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Integral and Peripheral Protein Composition of the Apical and Basolateral Membrane Domains in MDCK Cells

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Summary. Selective biotinylation of the apical or basolateral domains of confluent MDCK monolayers grown on polycarbonate filters with a water soluble biotin analog, sulfo-NHS-biotin, was employed to reveal strikingly distinct patterns of endogenous "peripheral" and "integral" membrane proteins. "Peripheral" proteins were found to be approximately fivefold more abundant with this procedure than "integral" membrane proteins, both on the apical and on the basolateral surface. The distinct apical and basal patterns were shown to depend upon the integrity of the monolayer; when the tight junctions were disrupted by preincubation in calcium-depleted medium, the patterns appeared practically indistinguishable. Two-dimensional gel electrophoresis demonstrated that only a very small percentage of the biotinylated proteins were found in similar amounts on both apical and basolateral domains. These results indicate that the sorting mechanisms that segregate apical and basolateral epithelial proteins are very strict. The simple procedure described here has clear advantages over other methods available to label apical and basal epithelial surface domains, namely, higher accessibility of the biotin probe to the basolateral membrane, possibility of purifying biotinylated proteins via immobilized streptavidin and minimal exposure of the researcher to isotopes. It should be very useful in characterizing the apical and basolateral protein compositions of other epithelial cells and in studies on the development of epithelial cell polarity.

Key Words epithelial cells · cell polarity · plasma membrane proteins · sulfo-NHS-biotin · streptavidin · Triton X-114

Introduction

The renal epithelial cell line, Madin-Darby canine kidney (MDCK), has provided an excellent model system for the in vitro study of the biogenesis of epithelial cell polarity [30, 38]. When grown on permeable supports (such as collagen-coated nylon nets or filters), MDCK monolayers exhibit a high degree of structural and functional asymmetry between the apical and basolateral cell surfaces [8, 13, 23]. A striking example of this asymmetry is the polarized budding of enveloped viruses [11, 22, 31–33]. Tight junctions, located at the boundary between the apical and basolateral cell surfaces, play an important role in the maintenance of such cell surface asymmetries by acting as a selective gate to ions and macromolecules and as a fence that prevents the mixing of both domains [7, 19, 20].

A previous report [29] described the total protein composition of the surface domains of MDCK cells grown on nitrocellulose filters using lactoperoxidase catalyzed iodination. The interpretation of these results is difficult because of the poor accessibility of the enzyme to the intercellular space and because of the large protein binding capacity of the nitrocellulose filters, which does not allow unequivocal distinction between adsorbed serum proteins and endogenous plasma membrane proteins.

In this report, we describe the integral and peripheral membrane proteins characteristic of both domains of MDCK cells, studied with a small and diffusible biotin probe which is, however, impermeable to the barrier of the tight junctions. For these experiments, the cells were grown on polycarbonate filters, which have much lower protein-binding capacity than nitrocellulose filters. Integral and peripheral membrane proteins were defined by their extractability with Triton X-114. The procedure described here offers a unique opportunity to study the polarity of endogenous or exogenous (infected or transfected) membrane glycoproteins in epithelial cell lines from a biochemical standpoint. A preliminary account of this work was presented at the 27th meeting of the American Society for Cell Biology [37].

Materials and Methods

Materials

Bovine serum albumin (BSA), Triton X-100 (TX-100), Triton X-114 (TX-114) were purchased from Sigma (St. Louis, MO).

Sulfo-N-hydroxy-succinimido-biotin (sulfo-NHS-biotin) was purchased from Pierce (Rockford, IL). Electrophoresis reagents were from Biorad (Richmond, CA).

Cell Culture

Madin-Darby canine kidney (MDCK), type II (passage 3–15) were maintained in Dulbecco's modified Eagle's medium (DME) (Gibco, Grand Island, NY) supplemented with 10% (vol/vol) horse serum (Hyclone Laboratories, Logan, UT) and antibiotics [31]. After dissociation with trypsin-EDTA, cells were plated at high density in 24.5 mm (0.4 μm polycarbonate, tissue culture treated) filter chambers (Transwell, Costar, Cambridge, MA [21]). The medium was changed every 2–3 days and experiments were performed 6–8 days after plating.

Monolayer Integrity

Filter-grown monolayers were assayed for impermeability to [^{3}H]-inulin or [^{3}H]-ouabain to assess the integrity of the monolayer as previously described [6]. Briefly, 1 ml complete media containing the above radioactive substances was added to the apical compartment of the filter chamber. After 2 hr incubation at 37°C, samples were collected from the basolateral compartment and counted. Monolayer permeability was expresses as a percentage of the total counts added to the apical compartment that leaked to the basolateral side. Monolayers showing permeability greater than 1% in 2 hr were discarded.

Selective Labeling of the Apical or Basolateral Surface

Biotinylation was performed similarly as described for leukocytes [15] or *D. discoideum* [16]. Time course and concentration dependence experiments indicated that optimal labeling of MDCK plasma membrane proteins was achieved with 0.5 mg/ml sulfo-NHS-biotin for 20 min at 4°C. Filter chambers were washed four times with phosphate-buffered saline (PBS), containing 1 mM $MgCl_2$, 0.1 mM $CaCl_2$ (PBS-C/M) and agitated for 30 min at 4°C. In some experiments, the cells were incubated in Moscona (glucose containing saline) in the absence or presence of Mg^{2+} and Ca^{2+}. Sulfo-NHS-biotin stock solutions (200 mg/ml in DMSO) were stored frozen at −20°C and thawed just before use. Sulfo-NHS-biotin was diluted to a final concentration of 0.5 mg/ml in ice-cold PBS-C/M and used immediately. Approximately 1 ml was added to either the apical or the basolateral compartment of the filter chamber. Compartments not receiving sulfo-NHS-biotin were filled with an equivalent volume of PBS-C/M alone. After 20–30 min of agitation at 4°C, filter chambers were washed with serum-free media (one time) and PBS-C/M (three times).

To visualize the pattern of biotinylated proteins, filters were excised from the chamber with a scapel and extracted with 1 ml of ice-cold lysis buffer (10 mM Tris, pH 7.4, 0.15 M NaCl, 1 mM EDTA, and 1% Triton X-114). After approximately 1 hr of extraction on ice with intermittent mixing, samples were transferred to Eppendorf tubes and clarified by centrifugation (14,000 × *g* for 5 min) at 4°C. Supernatants were collected and subjected to phase separation as described [9]. Phase separation with TX-114 can be used to separate hydrophilic "peripheral" membrane proteins, from hydrophobic "integral" membrane proteins [5, 28].

Alternatively, cells grown on plastic petri dishes (35 mm), were labeled with sulfo-NHS-biotin as described above, lysed in 10 mM Tris, pH 7.4, 0.15 M NaCl, 5 mM EDTA, 1% Triton X-100 (TNET) and extracted for 1 hr at 4°C with agitation.

Viral Infection and Immunoprecipitation

Vesicular stomatitis virus (VSV), wild type (wt) and ts045 mutant, were grown and titered as described previously [31] and used for cell infections. Briefly, subconfluent MDCK cells (in 35-mm petri dishes) were inoculated at a multiplicity of infection (moi) of 10 and 30 for wt and ts045 VSV, respectively. The inoculum was removed and replaced after 1 hr of incubation at 37°C. In certain experiments, virally infected cells were metabolically labeled 3–5 hr postinfection with methionine ([^{35}S] Translabel; ICN, Irvine, CA) at a concentration of 100 μCi/ml in methionine-, cysteine-free DME. Cells inoculated with the ts045 mutant, were incubated first for several hours at the nonpermissive temperature, 39.5°C, to allow accumulation of G-protein in the endoplasmic reticulum and then transferred to the permissive temperature, 32°C, to allow synchronous transport of G to the cell surface [31]. At the expected time of arrival of G-protein at the cell surface, cells were labeled with sulfo-NHS-biotin and lysed in TNET. After centrifugation for 10 min at 4°C (14,000 × *g*), supernatants were subjected to immunoprecipitation with rabbit antiserum against whole *VSV* (contains antibodies against G, M, and N proteins). Immune complexes were precipitated with protein A-Sepharose and processed as described [2].

Detection of Biotinylated Proteins (Electrophoresis and Electroblotting)

Cell extracts precipitated with five volumes of acetone (−20°C for 30 min) or immunoprecipitates, were resuspended in 100 μl Laemmli sample buffer, boiled for 2–3 min, and electrophoresed under reducing conditions in SDS polyacrylamide (7.5, 10 or 6–16%) slab gels [18]. After electrophoresis, proteins were transferred to nitrocellulose as described by Towbin et al. [39] in a Transblot apparatus (Biorad) at constant voltage (60 V) for 14–16 hr. Two-dimensional gel electrophoresis was performed as previously described by O'Farrell [26].

Nitrocellulose sheets were incubated with [^{125}I]-streptavidin under conditions that reduce nonspecific binding to nitrocellulose [4]. Briefly, blots were blocked with 3% BSA/1% non-fat dry milk (Carnation) for 1 hr in PBS containing 0.5% (vol/vol) Tween 20, 10% (vol/vol) glycerol, 1 M glucose (TGG) at room temperature. [^{125}I]-streptavidin (1–2 × 10^6 cpm/ml in TGG containing 0.3% BSA) was allowed to bind for 2 hr at room temperature, followed by washing with PBS containing 0.5% Tween 20 (3 times 10 min each). Blots were dried and autoradiographed (2–12 hr at −70°C with an intensifying screen) on Kodak XAR-5 film.

Immunofluorescence and Semithin Frozen Sections

Monolayers were fixed with 2% (wt/vol) paraformaldehyde and semithin (0.5 μm) frozen sections were prepared according to Salas et al. [35]. Biotinylated proteins were visualized by incubation with Texas-Red conjugated streptavidin (Cappel, Cooper Biomedical, Malvern, PA) at a dilution of 1/50.

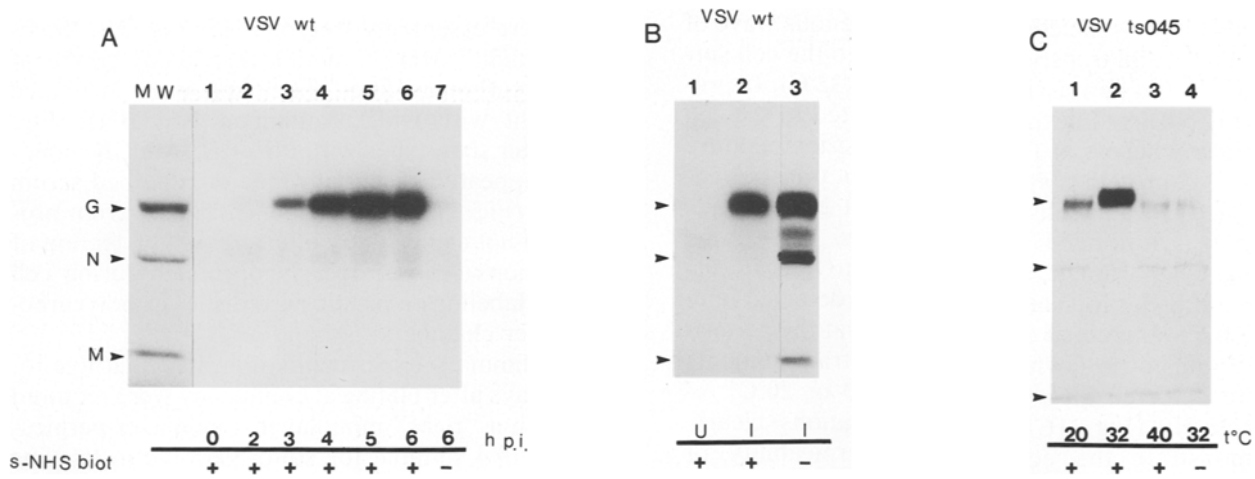

Fig. 1. The only VSV protein biotinylated in infected MDCK cells is G-protein. (*A*) Subconfluent MDCK cells grown on plastic petri dishes were infected with VSV (multiplicity of infection = 10) and biotinylated (0.5 mg/ml sulfo-NHS-biotin, 20 min, 4°C) at various times postinfection (*p.i.*). Cells were lysed and subjected to immunoprecipitation with antiserum directed against whole virus (recognized G, M and N proteins). After SDS-PAGE and transfer to nitrocellulose, biotinylated proteins were visualized with [^{125}I]-streptavidin. Lane 1, ^{35}S-labeled VSV standard; lanes 2–7, VSV-infected MDCK cells biotinylated at various times after infection. (*B*) MDCK cells infected with VSV (moi = 10) were metabolically labeled (^{35}S) or biotinylated 6 hr *p.i.* and subjected to immunoprecipitation as in *A*. Lane 1, mock-infected (*U*), biotinylated cells; lanes 2 and 3, VSV-infected (*I*) cells, either biotinylated (lane 2) or metabolically labeled (lane 3). The antiserum precipitates G, N and M, but biotin label is observed only on G-protein. (*C*) MDCK cells infected with VSV-ts045 (moi = 30) were incubated at the nonpermissive temperature (40°C) for 4 hr *p.i.* and at the indicated temperatures for 1 hr. Cells were metabolically labeled (^{35}S) 2–4 hr *p.i.*, biotinylated 5 hr *p.i.* and immunoprecipitated as in *A*. Lane 1: 20°C-Golgi block; lane 2: permissive temperature (32°C); lane 3: nonpermissive temperature (40°C); lane 4: permissive temperature (32°C), unbiotinylated control

Radio-Iodination

Streptavidin (BRL) was radio-labeled with Na[^{125}I] (NEN, Chadds Ford, PA) using chloramine T and separated from unreacted material by size exclusion chromatography on Sephadex G-50. Typically, 100–200 μg of streptavidin was iodinated with 1–1.5 mCi Na[^{125}I], yielding a specific activity of approximately 5–10 μCi/μg.

Preparation of Biotinylated Horse Serum (BHS)

Horse serum (HS; 1 ml; 80 mg/ml) was chromatographed on G-50 Sephadex to eliminate free amino acids. Elution was monitored by inclusion of [^{125}I]-protein A. Peak fractions were pooled (80% recovery) and HS was biotinylated with sulfo-NHS-biotin at a fivefold molar excess biotin : protein, assuming an average protein molecular weight of 60 kD.

Results

Labeling with Sulfo-NHS-Biotin is Confined to the Cell Surface

The impermeability of the cell membrane to sulfo-NHS-biotin was tested using VSV-infected MDCK cells grown on plastic petri dishes. This system provides an excellent means of evaluating the impermeability of sulfo-NHS-biotin, since G-protein (the viral envelope glycoprotein of VSV) is transported to the cell surface, whereas other viral proteins remain within the cytoplasm (M, N) or on the cytoplasmic side of the plasmalemma (M). Transport to the cell surface can also be manipulated by employing ts045, a temperature-sensitive mutant of VSV in which the exit of G from the endoplasmic reticulum is reversibly inhibited by incubation at 39–40°C [31].

VSV-infected MDCK cells were labeled with sulfo-NHS-biotin at various times postinfection and immunoprecipitated with antiserum directed against whole virus (recognizes G, M, and N proteins). Increasing amounts of G-protein became accessible to biotinylation with time of infection, consistent with the known time course required for synthesis and transport of G-protein to the cell surface; no M or N proteins were biotinylated in the same cells (Fig. 1*A*). That the antiserum used could precipitate M and N proteins, in addition to G, was shown by parallel immunoprecipitation of metabolically labeled monolayers infected with VSV (Fig. 1*B*).

Finally, MDCK cells were infected with the temperature-sensitive mutant of VSV ts045, which

readily allows the control of a synchronous wave of synthesis and transport of G-protein to the cell surface. At the permissive temperature (32°C), G-protein is both synthesized and transported to the cell surface whereas at the nonpermissive temperature (40°C) G-protein is synthesized but not transported. Similarly, when cells are incubated at 20°C, G-protein is synthesized and concentrated in a trans-Golgi compartment, with a slow leak to the cell surface [12]. Biotinylated G-protein was detected only when VSV-infected cells were placed at the permissive temperature, whereas no or only trace amounts were detected after incubation at 40 or 20°C, respectively (Fig. 1*C*). These observations clearly demonstrate the cell surface impermeability of sulfo-NHS-biotin, an important prerequisite of the experiments to be described.

Tight Junctions Constitute a Barrier to the Diffusion of Sulfo-NHS-Biotin

To study the polarized distribution of viral or endogenous membrane proteins, MDCK cells can be grown on permeable supports (nitrocellulose or polycarbonate filters), which allow separate access to the apical or basolateral membrane. Selective labeling of either the apical or basolateral surface with sulfo-NHS-biotin appeared feasible, since MDCK cells grown on such permeable supports form "tight" monolayers with cationic selective permeability given by the presence of tight junctions [7].

An initial step was to evaluate permeable supports for their ability to nonspecifically adsorb serum proteins, a property that could potentially hinder the visualization of endogenous cell surface glycoproteins or cause uncertainty as to the cellular origin of labeled proteins. Experiments in which nitrocellulose and polycarbonate filters were exposed to horse serum indicated that nitrocellulose-based filter chambers (Millipore) adsorbed an amount of serum proteins comparable with the amount of endogenous proteins detected on the MDCK cell surface. Polycarbonate filter chambers (Transwell, Costar), on the other hand, appeared essentially free of nonspecific protein binding (Fig. 2*A*). Thus, subsequent experiments were restricted to the use of polycarbonate filter chambers.

We also evaluated the ability of MDCK cells, grown on plastic or on filters, to nonspecifically adsorb biotinylated serum proteins. Subconfluent MDCK cells grown on plastic petri dishes were incubated with DME, containing 10% biotinylated horse serum (BHS). After three to five washes with PBS-CM, the normal protocol used during labeling of the cell surface with sulfo-NHS-biotin, MDCK cells were essentially free of BHS (Fig. 2*B*). Similarly, "tight" MDCK monolayers, 6-day confluent on polycarbonate filter chambers, were incubated overnight with DME containing 10% BHS (Fig. 2*C*). After six washes with PBS-CM, MDCK monolayers appeared essentially free of adsorbed serum protein (Fig. 2*C*, lanes 2 and 4). Thus, serum proteins do not significantly interfere with detection of endogenous cell surface glycoproteins during cell surface labeling on plastic petri dishes or polycarbonate filter chambers.

Preliminary experiments indicated that five-to-seven days after plating at confluency were required to obtain a "tight" monolayer (monolayer permeability $<1\%$) suitable for sulfo-NHS-biotin labeling (*see* Materials and Methods for details). MDCK monolayers were then labeled by the addition of sulfo-NHS-biotin (0.5 mg/ml in PBS) to either the apical or basolateral compartment of the filter chamber.

Two lines of evidence indicated that tight junctions were effective barriers to the diffusion of sulfo-NHS-biotin. First, monolayers grown on polycarbonate filters, labeled from the apical or the basolateral side, were fixed, frozen sectioned (0.5-μm sections) and stained with Texas Red-conjugated streptavidin. Fluorescence was found to be confined exactly to the side from which the monolayers were labeled; when added from the basal side, biotin labeling was observed up to the level of the tight junctions (Fig. 3). Second, an apical marker, γ-glutamyl transpeptidase (γGT) [38] and a basolateral marker, VSV G-protein [30, 32], were immunoprecipitated after selectively labeling with sulfo-NHS-biotin the apical or basolateral surface of the monolayer and extraction with Triton X-100 (Fig. 4). γ-GT was largely detected on the apical membrane and VSV G-protein on the basolateral membrane; only trace amounts of the markers were detected on the opposite surface domains.

These experiments indicated that sulfo-NHS-biotin selectively labeled the apical or the basolateral surface domains of tight epithelial monolayers.

Apical and Basolateral Membrane Proteins of MDCK Cells

In order to selectively label apical or basolateral cell surfaces, MDCK monolayers confluent for 5–7 days were exposed to sulfo-NHS-biotin added to the apical or to the basal compartment, as described before. Proteins from labeled monolayers were subsequently extracted with TX-114 and partitioned into hydrophilic "peripheral" and amphipathic "integral" membrane proteins by a temperature-in-

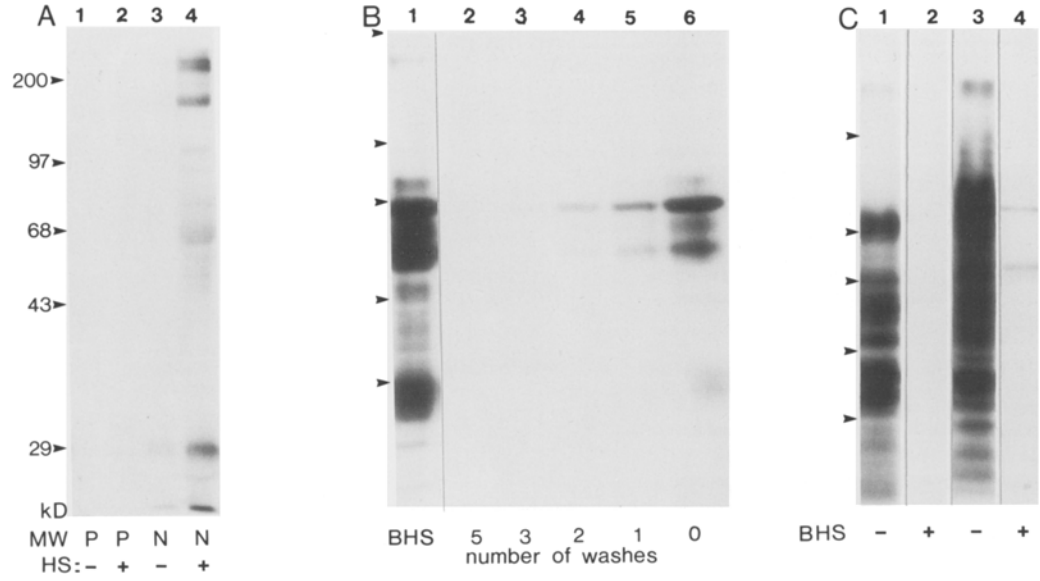

Fig. 2. Serum proteins do not interfere during cell surface labeling on polycarbonate filters. (*A*) Nonspecific adsorption of serum proteins to nitrocellulose filters. Polycarbonate (*P*) or nitrocellulose (*N*) filter chambers (24.5-mm diameter) were incubated in DME in the absence (−) or presence (+) of 10% (vol/vol) horse serum (HS) overnight at 37°C. Filters were washed extensively (five times) with PBS-C/M, biotinylated (0.5 mg/ml, 20 min at 4°C) and extracted with 1% Triton X-100. After SDS-PAGE and transfer to nitrocellulose, adsorbed biotinylated proteins were visualized with [^{125}I]-streptavidin and autoradiography. Lanes 1 and 2, polycarbonate (*P*) filter chambers; lanes 3 and 4, nitrocellulose (*N*) filter chambers. Note the absence of nonspecific protein adsorption by polycarbonate filter chambers. (*B*) Nonspecific adsorption of serum proteins to plastic grown MDCK cells is removed during repeated washes with PBS-C/M. Subconfluent MDCK cells grown on 24-well plastic petri dishes were incubated with 0.5 ml DME supplemented with 10% (vol/vol) biotinylated HS (BHS) for 1 hr at 4°C, and washed for the indicated number of times with 1.0 ml of PBS-C/M. After extraction with TX-100, the samples were analyzed by SDS-PAGE (6–16% linear gradient), transferred to nitrocellulose and visualized with [^{125}I]-streptavidin (lanes 2–6). An aliquot (0.1 ml) of DME, containing 10% BHS was acetone precipitated for comparison (lane 1). (*C*) Nonspecific adsorption of serum proteins to filter-grown MDCK cells is removed during repeated washes with PBS-C/M. Tight MDCK monolayers grown on polycarbonate chambers were incubated overnight with DME containing 10% (vol/vol) biotinylated HS (BHS). Filter chambers were washed with PBS-C/M (six times); filters were excised and extracted with TX-114. Alternatively, filters were reacted with sulfo-NHS-biotin (0.5 mg/ml, 20 min at 4°C). After temperature-induced phase separation, proteins in detergent (lanes 1 and 2) and aqueous (lanes 3 and 4) phases were separated by SDS-PAGE (6–16% gradient) and visualized as described above. Lanes 1 and 3: control MDCK monolayers biotinylated both apically and basolaterally. Lanes 2 and 4: MDCK monolayers incubated overnight with BHS and washed with PBS-C/M

duced phase separation. Peripheral (hydrophilic) membrane proteins appeared more abundant than integral (hydrophobic/amphipathic) membrane proteins, exceeding their concentration by approximately fivefold. Integral apical proteins were grouped into four major sets with approximate M_r 100, 70, 40–60 and 30 kD while the corresponding basal proteins were most abundant around M_r 40–70 kD. Major apical proteins partitioning with the aqueous phase had M_r 180, 110, and 32 while the majority of the corresponding basal proteins migrated with M_r 90–150 kD; a 45 kD band was observed at approximately equal levels on both surfaces (*see* Fig. 5, lanes 1 and 2).

To evaluate whether the differences observed were dependent on the tight junctional barrier, monolayers were labeled under conditions which disrupt tight junctional integrity. "Tight" monolayers (<0.5–1% monolayer permeability) grown on filter chambers were washed and incubated in the absence of divalent cations (Ca^{2+} or Mg^{2+}). Increases in monolayer permeability were monitored by the diffusion of [^{3}H]-inulin from the apical to the basolateral compartment (Fig. 5*C*). A level of approximately 11.5% monolayer permeability was observed after 2.5 hr incubation in divalent cation-free medium at 37°C ("leaky" monolayers). "Leaky" monolayers were then apically or basolaterally labeled with sulfo-NHS-biotin and subsequently extracted with TX-114. As seen in Fig. 5 (lanes 3 and 4), biotinylated protein patterns from apically or basolaterally labeled "leaky" monolayers appeared practically indistinguishable, although some differences in amphipathic protein patterns were retained. Thus, the distinct apical and basolateral protein patterns were clearly dependent upon the degree of monolayer "tightness," i.e., the integrity of the tight junctional gate.

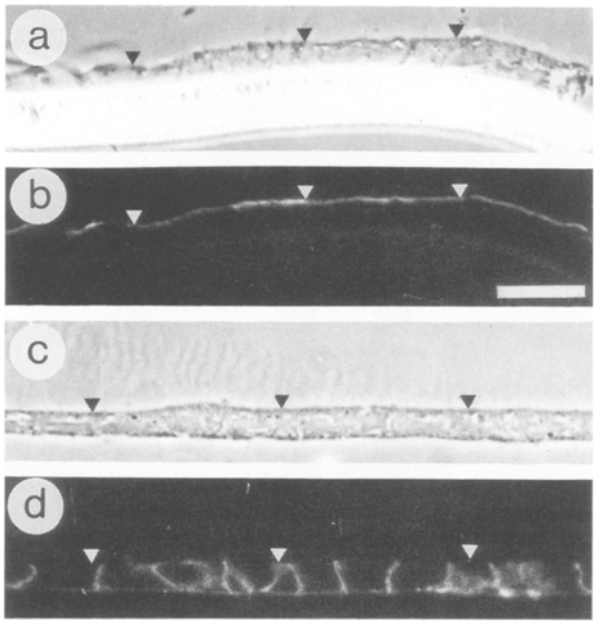

Fig. 3. Selective labeling with sulfo-NHS-biotin of apical or basolateral MDCK surfaces: immunofluorescence on semithin frozen sections. MDCK monolayers confluent for 5 days in polycarbonate filter chambers were labeled with sulfo-NHS-biotin (0.5 mg/ml, 20 min at 4°C) from the apical or basal side, fixed with 2% formaldehyde (freshly prepared from paraformaldehyde) in PBS-C/M and processed for semithin frozen sections. 0.5-μm sections were stained with Texas-Red conjugated streptavidin. (*a, b*) Phase and fluorescence image of monolayers that were exposed to sulfo-NHS biotin added from the apical side. (*c, d*) Phase and fluorescence images of monolayers exposed to sulfo-NHS-biotin from the basal side. Arrowheads point at the apical surface. The white bar represents 10 μm

Fig. 4. Selective biotinylation of apical and basolateral surfaces of MDCK monolayers: immunoprecipitation of apical and basolateral markers. Confluent MDCK monolayers were labeled from the apical (*A* lanes) or from the basal (*B* lanes) side with sulfo-NHS-biotin, extracted with Triton X-100 and immunoprecipitated with antibodies against the light chain of γ-glutamyl transpeptidase (25 kD) or VSV G-protein (68 kD). *C* lanes represent unbiotinylated controls. Panel *A* (left): γ-glutamyl transpeptidase. Panel *B* (right): VSV G-protein, monolayers infected for 6 hr

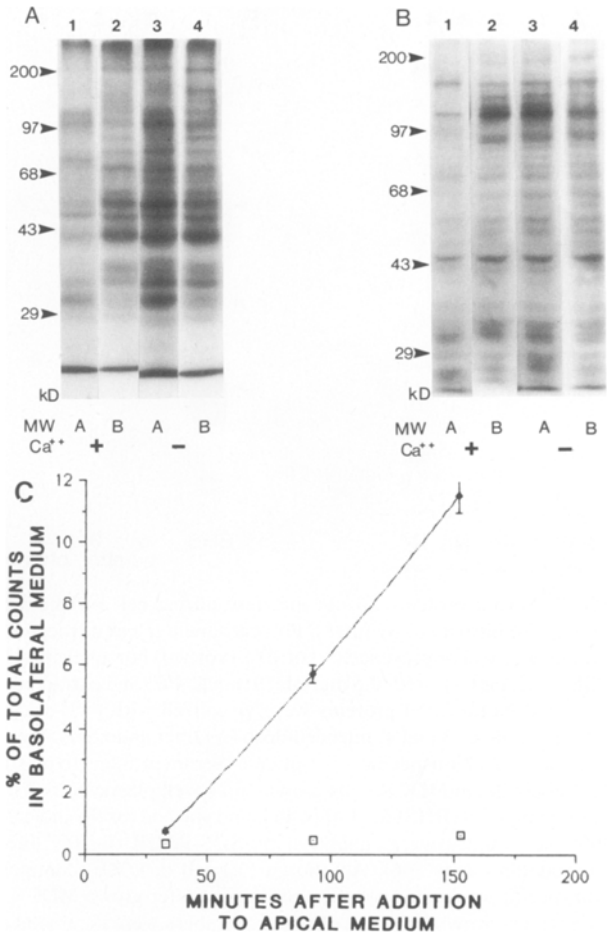

Fig. 5. Selective biotinylation of the apical or basolateral surfaces of MDCK monolayers depends upon monolayer "integrity." MDCK monolayers, 6–7 days confluent on polycarbonate filters, were incubated at 37°C in the absence (filled diamonds) or presence (open squares) of divalent cations (Ca^{2+}, Mg^{2+}). Increases in monolayer permeability were monitored by the addition of [^{3}H]-inulin to the apical compartment of the filter chamber (*see* Materials and Methods). At the times indicated, samples were withdrawn from the basolateral compartment and counted (*C*). Each time point represents the mean of four individual determinations. Panels (*A, B*) After 2.5 hr incubation (*see* graph in panel *C*), "leaky" and "tight" monolayers were biotinylated apically (lanes *A*) or basolaterally (lanes *B*) and extracted with Triton X-114 and phase separated. Biotinylated proteins in detergent (panel *A*) and aqueous (panel *B*) phases were analyzed by SDS-PAGE (7.5% acrylamide gel) and by [^{125}I]-streptavidin blot, as described in Materials and Methods. Lanes 1 and 2: "tight" monolayers; lanes 3 and 4: "leaky" monolayers. Note the large differences between apical and basolateral patterns of integral and peripheral plasma membrane proteins. These differences largely disappear in leaky monolayers. The amounts of peripheral proteins loaded on the gel were 5 times lower, since they were about fivefold more abundant than the integral proteins, as detected by this labeling procedure

Two-dimensional electrophoresis was performed to determine which, if any, membrane proteins were common to both apical and basolateral cell surfaces. As seen in Fig. 6, very few proteins were found to be common to both apical and basolateral surfaces by this procedure. This finding has important consequences for the biogenesis of epithelial polarity; it suggests that the mechanisms that control the targeting and retention of membrane proteins at the two poles of epithelial cells must be exquisitely selective.

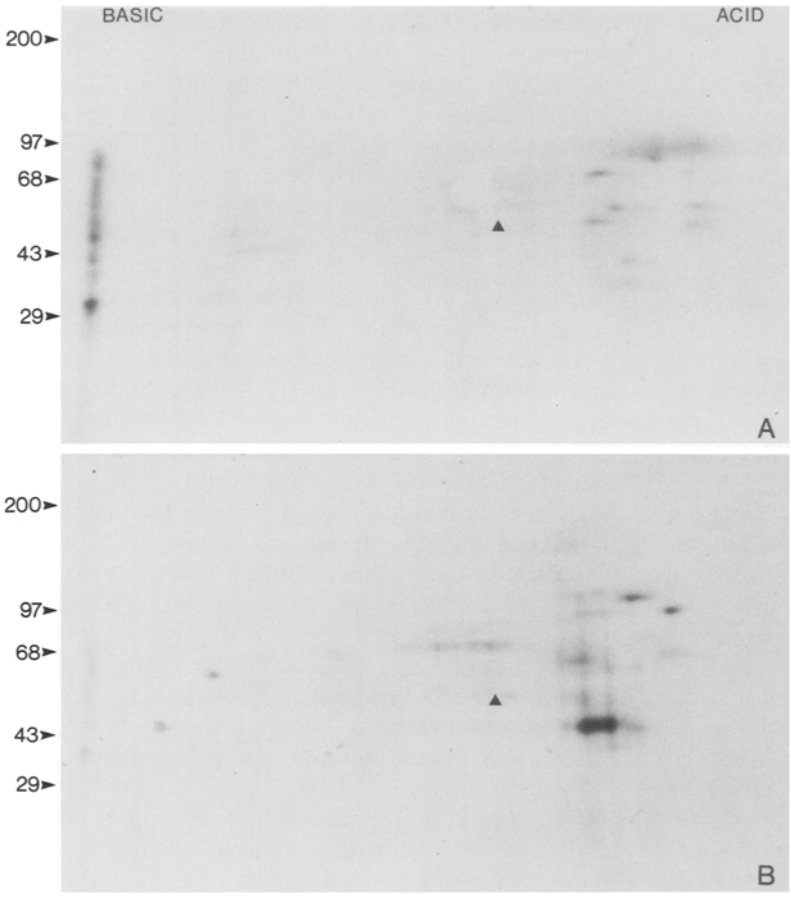

Fig. 6. Two-dimensional electrophoretic analysis of apically and basolaterally biotinylated MDCK monolayers. Confluent MDCK monolayers were biotinylated from the apical or from the basolateral side (0.5 mg/ml, 20 min at 4°C), extracted with Triton X-100 and precipitated in cold acetone (−20°C). Samples were solubilized in the sample buffer of O'Farrell and analyzed by two-dimensional electrophoresis followed by [^{125}I]-streptavidin blot. Note that the apical (*A*) and basolateral (*B*) protein patterns are very different. The arrowhead denotes the position of actin as visualized by Ponceau red staining. The first dimension is an isoelectrofocusing gel with a 5–8 pH gradient; the second dimension is a 6–16% polyacrylamide gel

Discussion

How different are apical and basolateral membranes of epithelial cells? A variety of enzymes and transport systems have been shown to be very polarized. [1, 17, 24, 30, 38]. Similarly, several endogenous plasma membrane antigens of unknown function are asymmetrically distributed in polarized MDCK monolayers [27, 34]. Viral envelope glycoproteins are highly polarized in epithelial cells infected with enveloped viruses [30, 38]. On the other hand, isolated examples exist of proteins that are present in equal amounts in both apical and basolateral surfaces [27, 34]. A similar situation is transiently observed during development of a polarized monolayer in culture; some surface antigens are unpolarized immediately after plating the cells but become asymmetrically distributed, at rates characteristic for each protein, as the monolayers become confluent [3, 14, 41]. In spite of this relative wealth of information on the polarity of specific markers, very little is known on how distinct are the "total" protein compositions of apical and basolateral membranes in different epithelia. A cell fractionation study in intestinal cells has shown that apical and basolateral membranes share very few proteins [10]. On the other hand, many lipids are shared by apical and basal surface domains, although some glycolipids appear to be concentrated in the apical surface [20, 21, 25, 40].

Epithelial cells in culture, either primary cultures or cell lines, have become preferred models to study the biogenesis of cell polarity; the MDCK line is the best characterized in this regard. Differently from native epithelia, fractionation methods are very difficult with cell lines. On the other hand, the possibility of growing the cells on inert substrata make labeling procedures attractive tools to study the composition of apical and basolateral membranes. Using sulfo-NHS-biotin to label the surface domains of MDCK monolayers grown on polycarbonate filters, we have identified in this report the integral and peripheral protein compositions of the apical and basolateral plasma membrane of this model cell line. The only previous report on the total protein composition of the surface domains of an epithelial cell line is by Richardson and Simmons [29], who used the lactoperoxidase-catalyzed iodination procedure on MDCK monolayers grown on nitrocellulose filters. The results of our work extend and improve their observations. Nitrocellulose filters bind large amounts of protein, about 20 $\mu g/cm^2$

[39]; MDCK monolayers grown on nitrocellulose filters contain about 200 μg protein/cm^2 [36] from which about 1% (or 2 μg/cm^2) may be estimated to be surface proteins. Thus, the amount of protein binding to the nitrocellulose filter is approximately 10 times higher than the amount of protein to be labeled on the cell surface. Therefore, it may be presumed that most of the protein labeled from the basolateral side of monolayers grown on nitrocellulose filters are proteins bound to the filter. Polycarbonate filters, on the other hand, bind exceedingly small amounts of protein. Thus, the compositions of apical and basolateral domains of MDCK cells reported here reflect the actual composition of the membrane much more accurately than previously reported.

The biotinylation procedure, in combination with extraction with the detergent Triton X-114, allowed us to identify integral and peripheral membrane proteins of apical and basolateral surface domains. Peripheral proteins, i.e., those partitioning with the aqueous phase, were approximately fivefold more abundant than the more hydrophobic integral membrane proteins that partition with the detergent phase. That all of these proteins are exposed on the exoplasmic aspect of the bilayer was proven by the lack of access of sulfo-NHS-biotin to cytoplasmic proteins, such as the M and N proteins of vesicular stomatitis virus in infected cells. It must be stressed that some of the "peripheral" proteins identified here may be shown, upon closer scrutiny, to belong to a class of integral membrane proteins with intermediate hydrophobicity levels. The aqueous phase remaining after separation from the higher density detergent phase contains about 0.7 mM TX-114, three to four times above the critical micellar concentration, which appears to be sufficient to solubilize some integral membrane proteins, leading to anomalous partitioning into the aqueous phase [*see* 28 for a review on TX-114]. As labeled with the biotin analog used here, integral apical proteins were grouped into four major sets with approximate M_r 100, 70, 40–60 and 30 kD while the corresponding basal proteins were most abundant around M_r 40–70 kD. Major apical proteins partitioning with the aqueous phase had M_r 180, 110, and 32 while the majority of the corresponding basal proteins migrated with M_r 90–150 kD; a 45 kD band was observed at approximately equal levels on both surfaces (*see* Fig. 5). Our studies open the way towards a characterization of the main surface components of the prototypic MDCK cell line in terms of their biogenesis, kinetics of polarization, recycling and other interesting biological properties.

The method utilized in this report to describe the MDCK plasma membrane proteins should prove very valuable in studying the composition of each domain in other epithelial cell types. The chemical properties of sulfo-NHS-biotin, a water-soluble analog of vitamin H, make it ideally suited for the selective labeling of apical or basolateral membrane domains. The sulfo-group, which confers an overall net negative charge to the molecule, prevents its diffusion across cell membranes thereby restricting the biotinylation reaction to the cell components embedded in or attached to the exoplasmic face of the plasma membrane. Furthermore, net negative charge, in addition to molecular size, impair its diffusion across the cationic-selective permeability barrier created by tight junctions. Thus, labeling can be confined to the exoplasmic face of specific cell surface domains (apical or basolateral), provided that the integrity of the tight junctions is maintained during the labeling procedure. The NHS-group, which confers reactivity to primary amino-groups, allows for the biotinylation of unblocked N-terminal amino acids, lysine, as well as amino lipids/sugars. Since lysine is a more abundant protein constituent than tyrosine, a wider variety of proteins can be detected using sulfo-NHS-biotin as compared with lactoperoxidase-catalyzed iodination [15, 16].

The biotin moiety offers high sensitivity of detection via reaction with streptavidin. Since the biotin-streptavidin association reaction is the strongest noncovalent interaction known ($K_D \approx 10^{-15}$), vanishingly small quantities (nanograms to picograms) of biotinylated protein can be detected under high stringency conditions, which minimize nonspecific binding [15, 16]. A rough comparison with the lactoperoxidase (LPO) catalyzed iodination procedure indicates that the biotin/[^{125}I]-streptavidin labeling method is considerably more sensitive, since only the equivalent of 1.0 cm^2 of MDCK monolayer is necessary for visualization of the surface proteins after 12–24 hr exposure, as opposed to 5 cm^2 (with considerably longer exposure times) for the lactoperoxidase-catalyzed iodination [29].

In addition to higher sensitivity, cell surface labeling with sulfo-NHS-biotin has additional practical advantages over lactoperoxidase-catalyzed iodination. Namely, (i) minimal exposure of the investigator to free radioactive iodine (a single batch of [^{125}I]-streptavidin can be used for over 40 experiments); (ii) biotinylated proteins can be recovered by precipitation with immobilized streptavidin; and (iii) biotinylated proteins can be visualized at the light and electron microscope level with the appropriate streptavidin conjugate.

In summary, we have carefully studied the protein composition of apical and basolateral domains of MDCK cells using a selective labeling procedure that employs a water soluble biotin analog. Periph-

eral and integral membrane proteins were found to be very different in each domain, indicating the existence of stringent sorting mechanisms in epithelial cells. The simple and sensitive biochemical procedure described here should prove extremely useful in studying the polarized distribution of endogenous proteins in other epithelial lines, as well as of proteins produced from transfected cDNAs in studies aimed at exploring the nature of the sorting signals involved in the biogenesis of polarity.

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We have recently employed domain-selective labeling with sulfo-NHS-biotin to study the polarized distribution of glycosyl-PI anchored proteins [M.P. Lisanti, M. Sargiacomo, L. Graeve, A.R. Salteil, E. Rodriguez-Boulan (1988), Polarized apical distribution of glycosyl-phosphatidylinositol anchored proteins in a renal epithelial cell line, *Proc. Natl. Acad. Sci. USA* **85:**9557–9561].