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Differential Arrival of Newly Synthesized Apical and Basolateral Plasma Membrane Proteins in the Epithelial Cell Line A6

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Abstract. The labeling of specific cell surface proteins with biotin was used to examine both protein distribution and delivery of newly synthesized proteins to the apical and basolateral cell surface in A6 cells. Steady-state metabolic labeling with [^{35}S]methionine followed by specific cell surface biotinylation demonstrated polarization of membrane proteins. The delivery of newly synthesized proteins to the apical or basolateral cell surface was examined by metabolic labeling with [^{35}S]methionine using a pulse-chase protocol in combination with specific cell surface biotinylation. Newly synthesized biotinylated proteins at the apical cell surface reached a maximum after a 5 min chase, and then fell over the remainder of a 2 hr chase. The bulk flow of newly synthesized proteins to the basolateral membrane slowly rose to a maximum after 90 min. The detergent Triton X-114 was used to examine delivery of hydrophilic and hydrophobic proteins to the cell surface. Delivery of both hydrophilic and hydrophobic proteins to the apical cell surface reached a maximum 5 to 10 min into the chase period. The arrival of hydrophilic proteins at the basolateral surface showed early delivery and a maximum peak delivery at 120 min into the chase period. In contrast, only an early peak of delivery of newly synthesized hydrophobic proteins to the basolateral membrane was observed.

Key words: Epithelial polarity — A6 cells — Biotin — Protein trafficking — Protein synthesis

The expression of epithelial cell surface proteins in a polarized manner is essential for the vectorial transport of solutes and the secretion of proteins. The intracellular pathways by which apical and basolateral membrane proteins reach their final destination after exiting the trans-Golgi network appear to be complex (*for review, see* Simons & Fuller, 1985; Rodriguez-Boulan & Nelson, 1989). For example, some proteins move directly from the trans-Golgi network to their appropriate cell surface location. Other proteins appear first at a surface site different from their final destination. They are relocated to the appropriate surface domain by moving across the cell, a process referred to as transcytosis (Simons & Fuller, 1985; Bartles et al., 1987; Simons & Wandinger-Ness, 1990). Alternatively, proteins insert randomly in both surface membranes. Missorted proteins are retrieved and then redirected to the appropriate membrane or degraded (Simons & Fuller, 1985; Matter et al., 1990; Hammerton et al., 1991).

The rates of delivery of newly synthesized proteins which reside in the apical and basolateral plasma membrane are similarly complex. This reflects differences in rates of protein synthesis, assembly, post-translational modification in endoplasmic reticulum and Golgi, differences in rates of movement between intracellular compartments, and finally, the intricacy of the pathways by which proteins reach the cell surface. For example, the newly synthesized membrane glycoprotein H-2K^k appears at the cell surface of murine B cell lymphoma line AKTB-1b within 1 hr, while the membrane glycoprotein H-2D^k which posseses 80% sequence identity with H-2K^k is transported to the cell surface with a half-time of 4 to 5 hr (Williams, Swiedler & Hart, 1985). The rate limiting step of intracellular trafficking was identified as the movement from the endoplasmic reticulum to the Golgi apparatus. Other

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proteins transit from the rough endoplasmic reticulum to the Golgi with differing rates, including albumin, α_1-antitrypsin, C3 complement, α_1-antichymotrypsin and transferrin in human hepatoma HepG2 cells (Lodish et al., 1983) or the viral membrane glycoproteins encoded by the *env* gene or *gag* gene in cells infected with murine leukemia virus (Fitting & Kabat, 1982). The time required for a protein to move from the Golgi to the cell surface domain will depend, in part, whether it takes a direct or an indirect pathway to the cell surface, as has been described in the trafficking of antigen 525, HLA-I, sucrase-isomaltase, aminopeptidase N, and alkaline phosphatase in Caco 2 cells (Le Bivic et al., 1990*b*; Matter et al., 1990; Gilbert et al., 1991).

The delivery rates of newly synthesized proteins to the cell surface in epithelia have been examined for a limited number of specific proteins and have been found to be quite variable (Bartles et al., 1987; Lisanti et al., 1989; Hughson & Hopkins 1990; Le Bivic et al., 1990*a,b*; Matter et al., 1990). To place this information in perspective, it is important to compare the rates of cell surface delivery of individual proteins with the overall bulk flow of newly synthesized proteins to the apical or basolateral cell surface. One study has examined the bulk flow of newly synthesized glycoproteins to the surface of MDCK cells (Lisanti et al., 1989), but similar studies have not been performed in other cell lines.

The amphibian cell line A6 was derived from the kidney of *Xenopus laevis* and serves as a model for the study of epithelial transport (Perkins & Handler, 1981). Metabolically labeled proteins can be tagged with the nonmembrane permeant sulfo-N-hydroxy-succinimido-biotin (sulfo-NHS-biotin) as they reach specific cell surface domains and are precipitated with streptavidin-agarose (Sargiacomo et al., 1989). This approach has allowed us to examine the delivery of newly synthesized proteins to the apical or basolateral plasma membrane of A6 cells.

Materials and Methods

Materials

Sulfo-NHS-biotin and streptavidin conjugated to agarose were purchased from Pierce (Rockford, IL). Collagen type I (Vitrogen 100) was purchased from Celtrix (Palo Alto, CA). Electrophoresis reagents were from Biorad (Richmond, CA). Amplify and [^{35}S]methionine were purchased from Amersham (Arlington Heights, IL). All other reagents were purchased from Sigma (St Louis, MO) and were reagent grade.

Cell Culture

A6 cells derived from *Xenopus laevis* kidney were obtained from the American Tissue Type Collection, previously cloned by limiting dilution, and used between passages 92 to 96. Cells were seeded on 4.7 cm^2 collagen-coated polycarbonate filters (Nucleopore) at 10^6 cells/cm^2 and maintained in a humidified 28°C incubator with 5% CO_2 in an amphibian medium supplemented with 5% fetal calf serum for 7 to 15 days (Kleyman et al., 1991). Cell resistance was measured at the beginning of the experiment and following biotinylation with a EVOM apparatus (World Precision Instruments, Sarasota, FL).

Biosynthetic Labeling

Cells were washed three times with methionine-free, serum-free medium. The filter supports were then inverted and 200 μl of methionine-free medium containing 1 mCi/ml [^{35}S] methionine (108 Ci/mmol) was placed on the surface of the filters for 15 min at 28°C. Following the 15 min incubation, cells were washed once and chased for different time periods in medium supplemented with 10 mM methionine at 28°C. In some experiments, steady-state labeling was performed by incubating cells for 16 hr at 28°C with 1.5 ml of serum-free medium containing 0.1 mCi/ml [^{35}S] methionine and 0.17 mM unlabeled methionine in the basolateral solution, and 1.5 ml of serum-free medium containing 0.17 mM unlabeled methionine in the apical solution.

Selective Apical or Basolateral Biotinylation

Following metabolic labeling with [^{35}S] methionine, cells were washed five times at 4°C with an amphibian Ringer's solution consisting of (in mM): NaCl 110.0; KCl 4.0; $NaHCO_3$ 2.5; K_2HPO_4 1.0; $CaCl_2$ 2.0; and glucose 11.0. The selective labeling of the apical or basolateral surface was performed with sulfo-NHS-biotin. A stock solution of sulfo-NHS-biotin was prepared at a concentration of 200 mg/ml in dimethylsulfoxide, stored frozen at −20°C and thawed just before use. Sulfo-NHS-biotin (0.5 mg/ml) prepared in amphibian Ringer's solution was added to the apical or basolateral solution and allowed to incubate for 20 min at 4°C with gentle agitation. Compartments not receiving sulfo-NHS-biotin received an equivalent volume of tissue culture medium containing 5% fetal calf serum to bind any sulfo-NHS-biotin that leaked across the epithelium. After 20 min, cells were washed 4 times with amphibian Ringer's solution containing protease inhibitors (1 μM antipain, 1 μM leupeptin, 1 μM pepstatin A, and 0.1 mM phenylmethylsulfonyl fluoride) and collected with a detergent buffer containing 0.4% (w/v) sodium deoxycholate, 1% (v/v) nonidet P40, 50 mM ethyleneglycol-bis-(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), 10 mM Tris-HCl, pH 7.4 and protease inhibitors (DNET buffer). Following a 2 min centrifugation (12,000 × *g*) to remove insoluble material, aliquots were removed from the supernatant for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), determination of protein concentration, and quantification of [^{35}S]methionine incorporation into protein. Proteins were precipitated twice with 10% trichloroacetic acid (30 min at 4°C) and resuspended in 1 M formic acid. Aliquots were removed for liquid scintillation counting and for protein determination using the method of Bradford (1976).

Separation of Hydrophilic and Hydrophobic Proteins with Triton X-114

In some experiments, proteins were subjected in phase separation with Triton X-114 (Bordier 1981; Conzelmann et al., 1986) follow-

ing metabolic labeling and biotinylation. Proteins were collected in a buffer containing 1% (v/v) Triton X-114, 10 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EGTA and protease inhibitors (TSE-T buffer). Triton X-114 solubilized proteins were agitated end over end for 30 min at 4°C. Following centrifugation to remove insoluble proteins (12,000 × *g*, 2 min) aliquots were removed for SDS-PAGE and trichloroacetic acid precipitation (*see above*). Hydrophilic proteins were separated from hydrophobic proteins by a temperature-induced phase separation. Proteins were warmed to 28°C for 7 min and centrifuged at 12,000 × *g* for 2 min at room temperature. The aqueous phase was removed and re-extracted once by addition of 100 μl of 11% (v/v) Triton X-114 and centrifuged at 12,000 × *g* for 5 min at 4°C. The supernatant was warmed to 28°C for 7 min and centrifuged at 12,000 × *g* for 2 min at room temperature. The aqueous phase was kept at 4°C until use. The detergent phases were combined and hydrophilic proteins were extracted three times with 10 volumes of 0.06% Triton X-114 by warming to 28°C for 7 min and phase separation by centrifugation for 2 min. Following the last extraction the volume was adjusted to 1 ml with DNET buffer.

Precipitation of Biotinylated Apical or Basolateral Proteins with Streptavidin-Agarose Beads

Biotinylated proteins were preabsorbed with 50 μl of anti-mouse IgG_1 bound agarose for 1 hr at 4°C. After a 3 sec centrifugation at 12,000 × *g* to pellet the agarose, 50 μl of streptavidin-agarose beads prewashed 2 times with the DNET buffer was added to the supernatant and incubated for 16 hr at 4°C. In preliminary experiments, the duration of incubation with streptavidin-agarose beads was varied (*see* Results). The beads were collected and washed 3 times with a buffer containing 150 mM NaCl, 5 mM EGTA, 1% (v/v) Triton X-100, 50 mM Tris, pH 7.4 and protease inhibitors, followed by two washes with a buffer containing 0.1% (w/v) SDS, 2 mM EGTA, 10 mM Tris-HCl, pH 7.4 and protease inhibitors. Bound proteins were eluted into 125 μl of a sample buffer containing 3% (w/v) SDS, 15% (w/v) sucrose and 92.5 mM Tris-HCl, pH 6.9 and sequentially incubated with 10 mM dithiothreitol for 20 min and 20 mM iodoacetamide for 15 min. Sample pH was adjusted with 1 μl of 375 mM Tris-HCl, pH 8.9 and reduced proteins were applied to 5–13% linear gradient gels (3.9% stacking gel). SDS-PAGE was performed as described previously (Kleyman et al., 1991). Gels were incubated for 30 min in Amplify prior to autofluorography. Each lane of the autoradiogram was individually scanned using a GS-300 scanning densitometer (Hoefer Scientific Instruments). The density (in arbitrary units) of all labeled proteins in the resolving lane of the autoradiogram was determined by integration using a GS 370 V2.3 Data System, and used as a measure of recovery of biotinylated (cell surface) radiolabeled proteins. In individual pulse-chase experiments, the density of labeled proteins recovered at 60 min was assigned a value of 100, and other time points a value relative to that at 60 min. The amount of protein recovered from nonbiotinylated cells which were labeled with [^{35}S]methionine (15 min pulse, 2 hr chase) and precipitated with streptavidin-agarose represented less than 3% of that recovered from cells which were subjected to biotinylation.

Analysis of Apical and Basolateral Hydrophilic Proteins by Two-Dimensional Electrophoresis

A6 cells were labeled with 0.1 mCi/ml of [^{35}S]methionine under steady-state conditions and apical or basolateral membrane proteins were biotinylated as described above. Cells were solubilized with TSE-T buffer and hydrophilic proteins were separated by a temperature-induced phase separation. Biotinylated proteins were precipitated with streptavidin-agarose beads, eluted into a sample buffer containing 9.5 M urea, 2.0% Triton X-100, 5% β-mercaptoethanol, 2% resolyte (4–8) ampholyte (Hoefer Scientific) and analyzed by two-dimensional electrophoresis. The first dimension was an electrofocusing gel with a 5 to 8 pH gradient. The second dimension is a 5–13% polyacrylamide gel. Labeled proteins were identified by autofluorography.

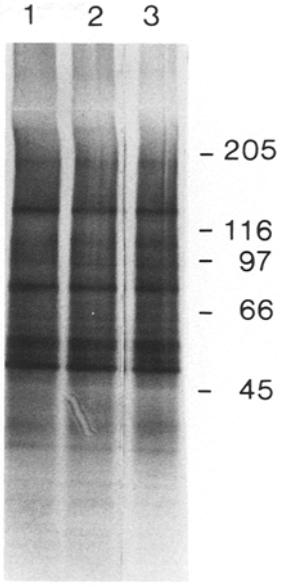


Fig. 1. Optimization of biotinylation. A6 cells grown on collagen-coated nucleopore membranes were labeled as described under Materials and Methods with [^{35}S]methionine for 15 min followed by a 30 min chase with unlabeled methionine. Cells were washed and incubated with 0.5 mg/ml sulfo-NHS-biotin in the apical compartment. (*Lane 1*) 20 min incubation with sulfo-NHS-biotin; (*Lane 2*) two successive 20 min incubations with sulfo-NHS-biotin; (*Lane 3*) three successive 20 min incubations with sulfo-NHS-biotin. Biotinylated proteins were precipitated with streptavidin-agarose and analyzed by 5–13% SDS-PAGE and autofluorography as described under Materials and Methods. Migration of various molecular weight standards is indicated at the right of the figure.

Statistics

Results are expressed as the mean ± standard error (SE).

Results

The time of arrival of newly synthesized proteins to the apical or basolateral plasma membrane has been defined for a limited number of distinct proteins (*see above*). However, the bulk flow of newly synthesized proteins to the plasma membrane in epithelia

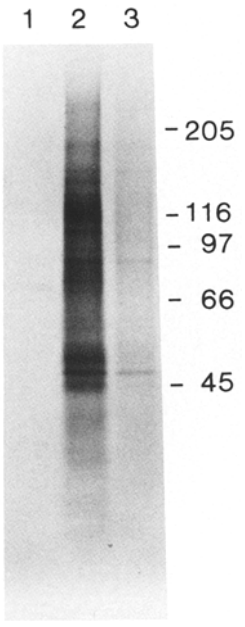


Fig. 2. Conditions for efficient recovery of radiolabeled biotinylated surface proteins. A6 cell proteins were pulse labeled 15 min with [^{35}S]methionine and chased for 1 hour with unlabeled methionine. Sulfo-NHS-biotin was added to the apical solution for 20 min, cells were then washed and solubilized in DNET buffer. Radiolabeled biotinylated proteins were precipitated with streptavidin-agarose and eluted. Nonprecipitated proteins were subjected to a second precipitation with streptavidin-agarose. Precipitated proteins were analyzed by 5–13% SDS-PAGE and autofluorography. Parallel control cells not exposed to sulfo-NHS-biotin were also examined. (*Lane 1*) Nonbiotinylated apical proteins precipitated with streptavidin-agarose. (*Lane 2*) Apical biotinylated protein precipitated with streptavidin-agarose. (*Lane 3*) Second precipitation of biotinylated proteins with streptavidin-agarose. Migration of various molecular weight standards is indicated at the right of the figure.

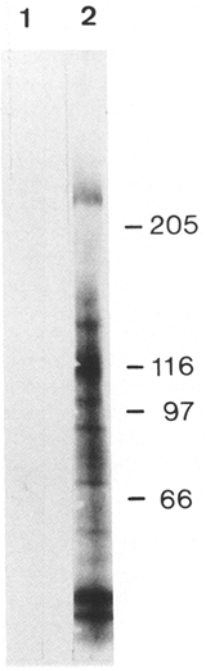


Fig. 3. Addition of free biotin prevents the precipitation of biotinylated proteins by streptavidin agarose. A6 cell proteins were labeled overnight with [^{35}S]methionine as described under Materials and Methods. Sulfo-NHS-biotin was added to the basolateral solution for 20 min and cells were then washed and solubilized in DNET buffer. Radiolabeled biotinylated proteins were precipitated with streptavidin-agarose in the absence or presence of 1 mM biotin. Precipitated proteins were analyzed by 5–13% SDS-PAGE and autofluorography. (*Lane 1*) Basolateral biotinylated protein precipitated with streptavidin-agarose in the presence of 1 mM biotin. (*Lane 2*) Basolateral biotinylated protein precipitated with streptavidin-agarose. Migration of various molecular weight standards is indicated to the right of the figure.

has received limited study. We have taken advantage of the water soluble N-hydroxysuccinimide derivative of biotin (sulfo-NHS-biotin) to identify newly synthesized proteins labeled with [^{35}S]methionine, by tagging them with biotin as they reach the cell surface. This nonpermeant reagent reacts specifically with free amino groups incorporating biotin into membrane proteins (Sargiacomo et al., 1989).

Previous studies suggest that two successive incubations of sulfo-NHS-biotin (0.5 mg/ml) for 20 min is sufficient to label membrane proteins (Graeve, Drickamer & Rodriguez-Boulan, 1989; Le Bivic, Real & Rodriguez-Boulan, 1989; Le Bivic et al., 1990*a*,*b*). We compared the efficiency of biotinylation of apical membrane proteins in A6 cells following an incubation for 15 min with [^{35}S] methionine and a chase of 30 min with excess unlabeled methionine. Cells were incubated with 0.5 mg/ml sulfo-NHS-biotin for 20 min in the apical compartment, or with successive 20 min incubations for a total of 40 or 60 min. Cells were then detergent solubilized. Biotinylated proteins were recovered by precipitation with streptavidin-agarose and analyzed by SDS-PAGE and autofluorography. The recovery of la-

Table 1. Epithelial resistance before and after the biotinylation procedure

Resistance ($\Omega \cdot cm^2$)	
Before biotinylation	After biotinylation
5,090 ± 921 $\Omega \cdot cm^2$*	3,915 ± 813 $\Omega \cdot cm^2$*

A6 cells were seeded at a density of 1×10^6 cells/cm^2 on collagen-coated polycarbonate filters (4.7 cm^2). Cell resistance was measured using an EVOM apparatus (World Precision Instruments) prior to metabolic labeling and following biotinylation. * (± SE, $n = 21$).

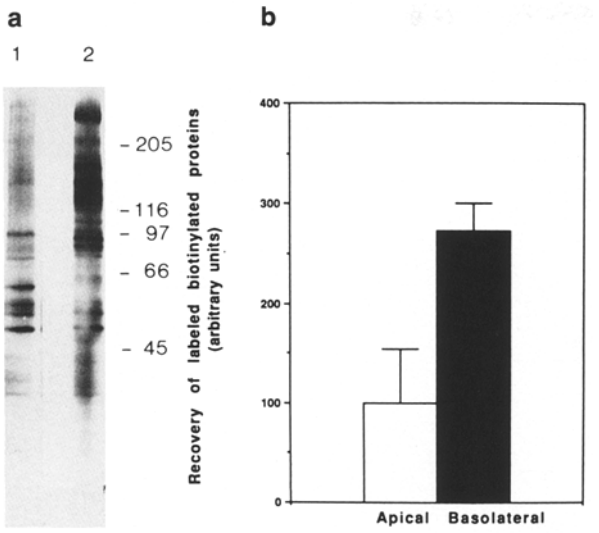
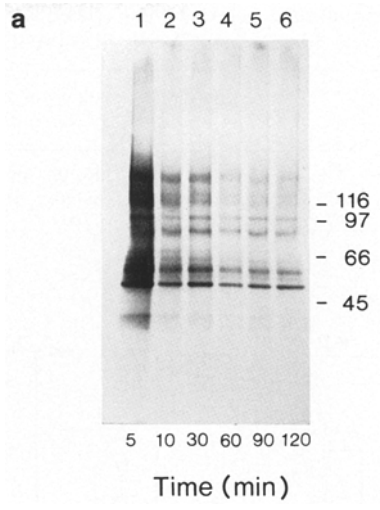


Fig. 4. Steady-state distribution of biotinylated apical and basolateral proteins. A6 cells were labeled 16 hr with [^{35}S]methionine as indicated under Materials and Methods. Sulfo-NHS-biotin (0.5 mg/ml) was added to the apical or basolateral compartment for 20 min at 4°C. Proteins were then solubilized in a DNET buffer, precipitated with streptavidin-agarose, analyzed by 5–13% SDS-PAGE, autofluorography (*a*), and quantitated by scanning densitometry (*b*). (*a*) Autofluorography of radiolabeled biotinylated apical (*lane 1*) and basolateral (*lane 2*) membrane proteins. Migration of various molecular weight standards is indicated at the right of the figure. (*b*) Comparison of amounts of radiolabeled biotinylated proteins obtained from densitometric scanning of autoradiograms as described under Materials and Methods. Results are expressed in arbitrary units ($n = 3$).

beled proteins was similar despite varying times of incubation with sulfo-NHS-biotin (Fig. 1). Few labeled proteins were recovered from cells which were not subjected to biotinylation (Fig. 2, *lane 1*). A single 20 min incubation with sulfo-NHS-biotin was used in subsequent experiments.

Efficiency of precipitation of biotinylated proteins with streptavidin-agarose beads was examined. Following an initial overnight incubation at 4°C with streptavidin-agarose and centrifugation to recover the biotinylated proteins, streptavidin-agarose beads were again added to the supernatant and a second precipitation was performed. Labeled, biotinylated proteins recovered with each successive precipitation were examined by SDS-PAGE and autofluorography. The efficiency of precipitation of labeled, biotinylated protein with one overnight incubation with streptavidin beads was greater than 90%, assuming that the entire pool was recovered with two successive precipitations (Fig. 2). The length of incubation of the radiolabeled, biotinylated proteins with streptavidin beads required for efficient precipitation was also examined. The recovery markedly diminished if the length of incubation was reduced to 1 or 3 hr (*data not shown*).



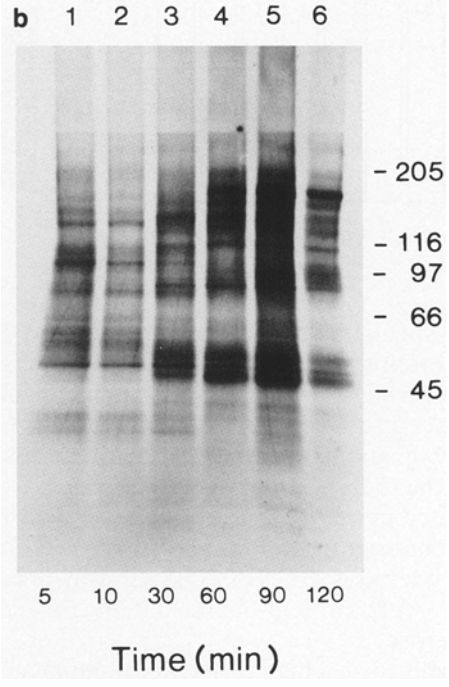


Fig. 5. Arrival of newly synthesized A6 cell proteins at the apical or basolateral surface. A6 cells were pulse labeled for 15 min with [^{35}S]methionine and chased with unlabeled methionine for a period of time varying between 0 and 120 min. Sulfo-NHS-biotin was then added to the apical or basolateral compartment; radiolabeled biotinylated proteins were solubilized in DNET buffer, precipitated with streptavidin-agarose, and analyzed by 5–13% SDS-PAGE and autofluorography. (*a*) Autoradiogram of biotinylated proteins appearing at the apical cell surface during the chase period. (*b*) Autoradiography of biotinylated protein delivery at the basolateral cell surface. The length of the chase (in min) is indicated at the bottom of each lane. Migration of various molecular weight standards is indicated at the right of each figure.

Table 2. Apparent molecular weights of biotinylated, [^{35}S]methionine (steady-state) labeled apical and basolateral membrane proteins identified by SDS-PAGE and autofluorography

Experimental condition	Apparent molecular weights (kD)
Apical	257, 232, 198, 145, 98, 88, 81, 66, 58, 54, 49, 39, 33, 30
Basolateral	244, 198, 165, 145, 127, 109, 101, 96, 91, 84, 70, 54, 49, 37, 35, 31, 30

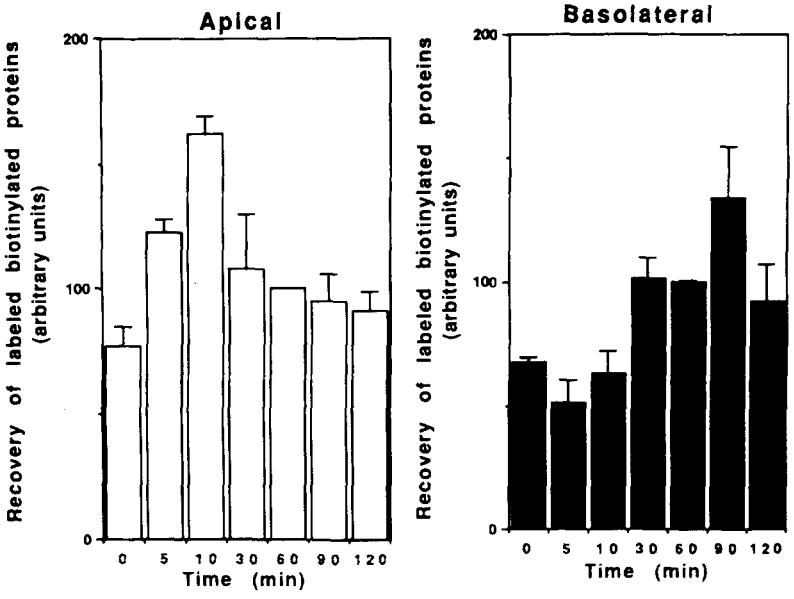


Fig. 6. Quantitation of the delivery of newly synthesized proteins to the apical and basolateral membrane. Densitometric scans of autoradiograms from five experiments, as represented in Fig. 5, were performed as described under Materials and Methods. Results are expressed in arbitrary units. The density of labeled proteins recovered at 60 min was assigned a value of 100. (*Left*) Apical biotinylation (open bars). (*Right*) Basolateral biotinylation (filled bars). The duration of the chase (min) is indicated at the bottom.

The specificity of the precipitation of biotinylated proteins was also examined. A6 cells were labeled overnight with [^{35}S]methionine. Basolateral cell surface proteins were biotinylated and, following solubilization, precipitated with streptavidin beads in the presence or absence of 1 mM free biotin. Precipitated proteins were examined by SDS-PAGE and autofluorography (Fig. 3). No labeled proteins were recovered if excess free biotin was present to compete with protein-bound biotin for streptavidin-agarose.

A6 cells form a high resistance monolayer when grown on semipermeable supports. The resistance fell only 23% following biotinylation of cell surface proteins to a value of 3,915 ± 813 $\Omega \cdot cm^2$ (Table 1), suggesting that when biotin is added to the solution bathing one of the cell surfaces, it will not leak across the epithelium.

The Distribution of Proteins at Apical and Basolateral Cell Surface Is Polarized

Proteins were labeled by incubating cells for 16 hr with [^{35}S]methionine (steady-state labeling) as described under Materials and Methods. Proteins at the apical or basolateral membrane were biotinylated and cells solubilized. Biotinylated proteins were precipitated with streptavidin-agarose, eluted, and analyzed by SDS-PAGE and autofluorography (Fig. 4*a*). The apparent molecular weights of these polypeptides are listed in Table 2. The profile of proteins identified at the apical membrane is distinct from that identified at the basolateral membrane, indicating that cell surface proteins in A6 cells are polarized. In addition, the recovery of labeled proteins present at the basolateral membrane is threefold greater than that recovered from the apical membrane (Fig. 4*b*).

The Arrival of Newly Synthesized Apical or Basolateral Membrane Proteins Is Sequential

Delivery of newly synthesized proteins to the apical or basolateral membrane was examined in pulse-chase studies by incubating cells with [^{35}S]methionine for 15 min (pulse), followed by an incubation with unlabeled methionine for varying periods of

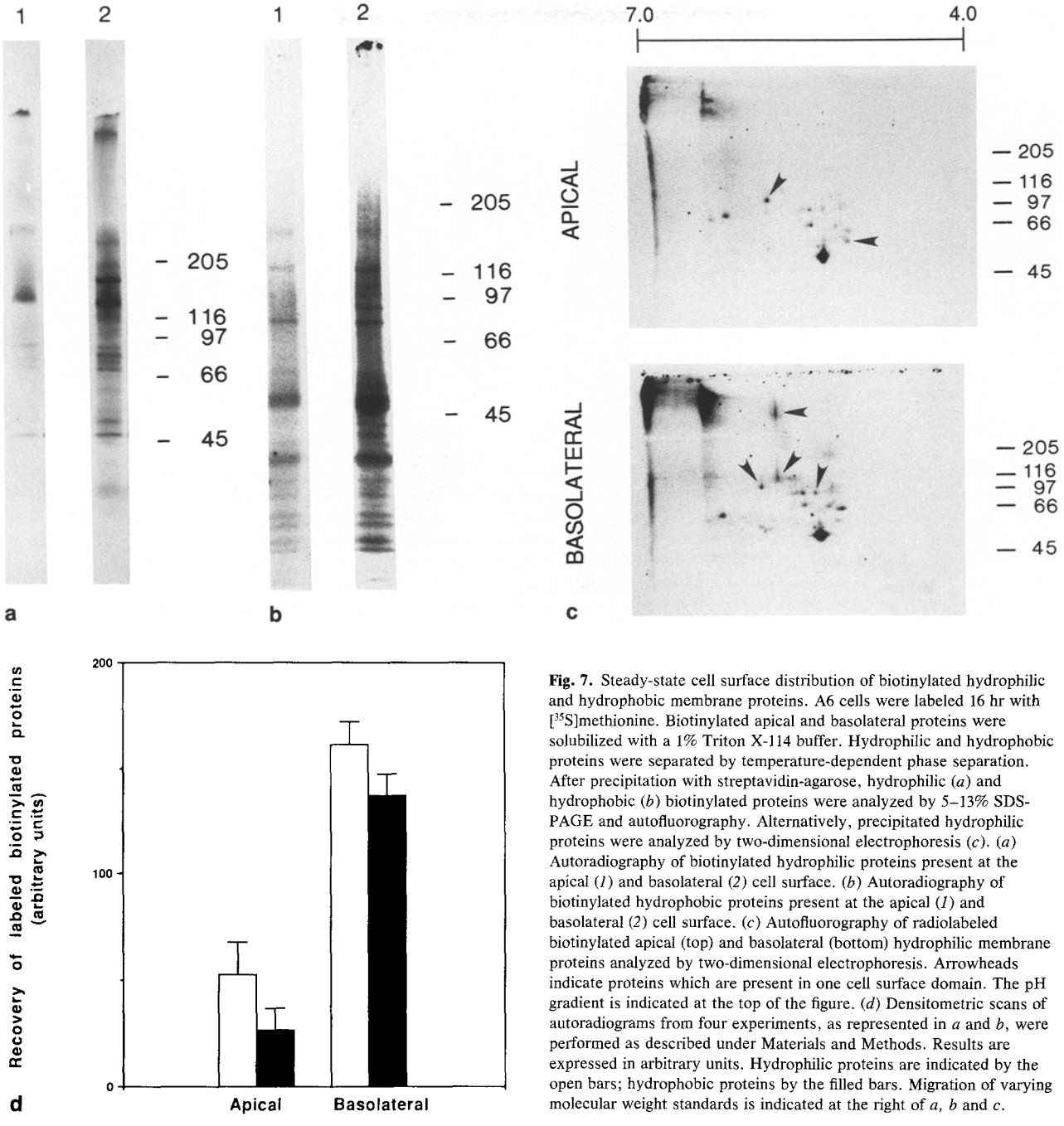


Fig. 7. Steady-state cell surface distribution of biotinylated hydrophilic and hydrophobic membrane proteins. A6 cells were labeled 16 hr with [^{35}S]methionine. Biotinylated apical and basolateral proteins were solubilized with a 1% Triton X-114 buffer. Hydrophilic and hydrophobic proteins were separated by temperature-dependent phase separation. After precipitation with streptavidin-agarose, hydrophilic (*a*) and hydrophobic (*b*) biotinylated proteins were analyzed by 5–13% SDS-PAGE and autofluorography. Alternatively, precipitated hydrophilic proteins were analyzed by two-dimensional electrophoresis (*c*). (*a*) Autoradiography of biotinylated hydrophilic proteins present at the apical (*1*) and basolateral (*2*) cell surface. (*b*) Autoradiography of biotinylated hydrophobic proteins present at the apical (*1*) and basolateral (*2*) cell surface. (*c*) Autofluorography of radiolabeled biotinylated apical (top) and basolateral (bottom) hydrophilic membrane proteins analyzed by two-dimensional electrophoresis. Arrowheads indicate proteins which are present in one cell surface domain. The pH gradient is indicated at the top of the figure. (*d*) Densitometric scans of autoradiograms from four experiments, as represented in *a* and *b*, were performed as described under Materials and Methods. Results are expressed in arbitrary units. Hydrophilic proteins are indicated by the open bars; hydrophobic proteins by the filled bars. Migration of varying molecular weight standards is indicated at the right of *a*, *b* and *c*.

Table 3. Apparent molecular weights of hydrophilic and hydrophobic biotinylated, [^{35}S]methionine (steady-state) labeled apical and basolateral membrane proteins following phase separation with Triton X-114 and identified by SDS-PAGE and autofluorography

Experimental condition	Apparent molecular weights (kD)	
	Hydrophilic proteins	Hydrophobic proteins
Apical	243, 138, 91, 42	151, 120, 81, 45, 29, 25, 22, 19, 18, 16, 15
Basolateral	225, 185, 164, 134, 119, 102, 93, 86, 80, 76, 49, 44	187, 158, 120, 99, 89, 80, 55, 51, 45, 38, 35, 29, 25, 22, 19, 18, 16, 15

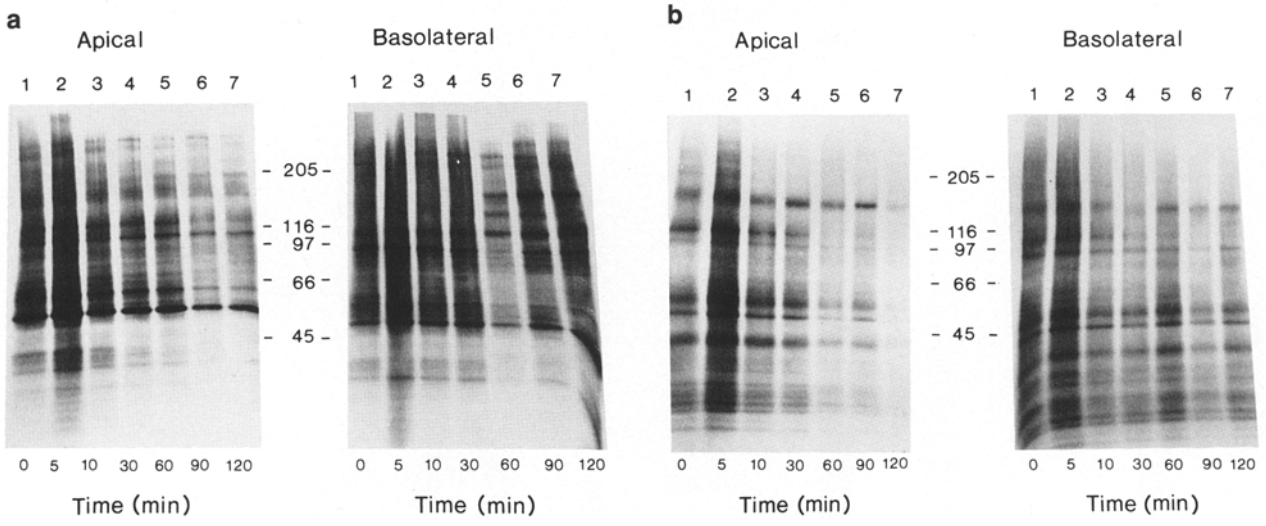


Fig. 8. Arrival of newly synthesized biotinylated hydrophilic and hydrophobic proteins at the apical and basolateral cell surface. A6 cells were pulse labeled for 15 min with [^{35}S]methionine and chased with unlabeled methionine for a period of time varying between 0 and 120 min. After selective biotinylation, cells were solubilized in TSE-T buffer and hydrophilic and hydrophobic proteins were separated. After precipitation with streptavidin-agarose, hydrophilic and hydrophobic biotinylated proteins were analyzed by 5–13% SDS-PAGE and autofluorography. (*a*) Autoradiography of biotinylated hydrophilic proteins appearing at the apical and basolateral cell surface during the chase period. (*b*) Autoradiography of biotinylated hydrophobic proteins appearing at the apical and basolateral cell surface during the chase period. The length of the chase (min) is indicated at the bottom of each lane. Migration of various molecular weight standards is indicated in the middle of each figure.

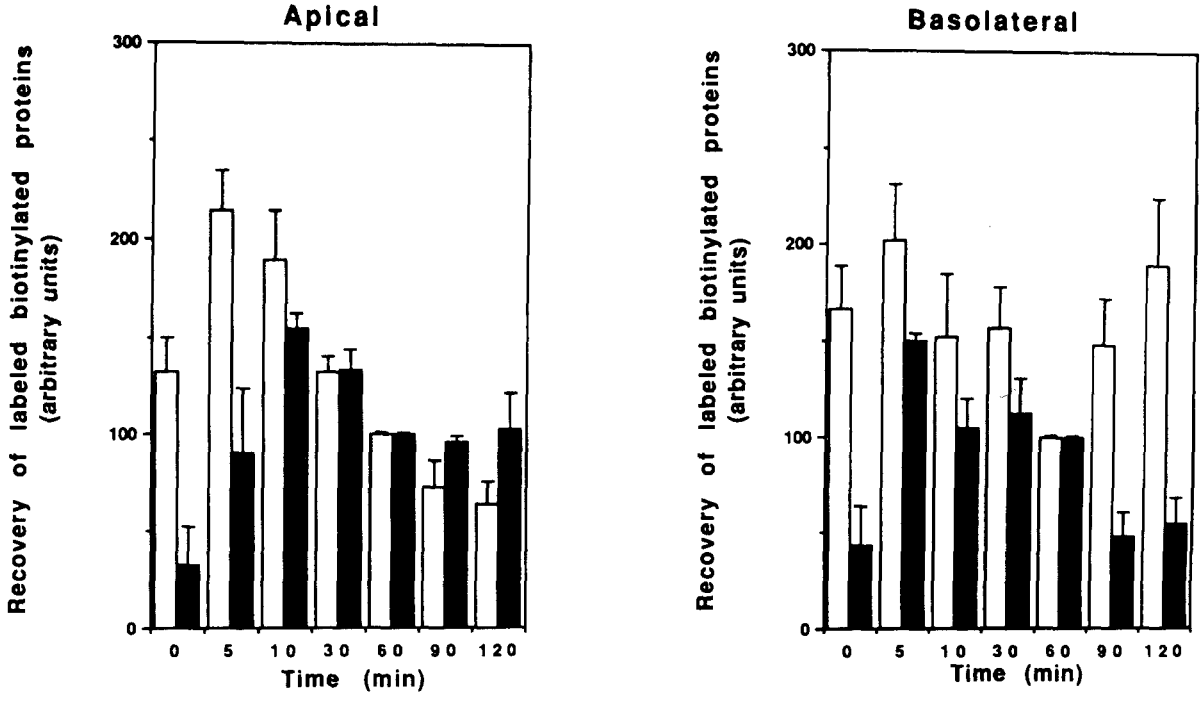


Fig. 9. Quantitation of delivery of newly synthesized biotinylated hydrophilic and hydrophobic proteins to the apical or basolateral cell surface. Densitometric scans of autoradiograms from five experiments, as represented in Fig. 8, were performed as described under Materials and Methods. Results are expressed in arbitrary units. The density of labeled proteins recovered at 60 min was assigned a value of 100. Hydrophilic proteins are indicated by the open bars; hydrophobic proteins by the filled bars.

time (chase). Proteins at the apical or basolateral cell surface were selectively labeled with sulfo-NHS-biotin, detergent solubilized, recovered with streptavidin-agarose, and analyzed by SDS-PAGE and autofluorography as described under Materials and Methods. Labeled proteins were detected at both cell surfaces following the 15 min pulse. Delivery of newly synthesized proteins to the apical surface reached a maximum value after a 10 min chase. The amount of biotinylated, radiolabeled protein at the apical membrane fell over the next 20 min and then remained relatively constant over the remainder of the 2 hr chase. Newly synthesized proteins detected at the basolateral membrane increased slowly during the chase period, reaching a maximum after 90 min of chase, and falling over the next 30 min (Figs. 5 and 6).

Partitioning of Hydrophilic and Hydrophobic Biotinylated Proteins with Triton X-114

Membrane proteins can be separated based on hydrophobicity (or hydrophilicity) with the detergent Triton X-114 (Bordier 1981; Conzelmann et al., 1986). This detergent undergoes a temperature-induced phase transition; hydrophilic proteins partition into an aqueous phase, whereas hydrophobic proteins partition into a detergent phase. The cell surface distribution of both hydrophilic and hydrophobic proteins was examined as described under Materials and Methods. Proteins were labeled with [^{35}S]methionine for 16 hr followed by biotinylation of apical or basolateral membrane proteins. Cells were solubilized in a buffer containing 1% Triton X-114. Following phase separation, biotinylated proteins were precipitated with streptavidin beads and labeled proteins identified by SDS-PAGE and autofluorography.

Hydrophilic proteins at the apical membrane differ, in part, from those at the basolateral membrane (Fig. 7*a*), confirming results described above using DNET buffer. Two-dimensional electrophoresis of [^{35}S]methionine-labeled, biotinylated proteins confirmed that several proteins were not present at both surface domains (Fig. 7*c*). In contrast, hydrophobic proteins identified at the apical membrane were similar in size to those identified at the basolateral membrane (Fig. 7*b*), although not all basolateral proteins were present at the apical surface. The apparent molecular weights of these polypeptides are listed in Table 3. In the presence of Triton X-114, the recovery of biotinylated hydrophilic proteins at the basolateral surface was three-fold greater than at the apical cell surface. Recovery of labeled hydrophobic proteins present at the basolateral membrane was five-fold greater than that recovered from the apical membrane (Fig. 7*d*).

The delivery of newly synthesized hydrophilic proteins and hydrophobic proteins was examined with pulse-chase labeling studies as described under Materials and Methods. Proteins were pulse labeled with [^{35}S] methionine for 15 min, chased for varying periods of time with unlabeled methionine, and the apical or basolateral cell surface proteins were subjected to biotinylation prior to solubilization with Triton X-114. Following phase separation, biotinylated hydrophilic and hydrophobic proteins were precipitated with streptavidin-agarose beads and analyzed by SDS-PAGE and autofluorography (Figs. 8 and 9). The maximal amounts of newly synthesized hydrophilic and hydrophobic apical proteins were noted 5 and 10 min, respectively, after the pulse and then fell over the next 50 to 80 min, remaining relatively stable for the remainder of the chase. Delivery of hydrophilic proteins to the basolateral membrane was relatively stable over the first 30 min of chase, fell over the next 30 min, but increased over the last hour of the chase. In contrast, hydrophobic basolateral proteins reached their maxima within 5 min of the chase, fell and stabilized over the first hour, but decreased over the last hour of the chase.

Discussion

The membrane protein composition at the apical surface of A6 cells obtained after cell solubilization with the DNET buffer clearly differs from that at the basolateral membrane (Fig. 4*a*). This is in agreement with studies which examined the composition of apical and basolateral membrane proteins in A6 cells using selective cell surface radioiodination to label apical or basolateral membrane proteins (Kleyman et al., 1991; Kleyman, Coupaye-Gerard & Ernst, 1992). Identification of hydrophilic proteins at the apical and basolateral cell surface with Triton X-114 confirmed that the composition of proteins at the two cell surface domains differs. However, common proteins at the two cell surface domains were identified in the hydrophobic phase with Triton X-114. While these results suggest that the distribution of A6 cell surface proteins is polarized, it is incomplete. Furthermore, recovery of radiolabeled biotinylated proteins from the basolateral surface was between three- to fivefold greater than at the apical cell surface and likely reflects a larger basolateral membrane surface area. One limitation of this study is that only proteins with accessible extracellular lysine residues will be labeled with sulfo-NHS-biotin and detected on the autoradiograms. A second limi-

tation is that proteins which are not biotinylated, but are tightly associated with biotinylated membrane proteins could be precipitated with streptavidin.

The bulk flow of newly synthesized proteins to the apical surface is rapid, reaching a maximum at 5 to 10 min into the chase period. This was observed using either DNET or TSE-T as detergent buffers. The bulk flow of newly synthesized proteins to the basolateral surface varied somewhat depending on the detergent used to solubilize cells. Results with DNET buffer suggest that delivery of newly synthesized proteins to the basolateral membrane is relatively slow, reaching a maximum at 90 min. Newly synthesized hydrophilic proteins, obtained following solubilization with TSE-T buffer and phase separation, arrived at the basolateral cell surface with a similar time course. In contrast, only an early peak of delivery of hydrophobic proteins to the basolateral cell surface was observed. The differences in delivery of newly synthesized proteins to the basolateral membrane may, in part, reflect variability in proteins recovered following solubilization with the different detergents. In addition, not all biotinylated proteins initially solubilized with Triton X-114 were subsequently analyzed by SDS-PAGE. Studies with Triton X-114 were performed using proteins separated based on hydrophilicity. Following the initial phase separation, subsequent extractions (with Triton X-114) were performed to ensure that only hydrophilic (or hydrophobic) proteins were analyzed and the extracted proteins were discarded. The Triton X-114 experiments do not examine the total pool of surface proteins, but a select pool of proteins which are labeled with [^{35}S]methionine, accessible to biotinylation, extracted with Triton X-114 and, following the initial phase separation, are not lost with subsequent Triton X-114 extractions.

Other investigators have examined the flow of proteins to the cell surface. An N-acyl-glycosyl-tripeptide transported by bulk flow to the cell surface (Wieland et al., 1987) and several peripheral and integral newly synthesized apical glycoproteins in MDCK cells (Lisanti et al., 1989) have been shown to possess a rapid transport to the cell surface. Glycoproteins were maximally present at the apical surface of MDCK cells immediately following a 15 min pulse labeling with [^{35}S]cysteine (Lisanti et al., 1989), suggesting that intracellular sorting and polarized delivery to the apical surface can be rapid. In contrast, basolateral glycoproteins moved slowly to the cell surface in MDCK cells, as it is the case in A6 cells where newly synthesized biotinylated basolateral proteins reach their maxima after a 90 to 120 min chase. Compared with protein transport, the bulk flow rate of lipid from endoplasmic reticulum to Golgi in CHO cells is rapid. Four minutes are required for half of the endoplasmic reticulum to be drained into the proximal Golgi at 30°C and sphingomyelin, a major component of the bulk of the lipid bilayer, is transported from the Golgi to the cell surface with a half-time of 14 min (Karrenbauer et al., 1990). Although our results and those of Lisanti et al. (1989) suggest that newly synthesized proteins can rapidly transit to the apical or basolateral cell surface, individual proteins which rapidly reach the cell surface have not been identified. This reflects, in part, the limited number of specific proteins whose delivery rates to the cell surface in epithelia have been examined. The differences in delivery of newly synthesized proteins to the apical and basolateral membrane may reflect the pathways used to transit from the Golgi to the cell surface. In addition, the relative proximity of the Golgi apparatus to the apical membrane in epithelia could account for differences in appearance of newly synthesized proteins at the apical or basolateral cell surface.

A6 cells serve as a model epithelium for the study of transepithelial Na^+ transport. In light of our results examining the bulk flow of newly synthesized proteins to the two surface domains in A6 cells, it will be of interest to examine the delivery of the epithelial Na^+ channel and Na^+, K^+-ATPase along with the intracellular pathways taken by these proteins to reach their final destination.

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