Steady-State Distribution and Biogenesis of Endogenous Madin–Darby Canine Kidney Glycoproteins: Evidence for Intracellular Sorting and Polarized Cell Surface Delivery

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Abstract. We used domain-selective biotinylation/125I-streptavidin blotting (Sargiacomo, M., M. P. Lisanti, L. Graeve, A. Le Bivic, and E. Rodriguez-Boulan. 1989. J. Membr. Biol. 107:277–286), in combination with lectin precipitation, to analyze the apical and basolateral glycoprotein composition of Madin–Darby canine kidney (MDCK) cells and to explore the role of glycosylation in the targeting of membrane glycoproteins. All six lectins used recognized both apical and basolateral glycoproteins, indicating that none of the sugar moieties detected were characteristic of the particular epithelial cell surface. Pulse-chase experiments coupled with domain-selective glycoprotein recovery were designed to detect the initial appearance of newly synthesized glycoproteins at the apical or basolateral cell surface. After a short pulse with a radioactive precursor, glycoproteins reaching each surface were biotinylated, extracted, and recovered via precipitation with immobilized streptavidin. Several basolateral glycoproteins (including two sulfated proteins) and at least two apical glycoproteins (one of them the major sulfated protein of MDCK cells) appeared at the corresponding surface after 20–40 min of chase, but were not detected in the opposite surface, suggesting that they were sorted intracellularly and vectorially delivered to their target membrane. Several “peripheral” apical proteins were detected at maximal levels on the apical surface immediately after the 15-min pulse, suggesting a very fast intracellular transit. Finally, domain-selective labeling of surface carbohydrates with biotin hydrazide (after periodate oxidation) revealed strikingly different integral and peripheral glycoprotein patterns, resembling the Con A pattern, after labeling with sulfo-N-hydroxy-succinimido-biotin. The approaches described here should be useful in characterizing the steady-state distribution and biogenesis of endogenous cell surface components in a variety of epithelial cell lines.

Polarized epithelial cells contain two distinct plasma membrane domains of unique protein and lipid composition (8, 30, 31, 43). Tight junctions, which mark the boundary between the apical and basolateral cell surfaces, provide a selective barrier to the passage of ions and macromolecules and act as a fence to prevent the mixing of cell surface components (10, 11, 19, 35).

The well-characterized Madin–Darby canine kidney (MDCK) cell line has provided an excellent in vitro model system for the study of the biogenesis of this polarized phenotype (2, 21, 38, 46, 52, 53, 58, 63). The observation that enveloped RNA viruses bud asymmetrically from polarized epithelial cells (49) has been exploited to study some of the questions surrounding the generation and maintenance of epithelial cell polarity. The viral envelope spike glycoproteins, influenza HA and vesicular stomatitis virus (VSV) G-protein, have served as model apical and basolateral membrane antigens, respectively (48). During viral infection, they are produced at levels 100-1,000-fold higher than corresponding plasma membrane proteins (46, 58). A synchronous wave of viral protein transport to each surface can be monitored by using temperature-sensitive viral mutants (45, 50, 54) or the 20°C Golgi block (18). Numerous studies with the MDCK cell line indicate that the sorting of these exogenous viral antigens occurs intracellularly in the transmost compartment of the Golgi apparatus, the trans Golgi network (17, 45), by incorporation into different “carrier vesicles” that are vectorially delivered to the respective surfaces (29, 34, 40, 44, 50).

An alternative route to the cell surface has been postulated, however, in other epithelial systems. In vivo metabolic labeling coupled with subcellular fractionation has been used to study the biogenetic pathways of endogenous membrane...
glycoproteins of the enteroctye (20, 28, 36, 41, 42) and the hepatocyte (4). Several apical antigens were transiently expressed in basolateral membrane fractions, suggesting that all membrane glycoproteins are transported first to the basolateral cell surface and that apical membrane antigens are sorted at the basolateral surface followed by rerouting to the apical cell surface. However, evidence for vectorial delivery of an apical glycoprotein has also been presented in intestinal cells (15, 26). Possible explanations for such apparently contradictory results may lie with the different approaches used to follow the targeting pathways, or with the nature of the different model systems (renal, intestinal, or hepatic). Furthermore, since the only apical antigen studied using the MDCK cell line was an exogenous viral glycoprotein, the possibility remains that endogenous apical antigens are sorted at the basolateral cell surface (3). As of yet, no evidence has been presented to support other possible sorting pathways, such as (a) random delivery and retrieval of apical and basolateral antigens to their correct domains; or (b) transport of all membrane glycoproteins to the apical cell surface with retrieval of basolateral antigens to their correct domain.

To provide a definite answer on whether different mechanisms operate in different epithelial cell types, it is of great interest to analyze the biogenesis of endogenous plasma membrane proteins in MDCK cells. To date, only one endogenous MDCK protein (Na÷/K÷-ATPase) has been characterized and found to be delivered vectorially to the basolateral membrane, using domain restricted labeling with an ouabain analogue and immunoprecipitation with an anti-ouabain antibody (9). Unfortunately, this approach is not applicable to other plasma membrane proteins and cell fractionation procedures do not provide sufficiently pure fractions in epithelial cell lines. However, we recently developed a method to selectively label the apical or basolateral surface proteins of filter-grown MDCK monolayers with a membrane/tight junction-permeant probe (sulfo-N-hydroxy-succinimido-biotin [sulfo-NHS-biotin]) that, in combination with Triton X-114 (TX-114) phase separation, allowed us to characterize the integral and peripheral membrane protein composition of each surface domain (27, 57). This method eliminates the need for cell fractionation, since domain-selective labeling provides essentially pure fractions of apical and basolateral membrane proteins.

Here, we describe the endogenous glycoprotein composition of the apical and basolateral cell surfaces of MDCK cells after domain-selective labeling and precipitation with immobilized lectins. In addition, we extend this approach to study the biogenesis and targeting of endogenous MDCK glycoproteins. Pulse-chase experiments were coupled with domain-selective biotinylation (and recovery with immobilized streptavidin), to follow the cell surface appearance of newly synthesized apical and basolateral glycoproteins. A host of endogenous MDCK glycoproteins were observed to be vectorially delivered to the respective cell surface.

Materials and Methods

Reagents

Sulfo-NHS-biotin, biotin hydrazide, and immobilized streptavidin were purchased from Pierce Chemical Co. (Rockford, IL.). immobilized lectins (Con A, wheat germ agglutinin [WGA], Ricinus communis agglutinin 1 [RCA-I], peanut agglutinin [PNA], Ulex europaeus agglutinin I [UEA], and Dolichos biflorus agglutinin [DBA]) were obtained from E.Y. Laboratories, Inc. (San Mateo, CA). Sodium periodate, protease inhibitors (leupeptin, pepstatin A, and antipain), TX-114, Triton X-100 (TX-100), BSA, and biotin were filled with an equivalent volume of ice-cold PBS-C/M. After 30 min of agitation at 4°C, filter chambers were washed with serum-free media (once) and PBS-C/M (three times).

Biotin Hydrazide. Approximately 1 ml of NaIO4 (10 mM in ice-cold PBS-C/M) was added either to the apical or basolateral compartment of the filter chamber (27, 57). Compartments not receiving sulfo-NHS-biotin (22, 24) or biotin hydrazide (5, 39, 51) as described below.

Cell Culture/Monolayer Integrity

MDCK cells, type II, were maintained in DMEM (Gibco Laboratories, Grand Island, NY) supplemented with 10% (vol/vol) horse serum (Hyclone Laboratories, Logan, UT) and antibiotics (47). For dissociation with trypsin-EDTA, cells were plated at high density in plastic petri dishes (35 mm) or in 24.5-mm (0.4 t~m polycarbonate, tissue culture-treated) filter chambers (Transwell, Costar, Cambridge, MA) (32). Media was changed every 2-3 d and filter-grown monolayers were assayed for impermeability to [3H]inulin (ICN Laboratories, Inc., Irvine, CA) 6-8 days after plating essentially as previously described (9). Monolayer permeability was expressed as a percentage of the total radioactivity added to the apical compartment that leaked to the basolateral compartment. Monolayers showing permeability >10% in 2 h were discarded.

Selective Biotinylation of the Apical or Basolateral Cell Surface

MDCK type II, filter-grown monolayers, were washed (four times) with ice-cold PBS containing 1 mM MgCl2, 0.1 mM CaCl2 (PBS-C/M) and agitated for 30 min at 4°C. After washing, monolayers were selectively labeled with sulfo-NHS-biotin (22, 24) or biotin hydrazide (5, 39, 51) as described below. Time-course and concentration-dependence experiments (data not shown) were used to determine the conditions which produced optimal labeling of MDCK plasma membrane proteins.

Sulfo-NHS-Biotin. Approximately 1 ml of sulfo-NHS-biotin (0.5 mg/ml in ice-cold PBS-C/M) was added either to the apical or basolateral compartment of the filter chamber (27, 57). Compartments not receiving sulfo-NHS-biotin were filled with an equivalent volume of ice-cold PBS-C/M. After 30 min of agitation at 4°C, filter chambers were washed with serum-free media (once) and PBS-C/M (three times).

Metabolic Labeling with [35S]Cysteine or Sulfate

Filter-grown, "tight" MDCK monolayers were washed (twice) with ice-cold PBS-C/M and labeled with [35S]cys in DME containing 10 mM Hepes, pH 7.3, 0.2% BSA (lacking antibiotics) for 30 min at 4°C. After washing, monolayers were selectively labeled with [35S]cys (New England Nuclear, Boston, MA) (62). Steady-state labeling, filters were labeled with 100 μCi/ml [35S]cys in DME containing 1/10 the normal concentration of met and cys, 10 mM Hepes, pH 7.3, 0.2% BSA for 15 min at 37°C and chased for various times (as indicated) in DME containing excess met and cys. More specifically, 1450 μl of labeling medium containing radiolabel was added to the basolateral side of each filter chamber that leaked to the basolateral compartment (placed inverted in a humidified chamber) (37, 62). Filters were then transferred after the pulse to six-well dishes and 1 ml of medium was added to the apical and basolateral compartments of the filter chamber.

Metabolic labeling with [35S]sulfate (New England Nuclear) was performed essentially as described above for labeling with [35S]cys (23). For steady-state labeling, filters were labeled with 100 μCi/ml [35S]sulfate in DME containing 10 mM Hepes, pH 7.3, 0.2% BSA (lacking antibiotics) for 12-16 h. For pulse-chase experiments, filter-grown monolayers were labeled with 1 μCi/ml [35S]sulfate (in the above media) for 15 min at 37°C.
and chased for various times in DME containing 10 mM Hepes, pH 7.3, 0.2% BSA, and antibiotics (penicillin/streptomycin/fungizone; Gibco Laboratories).

**Cell Lysis and Lectin Precipitation**

Filters were excised from the chamber with a razor blade and extracted with 1 ml of ice-cold TBS (10 mM Tris, pH 7.4, 0.15 M NaCl) containing 1 mM EDTA and 1% (vol/vol) TX-114 for 30–45 min on ice. Cell extracts were transferred to Eppendorf tubes and clarified by centrifugation (14,000 g for 10 min). Supernatants were collected and subjected to a temperature-induced phase separation as previously described (7, 13). It should be noted that all solutions used for cell extraction and phase separation contained 10 µg/ml of each of the following protease inhibitors: leupeptin, pepstatin A, and antipain. Resulting aqueous and detergent phases were readjusted to 1% (vol/vol) TX-114 and precipitated with immobilized lectins, or directly precipitated with either 5% of acetone (–20°C for 30 min) or immobilized streptavidin.

For precipitation with immobilized lectins, aqueous and detergent phases were precleared with 40 µl/ml Sepharose CL-4B (Pharmacia Fine Chemicals, Uppsala, Sweden) for 30 min at 4°C. (Note that Sepharose CL-4B was washed three times with TBS, pH 8.0, containing 1% [vol/vol] TX-100 [TBST] before use.) Approximately 50 µl of immobilized lectin gel was added to each 1-ml sample and allowed to bind overnight (12–16 h) at 4°C while rotating end over end. After binding, the immobilized lectins were collected by centrifugation (14,000 g for 30 s) and washed (five times) with TBST.

Bound proteins were eluted with 200 µl of the specific hapten sugar (0.05–0.2 M in TBST depending on the lectin) overnight (16–24 h) at 4°C while rotating end over end. Released proteins were precipitated with 5 vol of acetone (–20°C for 30 min) and collected by centrifugation (14,000 g for 15 min) at 4°C or diluted fivefold with TBST and precipitated with immobilized streptavidin (see Streptavidin Precipitation). Acetone precipitates were resuspended in 100 µl Laemmli sample buffer, boiled for 2–3 min, and stored frozen at –20°C.

Serial lectin precipitations were performed essentially as above. However, after bound proteins were eluted with the specific hapten sugar, the eluate was diluted fivefold with TBST and subjected to a second round of lectin precipitation with a different immobilized lectin gel. It should be noted that all solutions used for Con A binding contained 2 mM CaCl2 and 2 mM MnCl2.

**Streptavidin Precipitation of Metabolically Labeled/Biotinylated Extracts**

Approximately 50 µl of immobilized streptavidin (6% cross-linked agarose) was added to each 1-ml sample and allowed to bind overnight (12–16 h) at 4°C while rotating end over end. After binding, immobilized streptavidin was recovered by centrifugation (14,000 g for 30 s) and washed (five to six times) with TBST. Bound proteins were eluted by the addition of 100 µl Laemmli sample buffer and repeated rounds of agitation and boiling (three times for 5 min each). Fresh 2-mercaptoethanol was added after each boiling step to a final concentration of 5% (vol/vol). Routinely, 90% of bound cpm's were eluted using the above scheme.

To estimate the recovery of metabolically labeled/biotinylated proteins with immobilized streptavidin, repeated rounds of streptavidin precipitation were performed using TX-114 extracts of 125I-labeled/biotinylated monoclonal antibodies. After the first round of incubation of aqueous and detergent phases with immobilized streptavidin (50 µl per round of incubation), roughly 80% and 95%, respectively, of streptavidin-precipitable counts were recovered (data not shown). Thus, only a single round of precipitation was necessary to recover the bulk of the metabolically labeled/biotinylated proteins.

**Detection of Biotinylated Proteins**

**Immunofluorescence and Semithin Frozen Sections.** Monolayers were fixed (2% [vol/vol] paraformaldehyde) and semithin frozen sections (0.5 µm) were prepared as described by Tokuyasu (59). Biotinylated proteins were visualized by incubation with a 1:50 dilution of Texas red–conjugated streptavidin (Cooper Biomedical, Inc., Malvern, PA.).

**Electrophoresis and Electroblothing.** Samples were subjected to SDS-PAGE (25) under reducing conditions (10% or 6–16% gradient acrylamide gels) and transferred to nitrocellulose (61). Nitrocellulose sheets were blocked with 3% BSA/1% nonfat dry milk (Carnation Co., Los Angeles, CA) for 1 h in PBS containing 0.5% (vol/vol) Tween 20, 10% (vol/vol) glycerol, 1 M glucose (6). After incubation with 125I-streptavidin (1–2 × 105 cpm/ml in Tween 20/glycerol/glucose containing 0.3% BSA) for 2 h, blots were washed (three times for 10 min each) with PBS containing 0.5% (vol/vol) Tween 20 and dried. Biotinylated proteins were visualized by autoradiography on Kodak XAR-5 film (–80°C with an intensifying screen).

Iodinated streptavidin was prepared using Na-25II (New England Nuclear) and chloramine T yielding a specific activity of 5–10 µCi/µl. Molecular mass markers used were as follows: myosin heavy chain, 200 kD; phosphorylase b, 97.4 kD; BSA, 68 kD; ovalbumin, 43 kD; carbonic anhydrase, 29 kD; b-lactoglobulin, 18.4 kD; and lysozyme, 14.3 kD.

**Electrophoresis and Na-Salicylate Treatment.** Metabolically labeled/biotinylated samples were subjected to SDS-PAGE (25) under reducing conditions (10% polyacrylamide). After fixation (40% methanol/10% acetic acid for 30–60 min) gels were rehydrated (10% glycerol for 30 min) and impregnated with Na-salicylate (1 M solution for 30 min) (12). Dried gels were subjected to autoradiography (12 h to 10 d at –80°C) with Kodak XAR-5 film.

**Results**

**Polarized Distribution of MDCK Lectin-binding Proteins (LBPs)**

The endogenous glycoprotein composition of apical and basolateral MDCK surface domains has not been fully characterized, partly because of the lack of methods to isolate pure apical and basolateral membrane fractions. A recent report used domain-selective galactosylation of a ricin resistant MDCK cell line, MDCK-RCA', to characterize a glycoprotein subset (8). Here, we use a more comprehensive approach which combines domain-selective biotinylation with TX-114 phase separation (57) and lectin precipitation, to analyze the polarized distribution of glycoproteins with affinity for a specific lectin (Fig. 1 a, scheme 1). The typical patterns obtained by 125I-streptavidin blotting after domain-selective biotinylation and phase separation are shown (Fig. 2 b).

Metabolic labeling with [3H]galactose was performed for comparison (Fig. 2 a). Relatively few glycoproteins incorporated [3H]galactose, as is the case with metabolic labeling with [3H]glucosamine (55). The most prominent bands included a peripheral membrane protein of 150–180 kD and an integral membrane protein of 100 kD (“peripheral” and “integral” are operationally defined throughout the text by TX-114 phase separation) (7). Metabolic labeling with [3H]sugars does not distinguish between apical or basolateral membrane proteins, nor can it distinguish between cell surface or internal glycoproteins (without additional manipulations) and requires long exposure times (2–4 wk).

In contrast, domain-selective labeling with sulfo-NHS-biotin followed by lectin precipitation and 125I-streptavidin blotting allowed the identification of cell surface glycoproteins confined to a specific domain (apical or basolateral) and could be visualized after hours of exposure (5–24 h). Six immobilized lectins recognizing different sugar moieties were chosen for evaluation. PNA was chosen to evaluate the polarity of O-linked glycosylation, since it recognizes a disaccharide (terminal galactose linked to N-acetyl galactosamine) not found in N-linked glycans, but present in O-linked glycans and glycosphingolipids (8). After domain-selective labeling and phase separation with TX-114, extracts were incubated with immobilized lectins (batchwise) and eluted with high concentrations of the competing hapten sugar. None of the lectins used solely recognized glycoproteins of either apical or basolateral origin, indicating that the sugar specificities tested, none was confined to either the apical or the basolateral cell surface (Fig. 3). Furthermore, several...
lectins appeared to recognize the same group of endogenous glycoproteins (Con A, WGA, and RCA-I).

To verify that certain glycoproteins were recognized by the three lectins that gave the strongest binding signals, a serial lectin precipitation was performed. Either Con A, WGA, or RCA-I was used for the first round of precipitation. After elution with the appropriate competing hapten sugars, the eluate was diluted and precipitated a second time with a different immobilized lectin (i.e., first Con A then WGA or RCA second, etc.). After the second round of precipitation, bound glycoproteins were eluted with the appropriate competing sugar and acetone precipitated. The results of these experiments (Fig. 4; summarized in Table I) confirmed our suspicion that the same group of glycoproteins was commonly recognized by Con A, WGA, and RCA-I and operationally define the "common" lectin-binding proteins.

Figure 1. Flow diagrams summarizing the different approaches developed to study the steady-state distribution and biogenesis of endogenous MDCK glycoproteins. (a) Tight MDCK filter-grown monolayers were either metabolically labeled (scheme 2, labeled with [35S]cys; scheme 3, with [35S]sulfate) or left unlabeled (scheme 1, no labeling). Subsequently, monolayers were surface labeled with sulfo-NHS-biotin; i.e., apically (Ap) or basolaterally (Bl). After domain-selective biotinylation, filters were then excised from the filter chambers and extracted with the detergent TX-114. Temperature-induced phase separation was used to separate hydrophilic peripheral from hydrophobic integral membrane proteins (partitioning into aqueous [aq] and detergent [det] phases, respectively). Glycoproteins could then be precipitated with immobilized lectins and eluted with the appropriate competing hapten sugars (schemes 1 and 2). Biotinylated cell surface glycoproteins were visualized by 125I-streptavidin blotting (scheme 1) or by recovery of metabolically labeled glycoproteins containing biotin via immobilized streptavidin precipitation (schemes 2 and 3). 125I- or 35S-labeling was visualized by autoradiography. (b) A similar approach, combining periodate oxidation and reaction with biotin hydrazide, was used to directly label cell surface glycoconjugates.

Recovery of Metabolically Labeled/Biotinylated LBPs with Immobilized Streptavidin: Evidence for Intracellular Sorting and Vectorial Delivery

The biogenesis and sorting of polarized membrane glycoproteins has long remained an area of interest and a source of great controversy. Two distinct mechanisms have emerged: (a) intracellular sorting and vectorial delivery of plasma membrane proteins; and (b) sorting of apical proteins at the level of the basolateral membrane (see Introduction). The first mechanism has been observed for viral glycoproteins (29, 34, 40, 45, 54) and Na, K-ATPase (9) in MDCK cells, whereas the second mechanism has been described in intestinal and liver cells (reviewed in reference 3). It is unclear to what extent the different mechanisms can be attributed to differences between epithelial cell types, different methods, or to the fact that only viral apical glycoproteins were analyzed in MDCK cells.

To study the surface appearance of endogenous MDCK glycoproteins during their biogenesis, we explored the possibility of recovering biotinylated glycoproteins (after metabolic labeling) via precipitation with immobilized streptavidin. Tight MDCK monolayers were metabolically labeled overnight with [35S]cys, followed by domain-selective biotinylation and precipitation with immobilized lectins. After elution with the appropriate hapten sugar, the fraction of radiolabeled glycoproteins containing biotin was recovered with immobilized streptavidin, and 35S-labeled proteins vi-
Figure 3. Identification of major MDCK LBPs: precipitation with immobilized lectins after domain-selective biotinylation. Tight MDCK filter-grown monolayers (7-8 d confluent) were apically (A) or basolaterally (B) biotinylated with sulfo-NHS-biotin (0.5 mg/ml; 30 min at 4°C). After temperature-induced phase separation using the detergent TX-114, glycoproteins present in the detergent (a, det) or aqueous (b, aq) phase were precipitated with six immobilized lectins of different specificity. After extensive washing, bound glycoproteins were eluted with high concentrations (0.05-0.2 M) of the appropriate competing hapten sugar. Eluted glycoproteins were recovered by precipitation with 5 vol of cold acetone and visualized by autoradiography after ~25I-streptavidin blotting. A prominent 100-kD apical integral protein (a, lanes 1, 3, 5, and 7) appeared to be recognized by a wide variety of lectins. Similarly, a prominent 20-25-kD apical peripheral membrane protein (b, lanes 1, 3, 9, and 11) was also recognized by several different lectins, but most specifically by PNA. Since PNA recognizes specific sugar linkage found only in O-linked sugar moieties and glycosphingolipids, these 20-25-kD glycoproteins (b, lane 9) represent the major apical MDCK O-linked glycoproteins. Molecular mass standards are as indicated in Fig. 2.

Figure 4. Identification of MDCK common LBPs via serial lectin precipitation. Tight MDCK filter-grown monolayers (7-8 d confluent) were subjected to domain-selective labeling with sulfo-NHS-biotin. Apically (A) or basolaterally (B) biotinylated monolayers were extracted with TX-114, partitioned into detergent (a, det) and aqueous (b, aq) phases, and precipitated with either immobilized Con A, WGA, or RCA-I (indicated by the number 1 at the bottom of the lanes). After elution with high concentrations of the appropriate competing hapten sugar, the eluate was diluted and subjected to a second round of precipitation with a different immobilized lectin (indicated by the number 2 at the bottom of the lanes). Eluted glycoproteins were precipitated with 5 vol of cold acetone and visualized by ~25I-streptavidin blotting/autoradiography. A prominent 100-kD apical glycoprotein and a 110-150-kD group of basolateral glycoproteins (all partitioning with the detergent phase) appeared to be commonly recognized by the three lectins used. Similarly, several apical glycoproteins (60-70, 35, and 20-25 kD) and a 85-150-kD group of basolateral glycoproteins (all partitioning with the aqueous phase) were also commonly recognized. Molecular mass standards are as indicated in Fig. 2. It should be noted that the N-acetyl glucosamine remaining after elution from immobilized WGA competitively inhibits subsequent binding to Con A (a and b, lanes 1 and 7).
both apical and basolateral glycoproteins. Thus, Con A was chosen for use in pulse-chase experiments aimed at follow-

When comparing ~25I-streptavidin blotting and metabolic la-

differences of relative intensities of glycoproteins observed the methods used for visualization probably account for the differences in the amino acid used for metabolic labeling. These differences in the methods used for visualization probably account for the differences of relative intensities of glycoproteins observed when comparing 35S-labeled with streptavidin blotting and metabolic labeling with 35S-labeled glycoproteins detected with this approach were observed first in the corre-

Table 1. Endogenous Glycoprotein Composition of the Apical and Basolateral Membrane Domains of MDCK Cells

| Aqueous phase | Detergent phase |
|---------------|----------------|
| Apical | Basolateral | Apical | Basolateral |
| kd | kd | kd | kd |
| Common | 120 | 85-150q | 100 | 110-150q |
| LBP | 85 | 50-60p | 70 | 40 |
| 60-70p | 35 | 30 | 30 |
| 100 | 70 |
| UEA | 85 | 50-60p | 30 | 35 |
| 30 | 30 |
| PNA | 85 | 50-60q | 30 | 30 |
| DBA | 85 | 50-60q | 30 | 30 |
| 20-25p | 30 |

Filter-grown MDCK monolayers (6-7 d confluent) were apically or basolaterally labeled with sulfo-NHS-biotin. After extraction and phase separation with TX-114, aqueous and detergent phases were incubated with immobilized lectins of different specificity. Bound glycoproteins were eluted with high concentrations of the appropriate competing hapten sugar and recovered by acetone precipitation. Biotinylated glycoproteins were visualized by autoradiography after 35S-labeled streptavidin blotting. The molecular masses of the various glycoproteins detected are listed above, p. Pair of polypeptides; t, triplet of polypeptides; q, quartet of polypeptides. 85-150q, quartet with M of 85, 100, 130, and 150 kD: 110-150q, triplet with M of 110, 130, and 150 kD.

Of the three lectins used for precipitation after steady-state labeling with 35S-labeled glycoproteins, Con A bound the widest variety of both apical and basolateral glycoproteins. Thus, Con A was chosen for use in pulse-chase experiments aimed at follow-

ing the biogenesis and targeting of a variety of endogenous MDCK glycoproteins. Tight MDCK monolayers were pulsed for 15 min with 35S-labeled glycoproteins, and chased for various times into medium containing excess cysteine (0, 20, 40, 80, and 160 min). Monolayers were subsequently biotinylated (apically or basolaterally), precipitated with immobilized Con A, and eluted with alpha-methyl-D-mannopyranoside. After elution, the fraction of radiolabeled glycoproteins containing biotin was recovered with immobilized streptavidin (Fig. 6).

Apical glycoproteins detected with this procedure (both integral and peripheral) exhibited extremely fast transit times to the cell surface. Certain apical glycoproteins (70-40- and 30-kD peripheral/30-kD integral) were present maximally at 0 min of chase, indicating that 15 min was sufficient for synthesis, intracellular sorting, and polarized delivery to the apical surface. The amount of these apical glycoproteins appeared to decrease drastically with increasing times of chase, suggesting transient association with the cell surface, followed by (a) secretion or shedding into the medium or (b) recycling to the cell’s interior. The apical 30-40-kD peripheral glycoproteins observed here may represent transient cell-associated forms of the 30-40-kD polypeptides previously found to be secreted preferentially into the apical medium of MDCK monolayers (62). In addition, a pair of apical glycoproteins (20- and 25-kD peripheral; recognized more specifically by PNA) was first detected on the apical cell surface at 40-80 min of chase. The apical 100-kD (integral) glycoprotein did not incorporate 35S-labeled cysteine or 35S-labeled methionine and was only faintly detected (after long exposures) after 40-80 min of chase.

Basolateral glycoproteins (both integral and peripheral) exhibited considerably slower transit times to the cell surface. Certain basolateral glycoproteins (85-kD peripheral and 45-kD integral) first appeared after only 20 min of chase, whereas others (40-, 110-, and 150-kD peripheral and the remaining integral glycoproteins) appeared only after 40-80 min of chase. Thus, the major apical and basolateral proteins observed with this approach were observed first in the corresponding (i.e., apical or basolateral) surface and were not detected in the opposite domain at earlier times of chase, suggesting that they were sorted intracellularly and delivered vectorially to the cell surface.
Figure 6. Biogenesis of major apical (A) and basolateral (B) LBPs. Tight MDCK filter-grown monolayers (7-8 d confluent) were pulsed with \[^{35}S\]cys (15 min; 1 mCi/ml) and chased for various times into medium containing excess cys (0, 20, 40, 80, and 160 min). After domain-selective labeling with sulfo-NHS-biotin, monolayers were extracted with TX-114 and partitioned into detergent (a, det) and aqueous (b, aq) phases. Glycoproteins were precipitated with immobilized Con A and eluted with \(\alpha\)-methyl-D-mannopyranoside. After elution, the fraction of metabolically labeled glycoproteins containing biotin was recovered with immobilized streptavidin. \(^{35}S\)-labeling was visualized by autoradiography. Certain samples (a and b, lanes 1 and 7) were metabolically labeled, but not biotinylated, to examine the possibility of nonspecific binding to immobilized streptavidin. Relatively little or no nonspecific binding was observed. Molecular mass standards are as indicated in Fig. 2. It should be noted that the apparent decrease in intensity observed in b, lane 4, is an artifact of underloading (half the sample was loaded).

Recovery of Metabolically Labeled Sulfated Glycoproteins with Immobilized Streptavidin: Additional Evidence for Intracellular Sorting and Vectorial Delivery

Protein sulfation is a fairly common posttranslational modification of the tyrosine residues or carbohydrate moieties of both secretory proteins and apical microvillar hydrolases (1, 14, 23, 62). Here, we used the biotinylation/immobilized streptavidin approach to explore the biogenesis of sulfated MDCK glycoproteins. For their initial identification, MDCK monolayers were labeled with \[^{35}S\]sulfate for 12–16 h, biotinylated apically or basolaterally, extracted with TX-100, and the fraction of metabolically labeled proteins containing biotin was recovered with immobilized streptavidin (Fig. 1 a, scheme 3). A single major apical glycoprotein of 150–180 kD and two minor basolateral glycoproteins (200 and 100 kD) were identified using this approach (Fig. 7 a). Additional experiments using extraction and phase separation with the detergent TX-114 indicated that these sulfated glycoproteins partitioned with the aqueous phase (operationally defining peripheral membrane proteins) (Fig. 7 b). No sulfated glycoproteins partitioning with the detergent phase were observed even on overexposure of the autoradiograph, suggesting that the major sulfated MDCK cell surface glycoproteins were peripherally associated with the plasma membrane. A similar apical glycoprotein (150–180 kD) was detected during steady-state metabolic labeling with \[^{3}H\]galactose (Fig. 2 a, lane 1) or \[^{35}S\]cys followed by lectin precipitation (Fig. 5 b, lanes 1, 3, and 5).

To tentatively localize the sulfation to either carbohydrate moieties or tyrosine residues, we took advantage of a lectin-resistant mutant cell line, MDCK II-RCA', which contains a pleiotropic defect in galactosylation (8, 16, 33). The nature of the defect (affecting both N- and O-linked glycosylation of proteins, as well as glycosphingolipid synthesis) apparently results from defective uptake of UDP-galactose at the level of the Golgi apparatus (8). MDCK II-RCA' were metabolically labeled with \[^{35}S\]sulfate and biotinylated (apically or basolaterally), and extracted with the detergent TX-114. Biotinylated proteins were recovered via precipitation with immobilized streptavidin. Only a 200-kD basolateral glycoprotein partitioning with the aqueous phase was observed (data not shown). Thus, the metabolic incorporation of

Figure 7. Recovery of metabolically labeled sulfated glycoproteins with immobilized streptavidin. Tight MDCK filter-grown monolayers (7-8 d confluent) were metabolically labeled overnight with \[^{35}S\]sulfate (100 \(\mu\)Ci/ml), and surface-labeled apically (A) or basolaterally (B) with sulfo-NHS-biotin. Monolayers were either extracted with TX-100 (a) or TX-114 (b). After extraction with TX-114, cell lysates were partitioned into aqueous (aq) and detergent (det) phases. In all cases, the fraction of metabolically labeled proteins containing biotin was recovered with immobilized streptavidin. \(^{35}S\)-labeling was visualized by autoradiography. A major apical glycoprotein of 150–180 kD and two minor basolateral glycoproteins (of 200 and 100 kD) were observed. No sulfated glycoproteins partitioning with the detergent phase were identified even on overexposure of the autoradiograph. (b) Unbiotinylated controls (–, lanes 3 and 6) indicated that little or no nonspecific binding was associated with immobilized streptavidin precipitation. Molecular mass standards are as indicated in Fig. 2.
during the biogenesis of these apical and basolateral MDCK opposite cell surface domain. Thus, intracellular sorting and direct targeting appears to be the primary pathway followed by steady-state labeling) without transient expression on the nal sugar residues (5, 39, 51), was evaluated as a candidate zide, a derivative of biotin which reacts with oxidized termi-

Figure 8. Biogenesis of apical (A) and basolateral (B) MDCK sulfated glycoproteins. Tight MDCK filter-grown monolayers (7-8 d confluent), were pulsed with [35S]sulfate (15 min; 1 mCi/ml) and chased for various times into media containing excess sulfate (0, 20, 40, 80, and 160 min). Monolayers were then subjected to domain-selective labeling with sulfo-NHS-biotin and detergent solubilization. The fraction of metabolically labeled sulfated glycoproteins containing biotin was recovered by precipitation with immobilized streptavidin. 35S-labeled sulfated glycoproteins were visualized by autoradiography. Certain samples (lanes 1 and 7) were metabolically labeled, but not biotinylated. The major apical sulfated glycoprotein (150-180 kD) was detected at 40-80 min of chase, whereas corresponding basolateral sulfated glycoproteins exhibited slightly faster transit times. Molecular mass standards are as indicated in Fig. 2.

[35S]sulfate into the 150-180-kD apical glycoprotein and 100-kD basolateral glycoprotein appeared to be dependent on glycosylation, suggesting that in these glycoproteins sulfation occurred predominantly on carbohydrate moieties.

To further study the biogenesis and targeting of these endogenous MDCK glycoproteins, tight MDCK monolayers were pulsed for 15 min with [35S]sulfate and chased for various times (0, 20, 40, 80, and 160 min) into media containing excess sulfate. Monolayers were subsequently biotinylated (apically or basolaterally), extracted with TX-100, and the fraction of radiolabeled glycoproteins containing biotin recovered via precipitation with immobilized streptavidin (Fig. 8). The apical sulfated glycoprotein (150-180 kD) was detected at 40-80 min of chase, whereas the minor basolateral sulfated glycoproteins (200 and 100 kD), exhibited slightly faster transit times and were detected at earlier times of chase (20-40 min). Both apical and basolateral sulfated glycoproteins appeared to arrive at their final destinations (as defined by steady-state labeling) without transient expression on the opposite cell surface domain. Thus, intracellular sorting and direct targeting appears to be the primary pathway followed during the biogenesis of these apical and basolateral MDCK endogenous glycoproteins.

**Discussion**

We have coupled domain-selective labeling (sulfo-NHS-biotin) with immobilized lectin precipitation to study the polarized distribution of endogenous MDCK membrane glycoproteins at steady state. Six distinct lectins with different specificities were used. None of the lectins recognized glycoproteins solely of either apical or basolateral origin, demonstrating that of the O- and N-linked sugar moieties detected, none were specific to a particular epithelial cell surface. To study the biogenesis of endogenous MDCK glycoproteins, we used a combination of metabolic-labeling and domain-selective biotinylation to detect the appearance of newly synthesized glycoproteins at the cell surface. Filter-grown monolayers were pulsed with radioactive precursors ([35S]cys or [35S]sulfate) and chased for various times to allow glycoprotein processing and arrival at the cell surface. Monolayers were then subjected to domain-selective labeling with sulfo-NHS-biotin. After biotinylation, cells were lysed and the fraction of metabolically labeled/biotinylated proteins recovered with immobilized streptavidin. In this system, the metabolic incorporation of radioactive precursors serves as the signal for the detection of newly synthesized proteins and the biotin moiety serves as a means of recovering the newly synthesized proteins that were transported to the cell surface within a given time.

Using this approach, endogenous apical and basolateral glycoproteins (including the major MDCK sulfated glycoproteins) were observed to be directly delivered to their
respective surface, making their initial appearance within 20–40 min of chase. Similar kinetics of vectorial delivery have been documented for exogenous viral glycoproteins (influenza HA and VSV G protein; see Introduction). In addition, several apical glycoproteins, peripheral and integral, were already present at maximal levels after the 15-min pulse, demonstrating a very fast transit to the apical cell surface. This unusually fast transport has only been previously reported for an acyl-glycosyl-tripeptide apparently transported by bulk flow to the cell surface (64). This report is the first description of the vectorial delivery of endogenous apical proteins in MDCK cells. The cell surface delivery of only one other endogenous MDCK protein, the α-subunit of Na⁺/K⁺-ATPase, has been characterized and found to be vectorial to the basolateral side (9). However, only a single time point, 90 min of chase, was studied in this case. Our findings with endogenous MDCK proteins contrast with those reported by Bartles et al. (4) in hepatocytes, where both apical and basal proteins were initially targeted to the basolateral membrane with similar kinetics (45 min of chase) followed by slow retrieval and transport (90-150 min) of apical proteins to the apical domain.

It may be argued that a very fast passage of apical glycoproteins through the basolateral membrane might have escaped detection by the biotin/streptavidin-agarose procedure reported here. This possibility appears unlikely upon comparison of this procedure with other methods that have been used to demonstrate expression of newly synthesized glycoproteins at a specific cell surface domain (including the procedures used to detect transient appearance of apical proteins

Figure 9. Domain-selective labeling of glycoconjugates with biotin hydrazide: an alternative to sulfo-NHS-biotin. (C) Either the apical (A) or basolateral (B) surfaces of tight MDCK monolayers were reacted sequentially with sodium periodate and biotin hydrazide. After labeling, filter-grown monolayers were fixed and processed for semithin frozen sections (0.5 μm) and biotin visualized with Texas red-conjugated streptavidin (a and b). Alternatively, filters were excised from the chamber and extracted with the detergent TX-114. After temperature-induced phase separation, integral (det) and peripheral (aq) membrane proteins were recovered via acetone precipitation (c). Biotinylated proteins were visualized by autoradiography after 125I-streptavidin blotting. Molecular mass standards (c) are as indicated in Fig. 2.
at the basolateral domain). All the procedures use a general scheme of metabolic labeling with radioactive precursors and immunoprecipitation, but differ in the manner in which the fraction of newly synthesized protein that reaches a specific cell surface is detected. Such approaches include (a) domain-selective trypsin/protease treatment, proteases are added to either cell surface allowing only the digestion of accessible (cell surface) newly synthesized glycoproteins (29). (b) Subcellular fractionation, cells are homogenized and the resulting population of apical and basolateral membrane vesicles, as well as intracellular membrane components, are separated by density gradient centrifugation (4, 15, 28, 36, 41, 42). (c) Photolysis with N-azido-ouabain, a photolyzable derivative of ouabain is added to either cell surface domain. After photolysis, ouabain is covalently attached to the α-subunit of the Na+/K⁺-ATPase and can be recovered by immunoprecipitation with anti-ouabain antibodies (9). Finally, (d) cell surface immunoprecipitation, antibodies are added directly to the cell surface and the preformed immune complexes are retrieved after cell lysis (40). We feel that the new approach described here has several clear advantages over the procedures outlined above, particularly when applied to cell lines. Domain-selective labeling with sulfo-NHS-biotin allows the direct visualization of the fraction of newly synthesized glycoproteins that reaches a given cell surface without the out-put of possible cross-contamination inherent in cell fractionation. Furthermore, the small diffusible biotin probe offers greater accessibility to the basolateral cell surface than proteases or antibodies and has a wide specificity, reacting with virtually any protein which contains exposed primary amino groups. Finally, the biotin/streptavidin–agarose procedure is sufficiently sensitive to easily detect the transient basolateral expression of polymeric Ig receptor in transfected MDCK cells (LeBivic, A., Y. Sambuy, K. E. Mostov, and E. Rodriguez-Boulan, manuscript in preparation). This receptor is translocated immediately after synthesis to the basolateral surface of a variety of epithelial cells (including transfected MDCK cells), where it binds polymeric IgA and IgM, and is then transcytosed to the apical surface (37).

More work is needed to determine whether the differences observed between different epithelia in the biogenetic pathways of apical glycoproteins are real or the result of different methodologies. Some epithelial cell types (e.g., kidney) may sort apical and basolateral proteins intracellularly, whereas others (e.g., liver and intestine) may sort them at the basolateral surface. To solve this question it will be necessary to characterize the biogenetic pathways of identical proteins in the two different epithelial cell environments, using comparable methods. This is currently possible through the use of the biotin/streptavidin–agarose procedure to analyze the targeting of endogenous or transfected proteins in available kidney and intestinal cell lines.

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