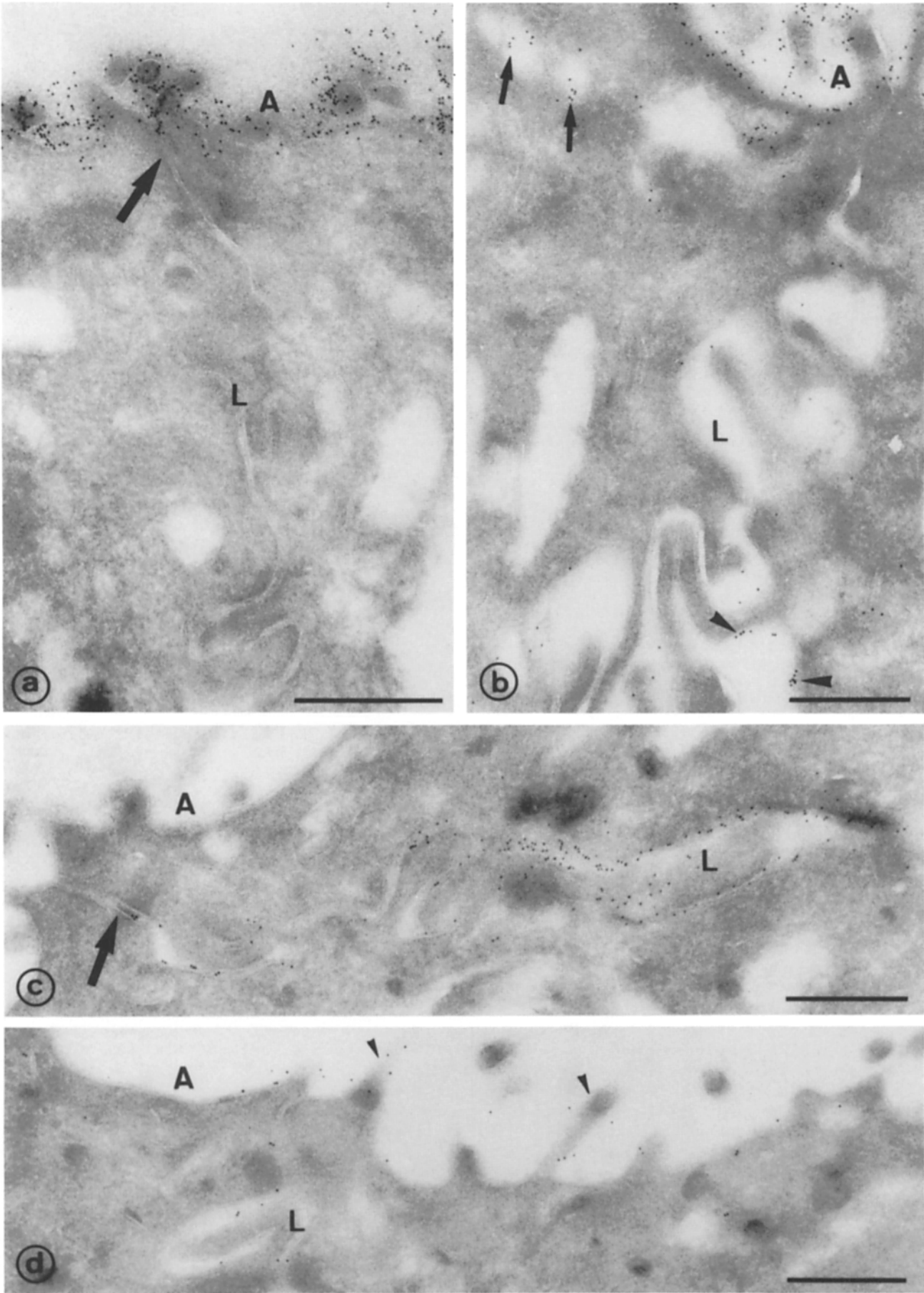
Figure 2. Experimental protocol used to detect apical cell surface glycoproteins transported by transcytosis to the basolateral plasma membrane in MDCKII-RCA^r cells. (●) GlcNAc; (□) Gal; B, biotin.

Figure 3. Delivery of apical glycoproteins to the basolateral membrane. Filter-grown MDCKII-RCA^r cells were exogalactosylated apically at 4°C, chased for 3 h at 37°C, and labeled basolaterally at 4°C with biotin (lanes b, d, and f). The 3-h chase at 37° was omitted from control cells (lanes a, c, and e). NHS-LC-biotin was used to biotinylate primary amines (lanes a–d). Sugar residues were oxidized with 10 mM NaIO$_4$ and reacted with BACH (lanes e and f). Cells were treated basolaterally with β-galactosidase from D. *pneumoniae* for 90 min at 4°C (lanes c and d) before cell lysis. Biotinylated proteins were adsorbed to streptavidin-agarose, and analyzed by SDS-PAGE. The molecular masses of the marker proteins indicated by arrowheads are: 200, 97, 69, 46, and 30 kD.

total cell surface area had to be taken into consideration. The basolateral-to-apical surface area ratio for MDCKII-RCA^r cells was determined by stereological analysis on Epon sections as described previously (Parton et al., 1989). The basolateral surface area was determined to be 2.7-fold larger than the apical surface area in these cells. Therefore, the gold particles per length of plasma membrane domain on sections were converted to absolute numbers of gold particles per cell (in arbitrary units) by multiplying the basolateral values by the basolateral to apical ratio (2.7:1).

Results

Quantification of Transcytosis of Membrane Proteins by Electron Microscopy

A novel electron microscopic approach was used to quantify transcytosis of cell surface molecules across MDCK cells. We decided to use a mutant cell line of MDCK cells, MDCK-II-RCA^r, for our studies. These cells polarize in culture, forming a tight monolayer with distinct apical and basolateral plasma membrane domains of unique protein composition (Meiss et al., 1982; Brändli et al., 1988). Due to the galactosylation defect exhibited by MDCKII-RCA^r cells, they are a convenient tool for studying cell surface glycoproteins (Brändli et al., 1988; Brändli and Simons, 1989).

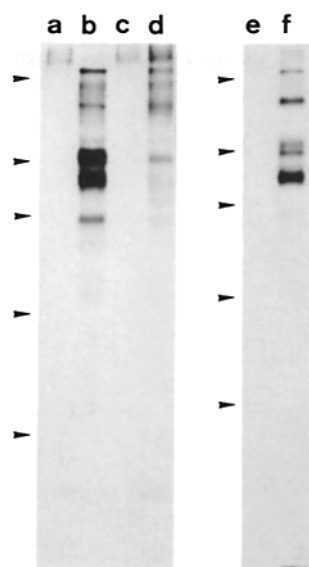Recently, it was shown that monolayers of epithelial cells

can be selectively biotinylated (Lisanti et al., 1988; Matter et al., 1990; Sargiacomo et al., 1989). We used filter-grown MDCKII RCA^r cells to derivatize cell surface molecules with a biotin reagent in a plasma membrane domain-specific manner. The biotin derivative NHS-LC-biotin is membrane impermeable, and reacts with primary amines. Molecules susceptible to NHS-LC-biotin include proteins as well as lipids, e.g., phosphatidyl serine and phosphatidyl ethanolamine. However, the majority of the aminophospholipids are predominantly found in the cytoplasmic leaflet of the plasma membrane bilayer and these should not be labeled (van Meer, 1989).

Biotin-derivatized cells were incubated at 37°C to reactivate membrane traffic. After incubation times ranging from 1 to 3 h, cells were fixed and processed for electron microscopy in order to determine the subcellular distribution of the biotinylated molecules. They were visualized by overlaying frozen sections with streptavidin-gold. Control cells fixed immediately after apical biotinylation showed heavy gold labeling restricted to the apical cell surface (Fig. 1 a). Similarly, basolateral biotinylation led to binding of gold particles all along the basolateral membrane up to the tight junctions. The absence of labeling of proteins of the opposite plasma membrane domain shows that NHS-LC-biotin does not penetrate tight junctions. Typically, a labeling density of 21.4 ± 4.5 gold particles per μm of apical membrane profile was detected. The density of gold particles on the biotinylated basolateral membrane, however, was about 7.3 ±

Figure 1. Electron microscopic analysis of transcytosis in MDCK cells. MDCKII-RCA^r cells were surface biotinylated either apically (a and b) or basolaterally (c and d). Cells were either fixed immediately after biotinylation (a and c) or were incubated for 3 h at 37°C before fixation (b and d). Frozen sections were overlaid with streptavidin-gold. In control cells (a and c), gold labeling is restricted to the surface domain subjected to biotinylation. Tight junctions (*large arrows*) form an impermeable barrier to the biotinylation reagent. In b, gold particles label internal structures (*small arrows*) and the basolateral membrane, where some gold particles are evident in clusters (*large arrowheads*). In d, gold labeling of the apical plasma membrane domain including the microvilli (*small arrowheads*) is apparent. A, apical surface; L, lateral space. Bars, 0.5 μm.
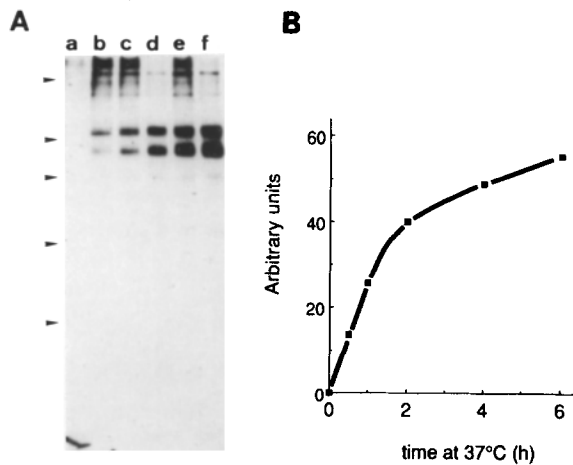
Figure 4. Kinetics of transcytosis of apical glycoproteins to the basolateral plasma membrane domain. (A) Filter-grown MDCK-II-RCA^r cells were exogalactosylated apically at 4°C, incubated for 0 (lane a), 0.5 (lane b), 1 (lane c), 2 (lane d), 4 (lane e), and 6 h (lane f) at 37°C. Subsequently, the cells were labeled with NHS-LC-biotin basolaterally at 4°C, lysed, and treated with streptavidin-agarose. Proteins adsorbed to streptavidin-agarose were analyzed by SDS-PAGE. Each time point represents material of pooled duplicate samples. The molecular masses of the indicated marker proteins are as in Fig. 3. B depicts the kinetics of basolateral delivery of the 85-kD protein determined by spectrophotometric quantification of the silver grains eluted from a duplicate of the fluorogram in A.

2.3 gold particles per μm of membrane profile. The reduced efficiency of biotinylation from the basolateral side is probably due to the diffusion barrier represented by the polycarbonate filter. Since the surface area of the basolateral membrane is 2.7-fold greater than the apical membrane in MDCKII-RCA^r cells, the number of gold particles detected per cell was about equal for both cell surface domains after biotinylation (Table I). Incubation of derivatized cells for 3 h at 37°C led to labeling of internal structures, and to the appearance of biotinylated apical molecules on the opposite plasma membrane. The gold particles were frequently seen as patches along the entire basolateral surface (Fig. 1 b). Biotinylated basolateral molecules were detected all over the apical surface including the microvilli (Fig. 1 d). Quantification of biotinylated molecules over the cell surface revealed a time-dependent decrease on the starting membrane domain and an increase on the opposite plasma membrane domain (Table I). After 3 h, ~10% of the biotinylated molecules had moved to the opposite plasma membrane. Thus, the amount of transcytosis seems to be equal in both directions with only a small fraction of the biotinylated molecules being transported across the cell.

A Biochemical Assay to Detect Glycoproteins Transported from the Apical to the Basolateral Plasma Membrane by Transcytosis

In MDCKII-RCA^r cells, N-linked glycans of glycoproteins terminate with GlcNAc rather than sialic acid residues due to the inability of the Golgi complex to translocate UDP-galactose into its lumen. ^3H-labeled galactose can be incorporated either into apical or basolateral glycoproteins by exogalactosylation of filter-grown MDCKII-RCA^r cells (Brändli

et al., 1988). This property of the cells was used to develop an assay to detect glycoproteins undergoing transcytosis (Fig. 2). Cell surface glycoproteins were galactosylated by incubation with UDP-[^3H]galactose and galactosyltransferase at 4°C. The cells were warmed to 37°C to allow internalization of galactosylated glycoproteins. Biotinylation at 4°C of the opposite plasma membrane domain was used to detect transcytosis of ^3H-labeled glycoproteins. Solubilized, biotinylated proteins were adsorbed onto streptavidin-agarose, and resolved on polyacrylamide gels. In this system, transcytosing molecules are defined as ^3H-labeled, biotin-bearing proteins.

A Group of Apical Glycoproteins Is Transported by Transcytosis to the Basolateral Surface

MDCKII-RCA^r cells were exogalactosylated apically, incubated for 3 h at 37°C, and subsequently biotinylated basolaterally on ice. Using NHS-LC-biotin, a number of ^3H-labeled polypeptides were visualized that were not detected in control cells (Fig. 3, lanes a and b). Major glycoproteins detected with NHS-LC-biotin had relative molecular masses of 85 and 100 kD. Minor transcytosing glycoproteins had relative molecular masses of 67, 155, and 215 kD.

After transcytosis, apically galactosylated glycoproteins should become sensitive to β-galactosidase added to the basolateral side. After a 3-h chase period, exogalactosylated MDCKII-RCA^r cells were biotinylated, and treated with β-galactosidase for 90 min at 4°C before cell lysis and streptavidin-agarose precipitation. Between 80 and 85% of the [^3H]galactose residues (as determined by spectrophotomet-
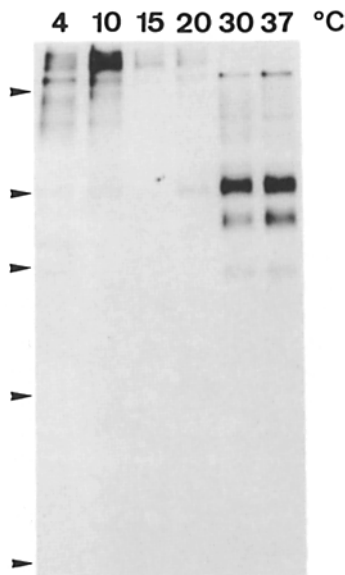

Figure 5. Basolateral delivery of apical glycoproteins by transcytosis is blocked at temperatures of 20°C and lower. MDCKII-RCA^r cells were labeled apically with [^3H]galactose, incubated for 3 h at temperatures ranging from 4 to 37°C, and then basolateral proteins were derivatized with NHS-LC-biotin at 4°C. Biotinylated proteins were precipitated by streptavidin-agarose, and analyzed by SDS-PAGE as described. The molecular masses of the indicated marker proteins (arrowheads) are as in Fig. 3.
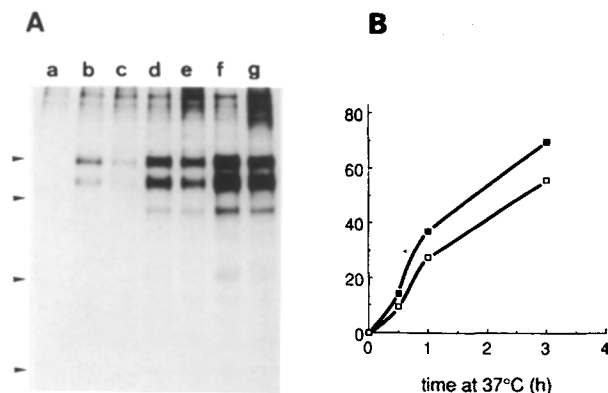
A

a b c d e f g

B

80
60
40
20
0

time at 37°C (h)

Figure 6. Effect of nocodazole on transport of apical proteins to the basolateral plasma membrane domain. (*A*) Filter-grown MDCKII-RCA[r] cells were exogalactosylated apically at 4°C. Incubations at 37°C were for 0 (lane *a*), 0.5 (lanes *b* and *c*), 1 (lanes *d* and *e*), and 3 h (lanes *f* and *g*). Nocodazole (33 μM) was added to the incubation medium (lanes *c*, *e*, and *g*). Basolateral proteins were derivatized with NHS-LC-biotin at 4°C, precipitated by streptavidin-agarose, and analyzed by SDS-PAGE. Each time point represents material of pooled duplicate samples. The molecular masses of the indicated marker proteins (*arrowheads*) are as follows: 97, 69, 46, and 30 kD. *B* depicts the kinetics of basolateral delivery of the 85-kD protein in the presence (*open squares*) or absence (*closed squares*) of nocodazole determined by spectrophotometric quantification of the silver grains eluted from a duplicate of the fluorogram in *A*.

ric quantification of silver grains eluted from autoradiograms) were removed from the proteins by β-galactosidase (Fig. 3, lane *d*). In addition, binding of biotinylated, ³H-labeled glycoproteins to streptavidin-agarose was inhibited by free streptavidin added basolaterally to the biotinylated cells before cell lysis (data not shown). These results suggest that a set of apical glycoproteins has indeed undergone transcytosis to the basolateral surface.

BACH, which reacts with sugar residues after oxidation with periodate (O'Shannessy et al., 1987), was used as an alternative biotinylation reagent. At 10 mM NaIO₄, all sugars containing vicinal hydroxyls (e.g., galactose) are oxidized (Van Lenten and Ashwell, 1971). Oxidation is surface domain selective, since NaIO₄ does not penetrate tight junctions (Brändli and Simons, 1989; Lisanti et al., 1989). Essentially the same set of polypeptides as with NHS-LC-biotin was identified (Fig. 3, lane *f*). The 100-kD protein detected with NHS-LC-biotin was resolved into two glycoproteins of 97 and 105 kD. Because the recovery of labeled glycoproteins was significantly lower, after biotinylation with BACH, we decided to use NHS-LC-biotin as biotinylation reagent for further experiments. Note that control cells biotinylated with NHS-LC-biotin showed some binding of ³H-labeled proteins of relative molecular masses >200 kD (see Fig. 3, lane *a* and lane *c*). However, this labeling was not seen in control cells treated with NaIO₄/BACH (Fig. 3, lane *e*).

Time, Temperature Dependence, and Sensitivity to Nocodazole of Transcytosis in Apical-to-Basolateral Direction

Apically exogalactosylated MDCKII-RCA[r] cells were incubated for up to 6 h at 37°C before basolateral biotinylation

to determine the kinetics of transcytosis. Fig. 4 *A* shows the fluorograph of such an experiment. Arrival at the basolateral plasma membrane was a time-dependent process with apparent half-times ranging from 60 to 90 min. The quantification for basolateral delivery of the 85-kD protein is represented in Fig. 4 *B*.

Transport of proteins by transcytosis is a temperature-dependent process. In MDCK cells expressing exogenous macrophage Fc receptors, apical-to-basolateral transcytosis of IgG mediated by Fc receptors ceases at 17°C (Hunziker and Mellman, 1989). Transcytosis is blocked at 15°C for EGF and at 18°C for ¹²⁵I-labeled ricin in MDCK cells (Maratos-Flier et al., 1987; Van Deurs et al., 1990). Simi-
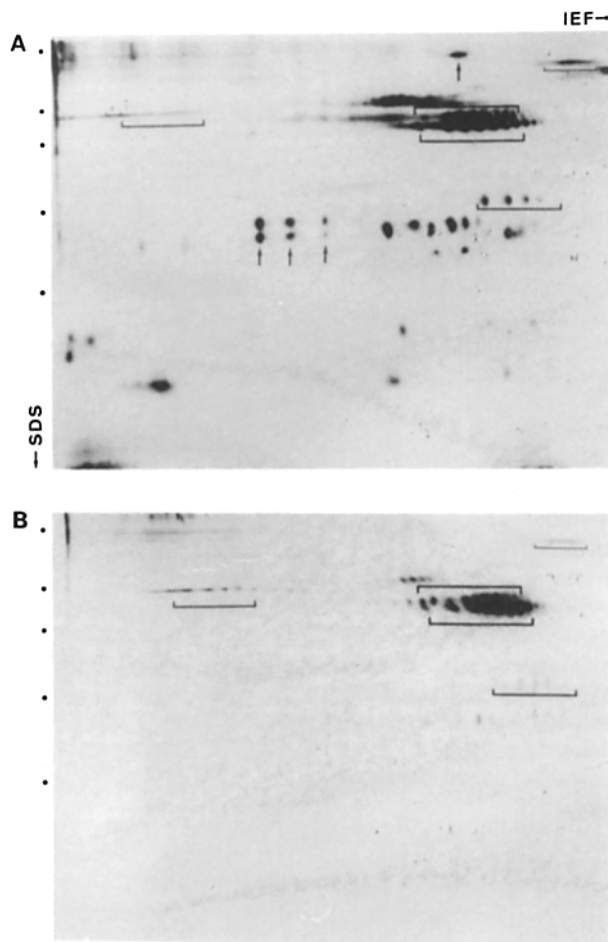

IEF→

A

− SDS

B

Figure 7. Analysis of glycoproteins transcytosing from the apical to the basolateral membrane by two-dimensional IEF/SDS-PAGE. (*A*) Analysis of total exogalactosylated apical glycoproteins. Filter-grown MDCKII-RCA[r] cells were exogalactosylated apically with [³H]galactose. Total cell lysates were prepared and analyzed by two-dimensional IEF/SDS-PAGE. (*B*) Analysis of apical transcytosing glycoproteins. Filter-grown MDCKII-RCA[r] cells were exogalactosylated apically at 4°C, chased for 3 h at 37°C, and labeled basolaterally with NHS-SS-biotin at 4°C. Cells were lysed, treated with streptavidin-agarose, and analyzed by two-dimensional IEF/SDS-PAGE. Brackets indicate examples of glycoproteins transported from the apical to the basolateral surface by transcytosis. Arrows denote examples of resident apical glycoproteins not undergoing transcytosis. The molecular masses of the indicated marker proteins (*closed circles*) are as in Fig. 3.

larly, temperatures between 15 and 20°C inhibit transcytosis in other model systems (Herzog, 1983; King and Johnson, 1985). In agreement with these observations, we found that transcytosis of apical glycoproteins was inhibited if exogalactosylated cells were incubated at temperatures of 20°C or lower (Fig. 5).

In hepatocytes, depolymerization of microtubules inhibits transcytosis of fluid phase markers from the basolateral to the apical membrane by >50% (Lowe et al., 1985; Scharschmidt et al., 1986). To test the effect of disruption of microtubules on transcytosis of apical glycoproteins to the basolateral plasma membrane domain in MDCKII-RCA^r cells, microtubules were depolymerized by a 60-min cold treatment during which the cells were exogalactosylated apically. Nocodazole (33 μM) was included in the incubation medium during the chase period at 37°C. This protocol results in dissociation of microtubules of filter-grown MDCK cells (Bacallao, R. L., and K. Simons, unpublished observation; Parczyk et al., 1989). Basolateral delivery was measured by biotinylation and streptavidin-agarose precipitation. After a 3-h chase period, we observed only a 20% decrease in the amount of the 85-kD protein delivered to the basolateral plasma membrane (Fig. 6). Similar results were obtained for the other glycoproteins undergoing transcytosis. At earlier time points, transport to the basolateral plasma membrane was slowed down more dramatically. After 30 min at 37°C, transcytosis was reduced by 35% for the 85-kD protein (Fig. 6 B) and by 60% for the 97/105-kD proteins (not shown). These results were confirmed by electron microscopic studies of biotinylated cells treated with nocodazole (data not shown). We conclude that disruption of the microtubular network delays transcytosis to the basolateral domain in MDCK cells.

Characterization of Apical Transcytosing Molecules by Two-dimensional Gel Analysis

Two-dimensional IEF/SDS-PAGE was used to analyze lysates of cells that had been apically exogalactosylated to reveal the complete pattern of apical glycoproteins (Fig. 7 A). This pattern was compared with the one obtained for glycoproteins transported by transcytosis from the apical to the basolateral plasma membrane (Fig. 7 B). A cleavable derivative of NHS-LC-biotin, NHS-SS-biotin, was used for the biotinylation reaction. Glycoproteins derivatized with NHS-SS-biotin can be solubilized from streptavidin-agarose through cleavage of the disulfide bond with DTT (Busch et al., 1989; Le Bivic et al., 1989). After an incubation of 3 h at 37°C, a set of biotinylated ³H-labeled glycoproteins showing extensive charge heterogeneity were resolved by two-dimensional IEF/SDS-PAGE (Fig. 7 B). Major transcytosing glycoproteins were identified in the molecular mass range of 80–110 kD, and most likely corresponding to the proteins of 85, 97, and 105 kD identified by one-dimensional SDS-PAGE (Fig. 3). Note that the transcytosing glycoproteins of 67 and 215 kD (see Fig. 3) could not be identified unambiguously. ³H-labeled glycoproteins were not detected on two-dimensional gels of control cells that had not been incubated at 37°C (not shown). All glycoproteins identified as molecules transported by transcytosis (Fig. 7 B, indicated by brackets) could be assigned in Fig. 7 A as apical glycoproteins. Slight differences in the isoelectric points of the glyco-
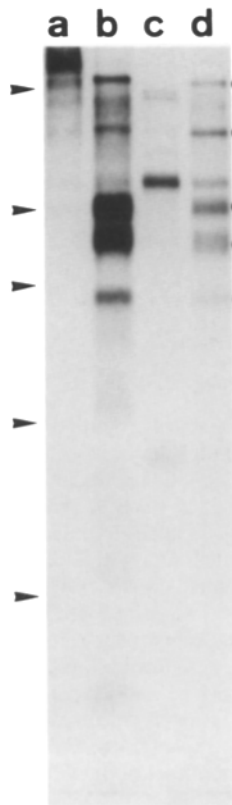

Figure 8. Delivery of basolateral glycoproteins to the apical cell surface. Filter-grown MDCKII-RCA^r cells were exogalactosylated apically (lanes a and b) or basolaterally (lanes c and d). Labeled cells were incubated for 3 h at 37°C, and opposite surface domain was labeled with NHS-LC-biotin (lanes b and d). The incubation at 37°C before biotinylation was omitted from control cells (lanes a and c). Cells were lysed, and treated with streptavidin-agarose. Proteins adsorbed to streptavidin-agarose were analyzed by SDS-PAGE. Basolateral glycoproteins detected on the apical surface after incubation at 37°C for 3 h are indicated (lane d, closed circles). The molecular masses of the marker proteins (arrowheads) are as in Fig. 3.

proteins in Fig. 7 B compared to their native mobility in Fig. 7 A are due to shifts to more acidic isoelectric points caused by the modification of primary amines by biotinylation. In addition to apical glycoproteins undergoing transcytosis, several resident glycoproteins were detected on the apical plasma membrane (Fig. 7 A, arrows). This suggests that the ability to undergo transcytosis to the basolateral plasma membrane is confined to a specialized group of apical glycoproteins.

The Major Apical Transcytosing Glycoproteins Are Also Transported from the Basolateral to the Apical Membrane

Transcytotic traffic from the basolateral to the apical surface also occurs in MDCK cells (von Bonsdorff et al., 1985; Mostov and Deitcher, 1986; Van Deurs et al., 1990). We asked whether endogenous basolateral membrane proteins moving to the apical surface could be detected using a modification of the experimental scheme outlined in Fig. 2. The assay was reversed by carrying out the exogalactosylation reaction on the basolateral plasma membrane domain of MDCKII-RCA^r cells, followed by incubation at 37°C, and subsequent biotinylation of the apical plasma membrane domain. The result of such an experiment is displayed in Fig. 8. In addition to those glycoproteins found in lysates of control cells (Fig. 8, lane c), a group of five additional glycoproteins was detected in lysates of cells that had been incubated at 37°C before biotinylation (Fig. 8, lane d). The identified glycoproteins undergoing transcytosis from the basolateral to the apical membrane have relative molecular masses of 67, 85, 97, 150, and 205 kD. Interestingly, the glycoproteins of 67, 85, and 97 kD were identical, by relative molecular mass,
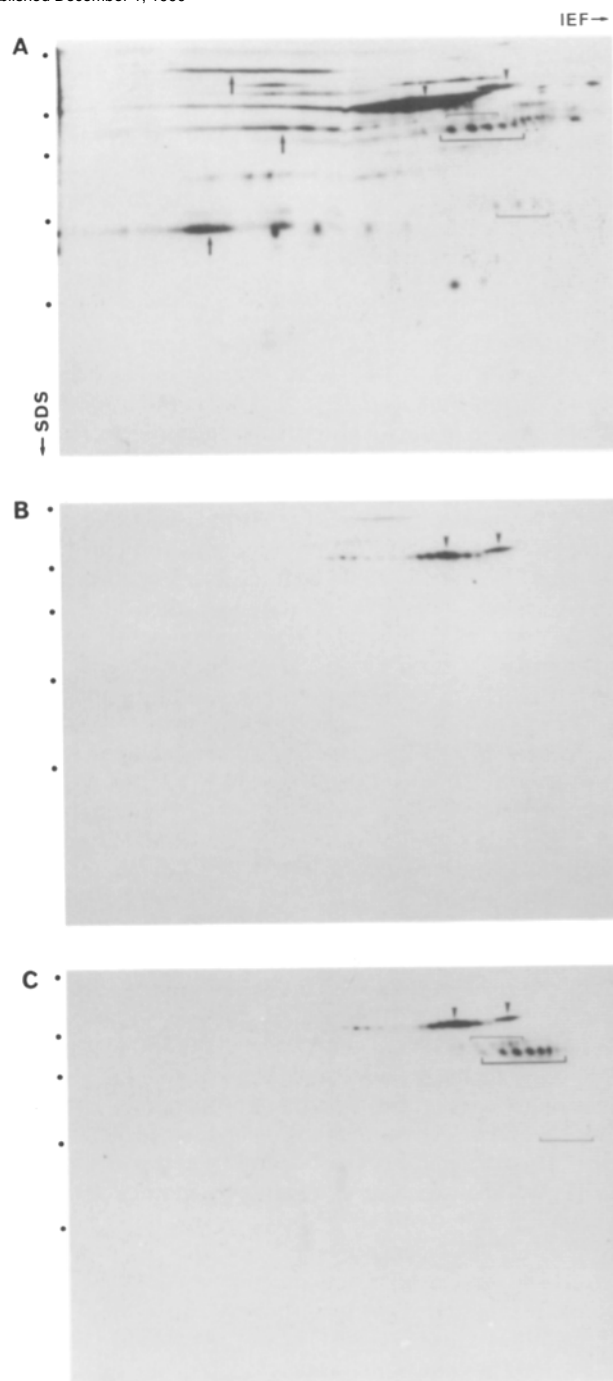
IEF→

A

↓SDS

B

C

A
a b

B
a b c d e f

Figure 10. Surface distribution and apical-to-basolateral transcytosis of the 85 kD protein. (*A*) Surface distribution of the 85-kD protein recognized by the monoclonal anti–114-kD antibody. Filter-grown MDCKII-RCA^r cells were exogalactosylated with [^{3}H]galactose either apically (lane *a*) or basolaterally (lane *b*). Detergent extracts were immunoprecipitated with the monoclonal anti–114-kD antibody. Immunoprecipitates were analyzed by SDS-PAGE. (*B*) Apical-to-basolateral transcytosis of the 85-kD protein. Filter-grown MDCKII-RCA^r cells were exogalactosylated apically at 4°C. Incubations at 37°C were for 0.5 (lane *c*), 1 (lane *d*), 2 (lane *e*), and 4 h (lane *f*). The incubation at 37°C was omitted from cells in lanes *a* and *b*. Cells were biotinylated apically (lane *a*) or basolaterally (*b–f*), lysed, and subjected to immunoprecipitation using the monoclonal anti–114-kD antibody. The immunoprecipitates were eluted with low pH. Neutralized eluates were treated with streptavidin-agarose (lanes *a–f*), and the adsorbed proteins were analyzed by SDS-PAGE. Due to lack of sensitivity, the 47-kD protein was not detected with this assay. The molecular masses of the indicated marker proteins (*arrowheads*) are as in Fig. 6.

Figure 9. Analysis of glycoproteins transcytosing from the basolateral to the apical membrane by two-dimensional IEF/SDS-PAGE. (*A*) Analysis of total exogalactosylated basolateral glycoproteins. Filter-grown MDCKII-RCA^r cells were exogalactosylated basolaterally with [^{3}H]galactose. Total cell lysates were prepared and analyzed by two-dimensional IEF/SDS-PAGE. (*B* and *C*) Analysis of apical transcytosing glycoproteins. Filter-grown MDCKII-RCA^r cells were exogalactosylated basolaterally, incubated for 0 (*B*) or 3 h (*C*) at 37°C and labeled apically with NHS-SS-biotin. Cells were lysed, treated with streptavidin-agarose, and analyzed by two-dimensional IEF/SDS-PAGE. Brackets indicate examples of glycoproteins transported from the basolateral to the apical surface by transcytosis. Arrows denote examples of resident apical glycoproteins not undergoing transcytosis. Glycoproteins detected in lysates from control cells (*B*) are shown with arrowheads. The molecular
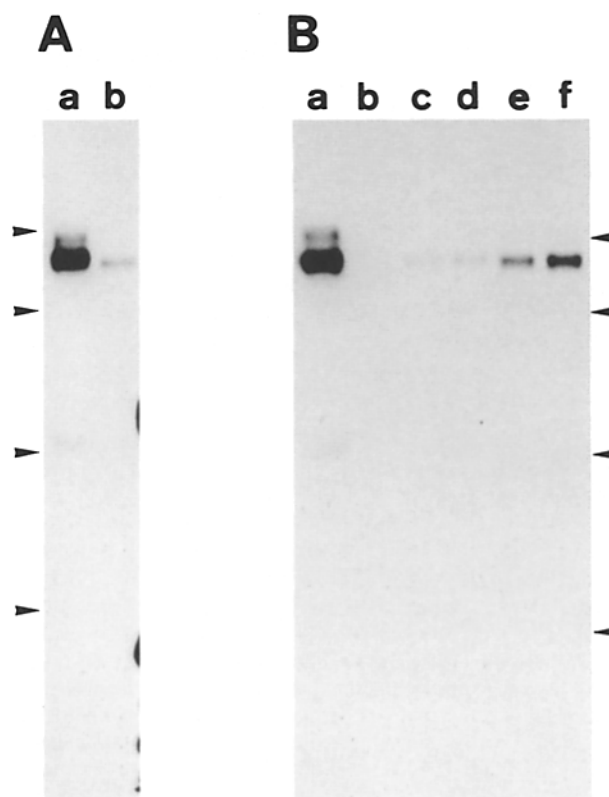
with glycoproteins transcytosing in the apical-to-basolateral direction (Fig. 8, lanes *b* and *d*).

We performed two-dimensional IEF/SDS-PAGE of the total pool of basolateral surface glycoproteins (Fig. 9 *A*) and the proteins transported by transcytosis as identified by the transcytosis assay (Fig. 9, *B* and *C*). Control cells that had been exogalactosylated basolaterally and biotinylated apically without 37°C incubation showed that several ^{3}H-

masses of the indicated marker proteins (*closed circles*) are as in Fig. 3.
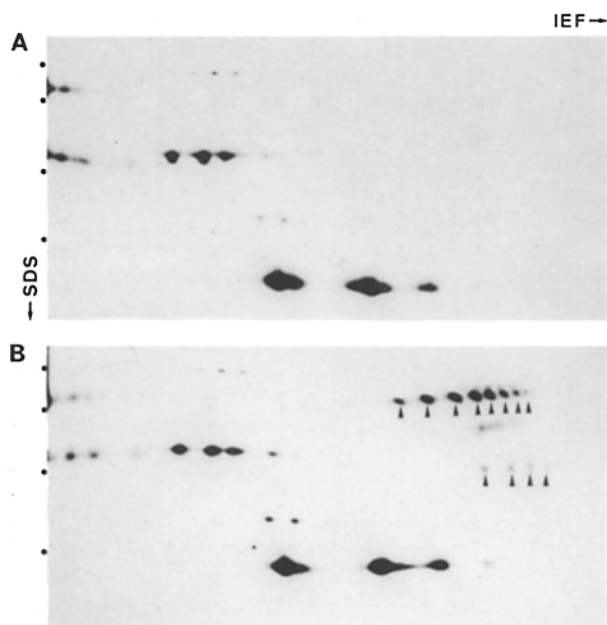
IEF→

—SDS→

A

B

Figure 11. Two-dimensional gel analysis of the 47- and 85-kD transcytosing glycoproteins by immunoprecipitation with the anti-114-kD antibody. A cell lysate was prepared from a filter of unlabeled MDCKII-RCA[r] cells, and subjected to immunoprecipitation using the monoclonal anti-114-kD antibody (*B*). For the control sample, the cell lysate was replaced by an equivalent volume of lysis buffer B (*A*). The immunoprecipitates were labeled with [³H]galactose and subjected to two-dimensional IEF/SDS-PAGE. Glycoproteins specifically precipitated by the antibody are indicated by arrowheads. The molecular masses of the indicated marker proteins (*closed circles*) are as in Fig. 6.

labeled glycoproteins (Fig. 9 *B*, *arrowheads*) had bound to streptavidin. These proteins are also detected in control cell lysates biotinylated by the NaIO₄/BACH method (not shown). Because β-galactosidase treatment (as in Fig. 3 *B*) or incubation with free streptavidin of control cells before cell lysis and streptavidin-agarose precipitation did not diminish the recovery of these proteins (not shown), we assume that the affinity of these glycoproteins to streptavidin-agarose is not mediated by biotin. However, we detect the appearance of two major glycoprotein species, and some minor glycoproteins in lysates of cells that had been incubated at 37°C for 3 h before apical biotinylation (Fig. 9 *C*). We were unable to identify the 67-, the 150-, and the 205-kD transcytosing glycoprotein with our two-dimensional gel system. By comparison with basolaterally exogalactosylated MDCK-II-RCA[r] cells (Fig. 9 *A*), the basolateral-to-apical transcytosing proteins constituted a subset of the entire pool of basolateral glycoproteins. Surprisingly, a group of the glycoproteins in Fig. 9 *C* (*brackets*) showed electrophoretic patterns virtually identical with some of the apical-to-basolateral transcytosing glycoproteins in Fig. 7. We conclude that these molecules represent a new class of cell surface molecules that are able to undergo bidirectional transcytosis in epithelial cells.

A Monoclonal Antibody Recognizes the 85-kD Transcytosing Glycoprotein

The characterization of a monoclonal antibody directed

against an apical 114-kD protein in MDCKII cells has previously been reported (Balcarova-Ständer et al., 1984). To determine the polarity of surface expression of the 114-kD protein in MDCKII-RCA[r] cells, filter grown cells were labeled with [³H]galactose either apically or basolaterally, lysed, and subjected to immunoprecipitation using the anti-114-kD antibody. As shown in Fig. 10 *A* (lane *a*), the precipitates from lysates of apically exogalactosylated cells contain three reactive glycoproteins, a major band of 85 kD and two minor ones of 90 and 47 kD. The altered mobility of the major precipitated glycoprotein compared to the form found in MDCKII cells is probably due to greatly reduced sialylation of the glycoprotein in MDCKII-RCA[r] cells (Brändli et al., 1988; Brändli and Simons, 1989). A fraction (~15%) of the 85-kD protein and of the 47-kD protein was also detected on the basolateral cell surface (Fig. 10 *A*, lane *b*).

The localization to both surface domains and the similarities in relative molecular masses to molecules identified earlier strongly suggested that the glycoproteins recognized by the anti-114-kD antibody could be transported by transcytosis across the cell. Therefore, we tested whether the 85-kD protein precipitated by the anti-114-kD antibody was undergoing transcytosis from the apical to the basolateral surface. MDCKII-RCA[r] cells were exogalactosylated apically, chased for up to 4 h at 37°C, and biotinylated with NHS-LC-biotin basolaterally. Cells were lysed and immunoprecipitations were carried out using the anti-114-kD antibody. The precipitated antigens were released from the antibody–protein A-Sepharose complex by low pH treatment. The eluate was neutralized and subjected to a second precipitation using streptavidin-agarose (Fig. 10 *B*). The fraction of the 85-kD protein sensitive to basolateral biotinylation increased with time showing essentially the same kinetics as seen earlier (see Fig. 3 *B*). At least 23% of the initially ³H-labeled 85-kD protein reached the basolateral surface within 4 h, demonstrating that a significant fraction of the 85-kD protein is transported to the basolateral plasma membrane. However, this number is probably an underestimation of the actual amount of transcytosis in this direction, since the 85-kD protein is also transported in basolateral-to-apical direction.

Finally, we analyzed the glycoproteins immunoprecipitated with the anti-114-kD antibody by two-dimensional IEF/SDS-PAGE. Comparison with Figs. 7 and 9 showed that the anti-114-kD monoclonal antibody precipitates the 85-kD glycoprotein (Fig. 11, *eight arrowheads*). Also, the 47-kD glycoprotein was detected (Fig. 11, *four arrowheads*). Therefore, the 47-kD protein, which coprecipitates with the 85-kD protein, is undergoing transcytosis in the apical-to-basolateral direction (Fig. 7 *B*) as well as in the basolateral-to-apical direction (Fig. 9 *C*, weakly staining).

Discussion

With a few exceptions (Herzog, 1983; King and Johnson, 1985), studies of receptor-mediated transcytosis have been carried out in vivo, rather than in cultured cells. Recent studies have shown that MDCK cells are a useful cell culture system to study transcytosis (von Bonsdorff et al., 1985; Mostov and Deitcher, 1986; Hunziker and Mellman, 1989). In this paper, we have analyzed transcytosis in MDCK cells by focusing on endogenous cell surface proteins. By using an electron microscopic approach, we have determined the

amount of transcytotic traffic of endogenous cell surface proteins across MDCK cells, and established a biochemical assay that permitted the identification of different classes of transcytosing molecules.

Both assays depend on two independent methods to introduce tags into cell surface proteins. First, glycoproteins of the glycosylation mutant, MDCKII-RCA^r, can be efficiently labeled with [^{3}H]galactose by exogalactosylation (Brändli et al., 1988). Second, a number of reagents are available to biotinylate cell surface molecules (Le Bivic et al., 1989; Lisanti et al., 1988, 1989; Sargiacomo et al., 1989). Both labeling methods have been applied to label selectively either apical or basolateral proteins. We have previously shown that exogalactosylation of filter-grown MDCKII-RCA^r cells results in plasma membrane-restricted labeling of glycoproteins (Brändli et al., 1988). The biotin derivatives used here, NHS-LC-biotin and NHS-SS-biotin, carry sulfo-groups that make these compounds water soluble and prevent diffusion across cell membranes. In MDCK cells, these derivatives are also impermeable to the barrier of the tight junctions (Sargiacomo et al., 1989). This is confirmed by our finding that labeled proteins are confined to the biotinylated surface domain. Finally, our observation that transcytosis was inhibited at 20°C or lower temperatures also argues against passive permeation of the tight junctions by the labeling reagents used in this study.

The fate of biotinylated cell surface molecules under conditions that allow membrane traffic to occur was followed by electron microscopic analysis of streptavidin-gold–labeled frozen sections. It was possible to show that transcytosis of cell surface molecules occurs in both directions across MDCK cells. The amounts of transcytosis in each direction was determined separately, and these values were correlated with the surface areas of the two membrane domains. Within 3 h at 37°C, about 10% of the cell surface molecules were transferred to the opposite side. The transcytosis rates appeared to be similar in both directions (Table I). However, the actual transcytosis rates might even be higher due to bidirectional transcytosis of some membrane proteins. In a previous study on MDCK cells, the rate of fluid-phase transcytosis was the same in either direction (von Bonsdorff et al., 1985). Our results demonstrating similar transcytosis rates of cell surface molecules in both directions across MDCK cells are consistent with this study.

The molecular identification of cell surface glycoproteins undergoing transcytosis in MDCK cells was achieved by a novel biochemical assay (Fig. 2). We demonstrated that a subset of apical proteins is transported by transcytosis in a time-dependent manner. Arrival at the basolateral membrane was already detectable after an incubation period of 30 min at 37°C. Depending on the glycoprotein, transport across the cell occurred with approximate half-times of 60–90 min. Basolateral-to-apical transcytosis of surface glycoproteins occurred with similar kinetics (Brändli, A. W., and K. Simons, unpublished observation). Since some of the glycoproteins are transported bidirectionally by transcytosis (e.g., 85- and 97-kD proteins), exact values for transcytosis are difficult to establish. However, our values are consistent with previous findings on transcytotic protein transport in MDCK cells, intestinal epithelial Caco-2 cells, and hepatocytes. EGF is transported with a half-time of 45–60 min from the basolateral to the apical side of MDCK cells (Maratos-Flier et al., 1987). In hepatocytes, transcytosis of

dimeric IgA and of newly synthesized apical proteins from the basolateral membrane to the apical membrane is half-maximal after 45–100 min (Sztul et al., 1983, 1985; Hoppe et al., 1985; Bartles et al., 1987). Similarly, transport of newly synthesized aminopeptidase N from the basolateral to the apical plasma membrane occurs with a half-time of ∼100 min in Caco-2 cells (Matter et al., 1990). Exogenous receptors expressed in MDCK cells show faster kinetics for transcytosis. Basolateral-to-apical transcytosis of dimeric IgA mediated by the polymeric Ig receptors occurs with half-time of 30–45 min (Mostov and Deitcher, 1986; Le Bivic et al., 1990). Half-times for apical-to-basolateral transcytosis of Fc receptors appear to range from 20 to 40 min (Hunziker and Mellman, 1989). Whether these differences are due to the experimental protocols used, or indeed reflect more efficient transcytosis of receptors carrying immunoglobulins remains to be determined.

Previous studies showed that disruption of microtubules interferes with the delivery of newly synthesized membrane and secretory proteins to the apical but not to the basolateral plasma membrane domain in epithelial cells (Achler et al., 1989; Eilers et al., 1989; Parczyk et al., 1989; Rindler et al., 1987). In hepatocytes, transport of fluid-phase markers to the bile caniculus by transcytosis was decreased by >50% in the presence of colchicine (Lowe et al., 1985; Scharschmidt et al., 1986). In MDCK cells, transcytosis of apical proteins to the basolateral surface is not critically dependent on an intact microtubular network (Fig. 6). Depolymerization of microtubules by cold treatment and nocodazole only delayed delivery to the basolateral membrane. This suggests that microtubules might help to facilitate transport across the cell by serving as tracks along which transcytotic vesicles are moved. This process could involve microtubule-based motors, like kinesin or cytoplasmic dynein (Vale, 1987; Sheetz et al., 1989; Scholey, 1990).

By one- and two-dimensional gel electrophoresis, we have established the existence of three classes of transcytosing molecules in MDCKII-RCA^r cells. The first class consisted of glycoproteins with relative molecular masses of 105, 155, and 215 kD that are transported in the apical-to-basolateral direction. Members of the second class were basolateral-to-apical transcytosing molecules of 150 and 205 kD. All these glycoproteins appeared to be transported unidirectionally across MDCKII-RCA^r cells. Similarly, transcytosis of Fc receptors and the polymeric Ig receptor is unidirectional and, in the latter case, is accompanied by proteolytic cleavage of the receptor during transport (Mostov and Deitcher, 1986; Breitfeld et al., 1989; Hunziker and Mellman, 1989). The third class of glycoproteins identified in this study consists of molecules which are transported bidirectionally across MDCKII-RCA^r cells. We have been able to characterize four glycoproteins of 47, 67, 85, and 97 kD which fall into this category. The possibility of cell surface glycoproteins shuttling back and forth between the apical and basolateral surfaces of epithelial cells has been suggested for the IgG receptor (Rodewald and Kraehenbuhl, 1984). We now provide biochemical evidence for the existence of such a class of cell surface glycoproteins.

Molecules transported by transcytosis across the epithelial cell represent a subset of the cell surface glycoproteins. This indicates that transcytosis is an endocytic event which requires special sorting signals. Transcytosing proteins seem to have endocytic sorting signals of the same type as recy-

cling plasma membrane proteins (Breitfeld et al., 1989; Hunziker and Mellman, 1989). The nature of the sorting signals responsible for transcytosis is still unknown. For the polymeric Ig receptor, phosphorylation has been shown to play a role in the process of transcytosis (Casanova et al., 1990). We detect no differences in the electrophoretic pattern for the 47- and the 85-kD transcytosing proteins whether they are expressed on the apical or basolateral surface. Therefore, covalent posttranslational modifications that introduce a change in the isoelectric point of the protein, e.g., phosphorylation, seem not to occur during transcytosis of these proteins. However, we cannot exclude the possibility that transcytosing proteins carry modifications that are transient. Alternatively, the proteins undergo reversible conformational changes before they transcytose. These conformational changes would mask or expose a domain of the polypeptide chain that would be recognized or excluded by the sorting machinery. The available evidence suggests that receptors that undergo transcytosis are first delivered to early endosomes by endocytosis, and are then sorted into transcytotic vesicles for transport across the cell (Geuze et al., 1984; Bomsel et al., 1989). The apical and basolateral early endosomes in MDCK differ in their functional characteristics (Bomsel et al., 1989). Therefore, it could very well be that the intraluminal environment in the early apical and basolateral endosomes is different as well. Structural changes of the transcytosing proteins could be regulated by the unique environment inside early endosomes. Reversibility of the conformational changes would allow continuous shuttling of such molecules across the cell.

At present, we can only speculate on the possible functions of the transcytosing molecules that were identified in MDCK cells. These cells are epithelial cells derived from the kidney tubule (McRoberts et al., 1981; Herzlinger et al., 1982). They are able to transport sodium and water in an apical-to-basal direction (Misfeldt et al., 1976) reflecting in vivo functions such as reabsorption of salts and urine concentration. Recently, receptors for the intrinsic factor–cobalamin (vitamin B_{12}) complex have been identified and immunolocalized to the apical surface of proximal tubular cells (Seetharam et al., 1988). In epithelial cells of the distal kidney tubule, transcytosing molecules might be involved in retrieval of compounds, such as hormones, growth factors, and vitamins, from the urine. Receptor-mediated apical-to-basolateral transcytosis across the epithelium would ensure reutilization of specific molecules that otherwise would be lost in the urine. The ability to undergo bidirectional transcytosis would ensure that the receptor could function for several cycles. It is also possible that some of the transcytosing molecules are part of the molecular machinery responsible for transcytosis. If this were the case, they should be expressed in other epithelial cells as well. The identification of glycoproteins transported by transcytosis in MDCK cells, may also allow insight into the mechanisms of invasion and penetration of epithelial cell layers by pathogens. Recently, MDCK cells have served as a model system to study penetration of pathogens through epithelial barriers (Finlay et al., 1988). In this system, *Salmonella* are transported by transcytosis in apical-to-basolateral direction across the monolayer. Specific apical adhesion of *Salmonella* to glycoproteins which are transported constitutively to the basolateral surface might be an important step in the penetration process. The availability of specific tools, such as antibodies, will allow further clarification of these issues.

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