

# Biogenetic Pathways of Plasma Membrane Proteins in Caco-2, a Human Intestinal Epithelial Cell Line

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**Abstract.** We studied the sorting and surface delivery of three apical and three basolateral proteins in the polarized epithelial cell line Caco-2, using pulse-chase radiolabeling and surface domain-selective biotinylation (Le Bivic, A., F. X. Real, and E. Rodriguez-Boulan. 1989. *Proc. Natl. Acad. Sci. USA*. 86:9313-9317). While the basolateral proteins (antigen 525, HLA-I, and transferrin receptor) were targeted directly and efficiently to the basolateral membrane, the apical markers (sucrase-isomaltase [SI], aminopeptidase N [APN], and alkaline phosphatase [ALP]) reached the apical membrane by different routes. The large majority (80%) of newly synthesized ALP was directly targeted to the apical surface and the mis-sorted basolateral pool was very inefficiently transcytosed. SI was more efficiently targeted to the apical membrane (>90%) but, in contrast to ALP, the mis-

sorted basolateral pool was rapidly transcytosed. Surprisingly, a distinct peak of APN was detected on the basolateral domain before its accumulation in the apical membrane; this transient basolateral pool (at least 60-70% of the enzyme reaching the apical surface, as measured by continuous basal addition of antibodies) was efficiently transcytosed. In contrast with their transient basolateral expression, apical proteins were more stably localized on the apical surface, apparently because of their low endocytic capability in this membrane. Thus, compared with two other well-characterized epithelial models, MDCK cells and the hepatocyte, Caco-2 cells have an intermediate sorting phenotype, with apical proteins using both direct and indirect pathways, and basolateral proteins using only direct pathways, during biogenesis.

**T**HE polarized distribution of epithelial plasma membrane proteins into apical and basolateral domains is a fascinating paradigm of molecular sorting in cell biology. Work in the last ten years has defined the general aspects of the biogenesis of plasma membrane proteins but has generated contradictory findings on the pathways followed by apical proteins during intracellular transport (for reviews see references 1, 8, 39, 47). Experiments carried out in native intestinal cells by Hauri et al. (16) and Massey et al. (27) and in hepatocytes by Bartles et al. (2) indicated that apical and basal glycoproteins are initially delivered to the basolateral cell surface, where sorting takes place: basolateral proteins remain there whereas apical proteins are transcytosed to the apical surface. On the other hand, experiments with the model epithelial cell line MDCK (39-42) have shown that viral envelope proteins introduced by infection, namely influenza HA (apical) and vesicular stomatitis virus G protein (basolateral) are sorted in the *trans*-Golgi Network and are vectorially delivered to the respective surface domain (14, 28, 32, 35, 38).

Because radically diverse methods were used, doubts arose as to whether the different biogenetic pathways ob-

served in native and cultured cells represent real differences between epithelial cell types, or are the consequence of the varying experimental approaches (9, 14, 28, 32, 35, 38). To solve this controversy, new techniques were developed to study the targeting of endogenous plasma membrane proteins in epithelial cell lines. Using a photoactivatable ouabain probe, Caplan et al. (9) showed that the alpha subunit of Na,K,ATPase is directly targeted to the basolateral surface of MDCK cells. A more general method, domain-selective biotinylation combined with lectin precipitation, was used by Lisanti et al. (24) to show that several endogenous glycoproteins of MDCK cells displayed surface expression kinetics consistent with vectorial targeting to the apical and basolateral domains. Domain-selective biotinylation was combined with immunoprecipitation and streptavidin precipitation by Le Bivic et al. (21, 22) to show that apical and basolateral proteins of MDCK cells and SK-CO-15 (a human intestinal cell line) were sorted intracellularly and vectorially targeted. This approach was sensitive enough to detect the transient basolateral appearance of polymeric Ig receptor (22), a transcytosing receptor (33). A cleavable biotin probe was used to directly show that the basolateral

pools of apical antigens were not precursors to the apical pools, thus providing a clear proof of the vectorial apical pathway (21, 22).

These results seemed to validate the vectorial biogenetic pathways; however, the two apical proteins for which the transcytotic pathway was originally proposed, aminopeptidase N (APN)<sup>1</sup> and sucrase-isomaltase (SI), were not characterized in cultured epithelial cells. We report here the use of the biotin targeting assays (mentioned above) to study the biogenesis of APN, SI, and other apical and basolateral proteins in the polarized human colon carcinoma cell line, Caco-2 (36). Our results clearly demonstrate that transcytotic and vectorial (apical and basolateral) biogenetic routes coexist in gut epithelial cells. Similar results were reported by Matter et al. (29) when this manuscript was in revision.

## Materials and Methods

### Reagents

Cell culture reagents were purchased from Gibco Laboratories (Grand Island, NY). Affinity-purified antibodies (rabbit anti-mouse IgG) were purchased from Cappel Laboratories (Westchester, PA). Protein A-Sepharose was from Pharmacia (Uppsala, Sweden); sulfo-*N*-hydroxyl-succinimido-biotin (S-NHS-biotin), sulfosuccinimidyl-2-(biotinamido) ethyl 1,3-dithiopropionate (S-NHS-SS-biotin), and streptavidin-agarose beads were from Pierce Chemical Co. (Rockford, IL). All other reagents were obtained from Sigma Chemical Co. (St. Louis, MO).

### Cells, Antibodies, and Cell Culture

Caco-2 cells (12) were grown in DME supplemented with 10% FBS, nonessential amino acids (1%), penicillin (50mU/ml), and streptomycin (50 µg/ml). For experiments, cells were grown on Transwells (Costar Data Packaging Corp., Cambridge, MA) and were used between 15 and 20 d after confluency to ensure optimal differentiation of the cells (36). Monoclonal antibody against HLA-DR A,B,C was purchased from AMAC (Westbrook, MI) and monoclonal antibody against human transferrin receptor (TFR) from Boehringer (Mannheim, FRG). Rabbit polyclonal antibodies against human placental alkaline phosphatase were from Accurate Chemical &

1. *Abbreviations used in this paper:* ALP, alkaline phosphatase; APN, aminopeptidase N; PIPLC, phosphatidylinositol phospholipase C; SI, sucrase-isomaltase; S-NHS, sulfo-*N*-hydroxy-succinimido; S-NHS-SS, sulfosuccinimidyl-2-(biotinamido) ethyl 1,3-dithiopropionate; TFR, transferrin receptor.

Scientific Corp. (Westbury, NY), and monoclonal antibody 525-5-4 against antigen 525 (Ag 525) has been described previously (20). Preparation and characterization of the mouse monoclonal antibodies HSI 9, specific for sucrase, and HSI 14, for isomaltase, have been described elsewhere (3). The monoclonal antibody HBB2/45, specific for human APN, was prepared by fusion of nSI myeloma cells with spleen cells obtained from a BALB/c mouse, immunized with human intestinal brush border membranes (200 µg protein/injection, three intraperitoneal injections, followed by harvesting of the spleen cells 3 d after the last boost). Hybridoma cells were selected and screened by immunofluorescence cell staining of jejunal frozen sections as previously described (3). The HBB2/45 hybridoma, producing antibodies staining specifically the luminal membrane of both crypt and villus cells in human jejunum, was cloned twice by dilution plating (3). It was found to produce immunoglobulins of the IgG1 subtype, which were purified from ascites fluids by affinity chromatography on a Sepharose 4B-protein A column and covalently linked to cyanogen bromide-activated Sepharose 4B. All techniques used in the determination of the antigen specificity of this antibody have been described previously (3). HBB2/45 specifically immunoprecipitated a protein of 145 kD from Triton X-100-solubilized, <sup>14</sup>C-labeled brush border membranes, which was found to have aminopeptidase activity, but lacked all other enzyme activities tested (lactase, dipeptidyl peptidase IV, alkaline phosphatase, sucrase, and maltase). Monoclonal antibody against human decay-accelerating factor, directly coupled to Sepharose beads, was a generous gift from M. Davidz (New York University, New York).

### Biotinylation and Reduction

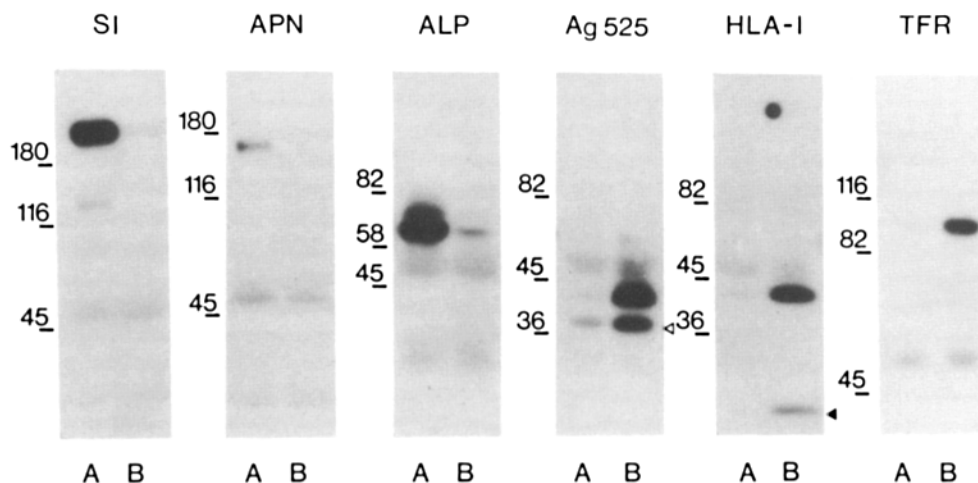
Biotinylation of monolayers on Transwells with S-NHS-biotin and S-NHS-SS-biotin was carried out twice in a row for 20 min each as described (43). After biotinylation, the surface exposed to S-NHS-SS-biotin was reduced twice with 50 mM glutathione (25 min each) at 4°C as described (7, 21).

### Pulse-Chase Experiments

Cells on filters were incubated for 30 min in DME without methionine or cysteine and pulsed for 20 min the same medium containing 0.8 mCi/ml of tran<sup>35</sup>S-label (ICN K&K Laboratories Inc., Plainview, NY) and 0.4 mCi/ml of <sup>35</sup>S-cysteine (New England Nuclear, Boston, MA) as described (33). After a wash with DME, cells were chased in DME containing 5X cysteine and methionine at normal concentrations and kept at 4°C in NaCO<sub>3</sub>H-free DME, 20 mM Hepes, 0.2% BSA before biotinylation.

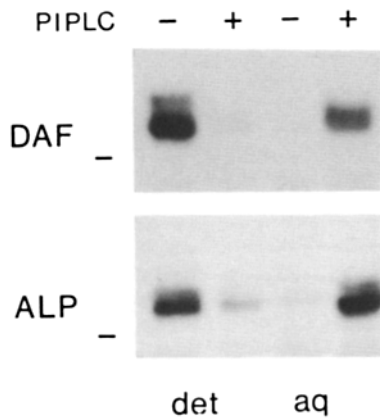
### Immunoprecipitation and Streptavidin Precipitation

After biotinylation cells were processed for immunoprecipitation as described previously (21) using dilutions of 1:100 for monoclonal antibodies (ascites fluid) and of 1:200 for polyclonal antibodies. SI was precipitated using HSI 9 against sucrase and HSI 14 against isomaltase in combination. To recover the immunoprecipitated biotinylated antigens, the beads were



**Figure 1.** Immunoprecipitation of SI, APN, ALP, Ag 525, HLA-I, and TFR after surface labeling of the apical (A) or the basolateral (B) sides of Caco-2 cells with S-NHS-biotin. The biotinylated proteins were revealed, after SDS/6–16% PAGE and transfer to nitrocellulose, by <sup>125</sup>I-streptavidin blotting. Molecular masses (in kilodaltons) are indicated. SI, APN, and ALP were mainly labeled from the apical side while Ag 525, HLA-I, and TFR were mainly detected from the basolateral side. The 45–50-kD band seen in each lane is due to the IgG from the im-

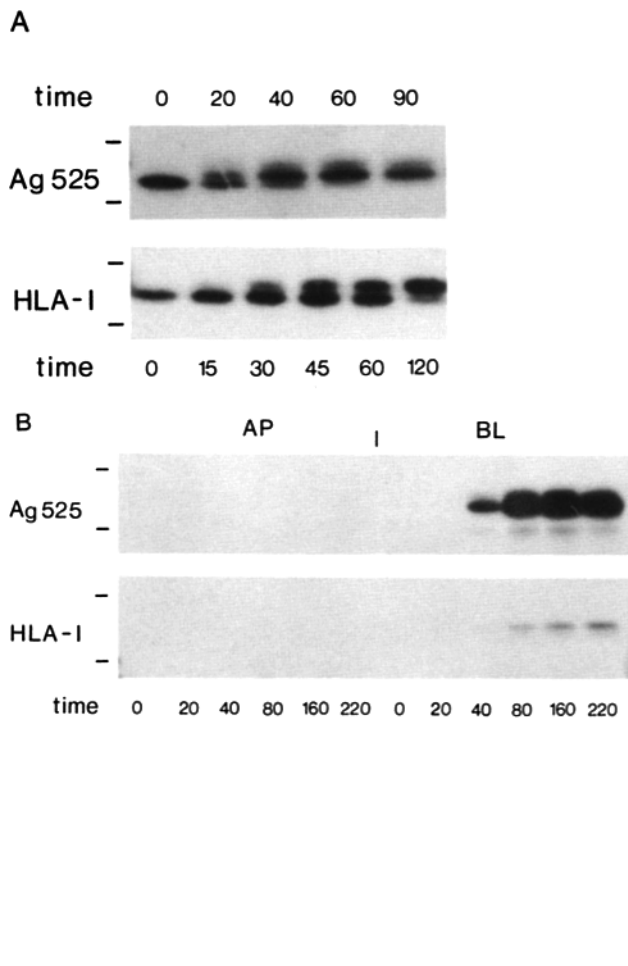
muno-precipitation that binds nonspecifically to <sup>125</sup>I-streptavidin. The open arrowhead points to a degradation product of Ag 525 and the solid arrowhead to beta 2-microglobulin coprecipitating with HLA-I.



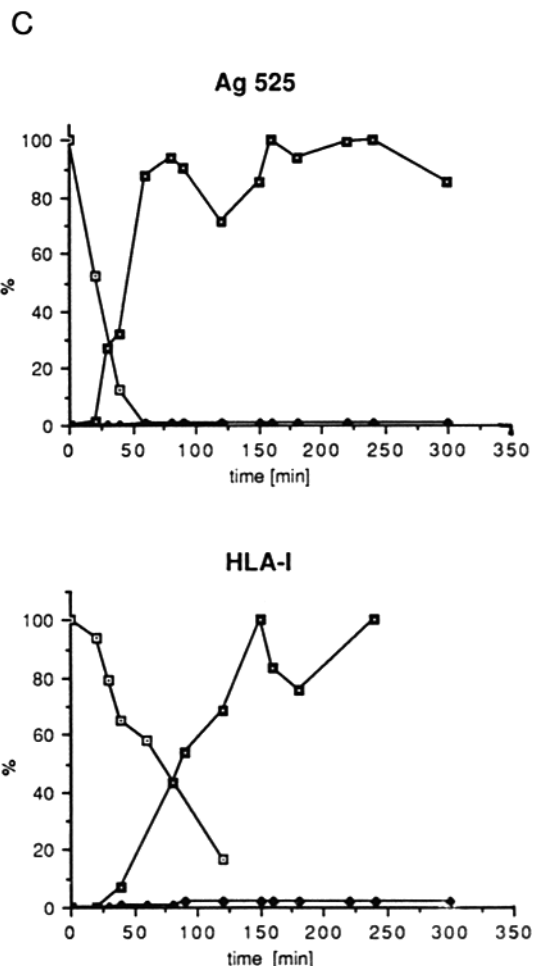
**Figure 2.** PIPLC digestion of the Triton X-114 phase after surface labeling of Caco-2 cells with S-NHS-biotin. Samples were treated with (+) or without (-) PIPLC and the resulting aqueous (*aq*) and detergent (*det*) phases were immunoprecipitated with anti-decay-accelerating factor (*DAF*) or anti-ALP antibodies. Immunoprecipitated decay-accelerating factor and ALP were then analyzed and revealed as in Fig. 1. Molecular mass marker is 58 kD.

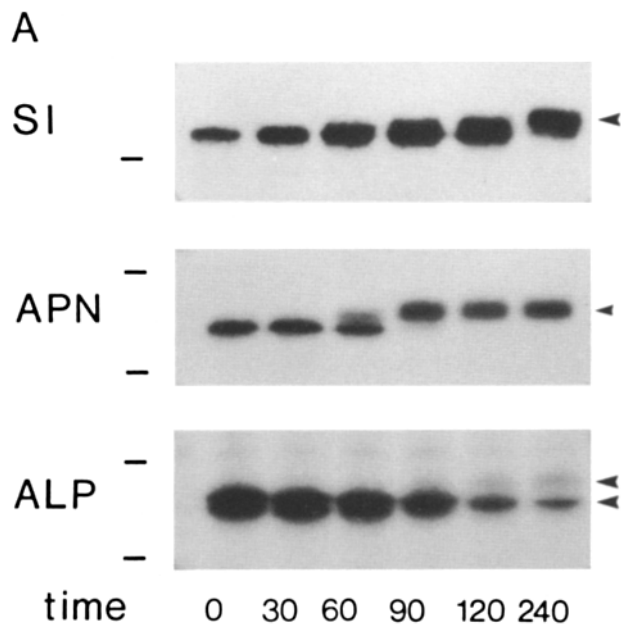
boiled with 10  $\mu$ l of 10% SDS for 5 min, diluted with lysis buffer, and centrifuged 1 min at 15,000 *g*. Biotinylated antigens present in supernatants were precipitated with streptavidin agarose beads as described (21). Finally the beads were boiled in SDS-PAGE sample buffer and analyzed by SDS-PAGE (19). Dried gels were processed as described (10) using preflashed films, and densitometry analysis was carried out within the limits of linearity. Recovery was estimated by metabolic pulse (45 min) followed by an overnight chase and cell surface biotinylation. Immuno- and streptavidin-precipitated samples were compared with samples submitted only to the immunoprecipitation. Values found were 22% for TFR, 24% for APN and SI, and 32% for Ag 525. Alternatively, immunoprecipitated antigens from biotinylated cells were directly analyzed by SDS-PAGE under reducing conditions for S-NHS-biotin or nonreducing conditions for S-NHS-SS-biotin, and blotted with  $^{125}$ I-streptavidin after transfer to nitrocellulose (43, 46).

For cell surface immunoprecipitation, cells grown on filters were washed once with culture medium and incubated with 1 ml of culture medium in each chamber. 10 or 20  $\mu$ l of anti-APN or anti-Ag 525 ascites were added to the apical or the basolateral medium and incubated for 2 h at 37°C. After incubation the filters were washed five times, 10 min each, with DME containing 0.5% BSA (2 ml in each chamber) and lysed as described (21). Extracts were precleared for 30 min with BSA coupled to Sepharose beads, and then centrifuged 10 min at 15,000 *g* and incubated for 2 h with protein A-Sepharose beads precoated with rabbit anti-mouse antibodies (21). To estimate the total amount of antigen, control wells incubated without antibodies were immunoprecipitated directly for 2 h. Antigen molecules not ac-

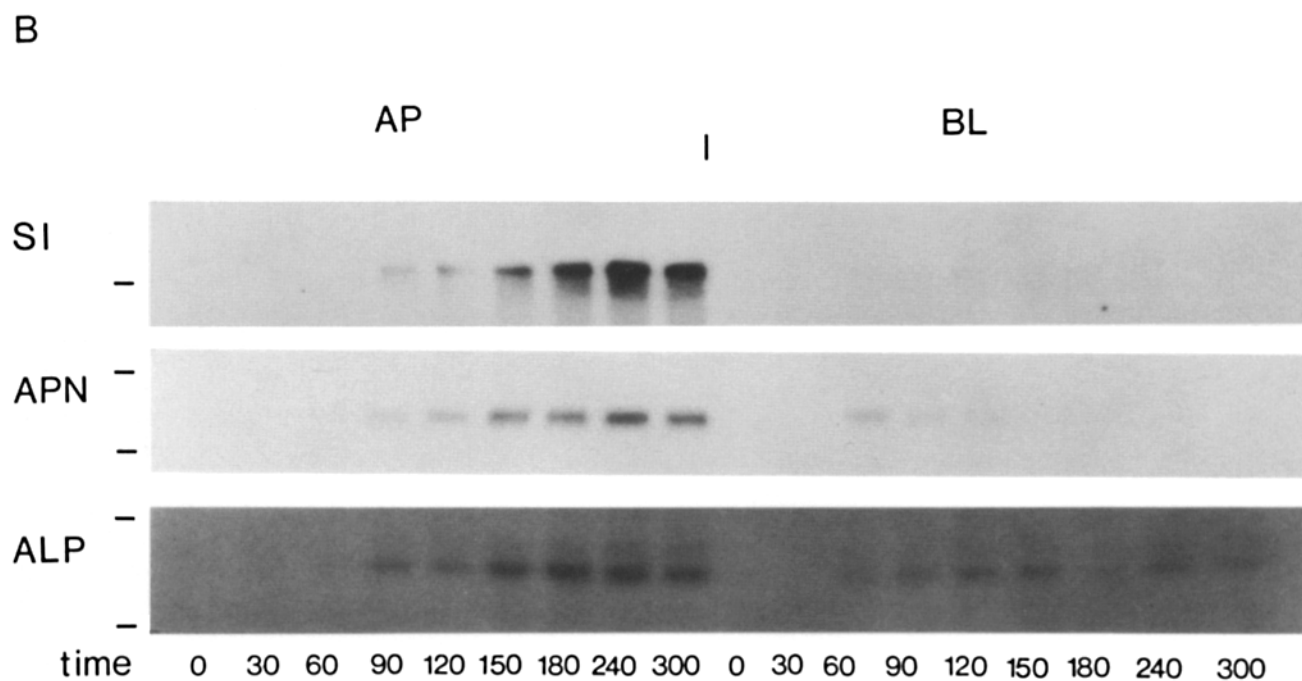


**Figure 3.** (A) Biosynthesis of Ag 525 and HLA-I in Caco-2 cells. Cells were pulsed with  $^{35}$ S-methionine for 15 min and chased for the time indicated. Ag 525 and HLA-I were immunoprecipitated, treated with endoglycosidase H, analyzed by SDS/6-16% PAGE, and revealed by fluorography. (B) Appearance at the cell surface of Ag 525 and HLA-I. Cells were pulsed for 20 min and chased for the times indicated. Newly synthesized Ag 525 and HLA-I were detected at the cell surface as described in Materials and Methods. Immunoprecipitated Ag 525 and HLA-I were analyzed by SDS/6-16% PAGE and fluorography. Molecular mass standards are, from top to bottom, 45 and 36 kD in A and B, and the time is expressed in minutes. (C) Biosynthesis and appearance at the cell surface of Ag 525 and HLA-I. Fluorograms of two independent experiments were quantitated and the results were expressed as a percentage of the amount at the time of maximal expression at the cell surface. Precursor ( $\square$ ), apical ( $\blacklozenge$ ), and basolateral ( $\blacksquare$ ) forms.





**Figure 4.** (A) Biosynthesis of SI, APN, and ALP in Caco-2 cells. Cells were pulsed for 20 min and chased for the times indicated. SI, APN, and ALP were immunoprecipitated, analyzed by SDS/6–12% PAGE, and revealed by fluorography. Arrowheads indicate the mature form of each protein. (B) Appearance at the cell surface of newly synthesized SI, APN, and ALP. Cells were pulsed for 20 min and chased for the times indicated. Newly synthesized SI, APN, and ALP were detected at the cell surface as described in Materials and Methods. Immunoprecipitated SI, APN, and ALP were analyzed by SDS/6–12% PAGE and revealed by fluorography. Time is in minutes. (C) Appearance at the cell surface of newly synthesized SI, APN, and ALP. Fluorograms of two independent experiments were quantitated and the results were expressed as a percentage of the amount at the time of maximal expression at the cell surface. Precursor (□), apical (◆), and basolateral (■) forms.



cessible at the cell surface were recovered by reincubating the extracts after the first immunoprecipitation with antibody-precoated protein A beads for an additional 2 h. Binding of HBB2/45 mAb to APN on fixed cells did not vary over a pH range of 5 to 7.5 (measured by cell surface ELISA), implying that the antibody would not be dissociated by endosomal pH levels.

### Enzymatic Digestions

Caco-2 cells were biotinylated with S-NHS-biotin, extracted with 150 mM NaCl, 20 mM Tris, pH 7.5, 1% Triton X-114, and 1 mM EDTA. The Triton X-114 phase (5) was treated with phosphatidylinositol phospholipase C (PIPLC), as described (23). Untreated controls and treated samples were immunoprecipitated as described (21). The purified PIPLC (1,500 U/ml) was kindly provided by M. Low (Columbia University, New York). Endoglycosidase H digestion was performed on immunoprecipitates as described (21).

## Results

### Apical and Basolateral Markers in Caco-2 Cells

To study the biogenetic pathways of plasma membrane proteins in Caco-2 cells we chose three apical and three basolateral membrane glycoproteins. We estimated their steady-state surface distribution in Caco-2 cells grown on polycarbonate filters by domain-selective biotinylation, immunoprecipitation, SDS-PAGE, and <sup>125</sup>I-streptavidin blotting (43) (Fig. 1). SI and APN were highly concentrated on the apical membrane (98.5 and 98%, respectively); alkaline phosphatase (ALP) appeared somewhat less polarized with 90% present on the apical domain. Ag 525, HLA-I, and TFR

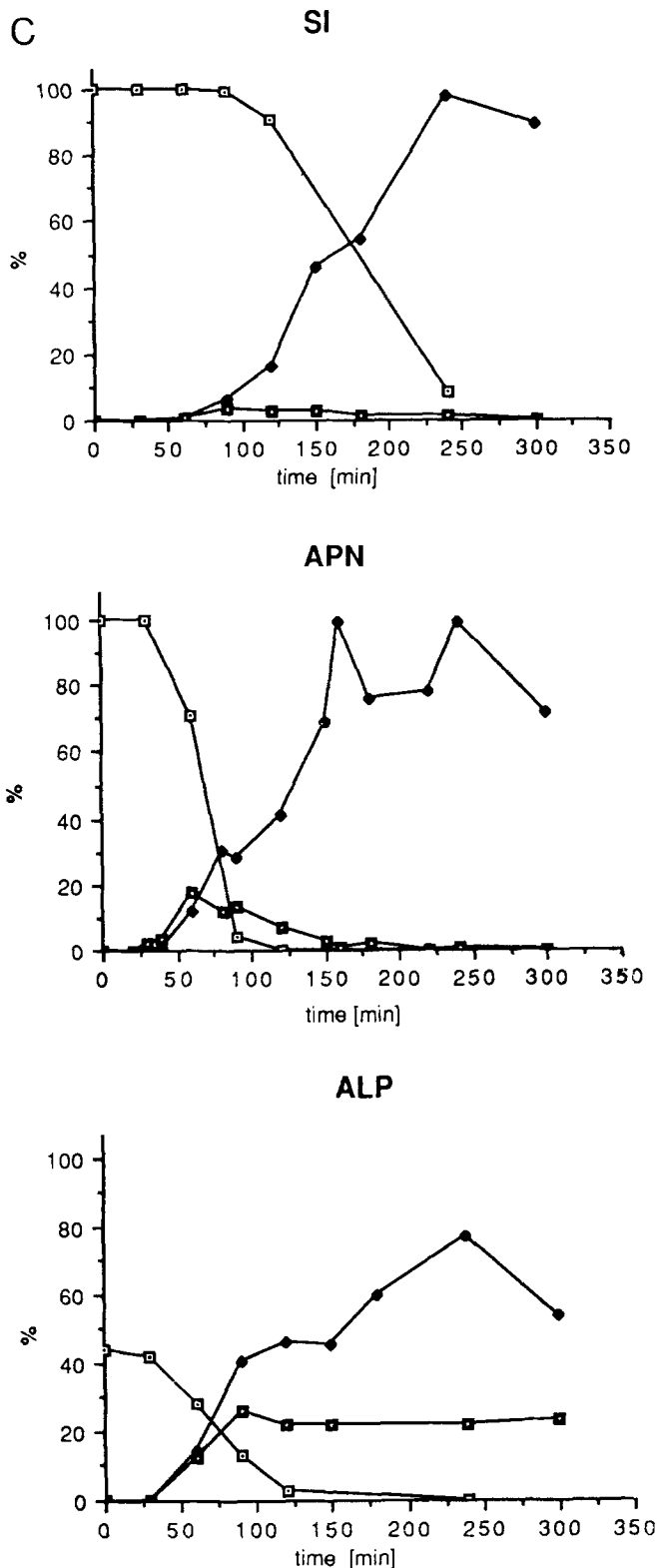


Figure 4 (continued).

were concentrated on the basolateral side (95.7, 92.1, and 96%, respectively). While SI and APN are type II proteins anchored by their NH<sub>2</sub>-terminal domain, the mode of anchoring of ALP into the membrane of human intestinal cells was not clear (4). To test the possibility that intestinal ALP

was anchored via glycosyl phosphatidylinositol, as shown for human placental ALP (25), we tested its sensitivity to PIPLC using decay-accelerating factor as a positive control (11, 31). Whereas control ALP partitioned with TX-114, after digestion with PIPLC the bulk of ALP was recovered from the aqueous phase (Fig. 2) indicating that most of ALP is anchored via a glycosyl phosphatidylinositol anchor (23, 26).

#### Transport of Basolateral Markers

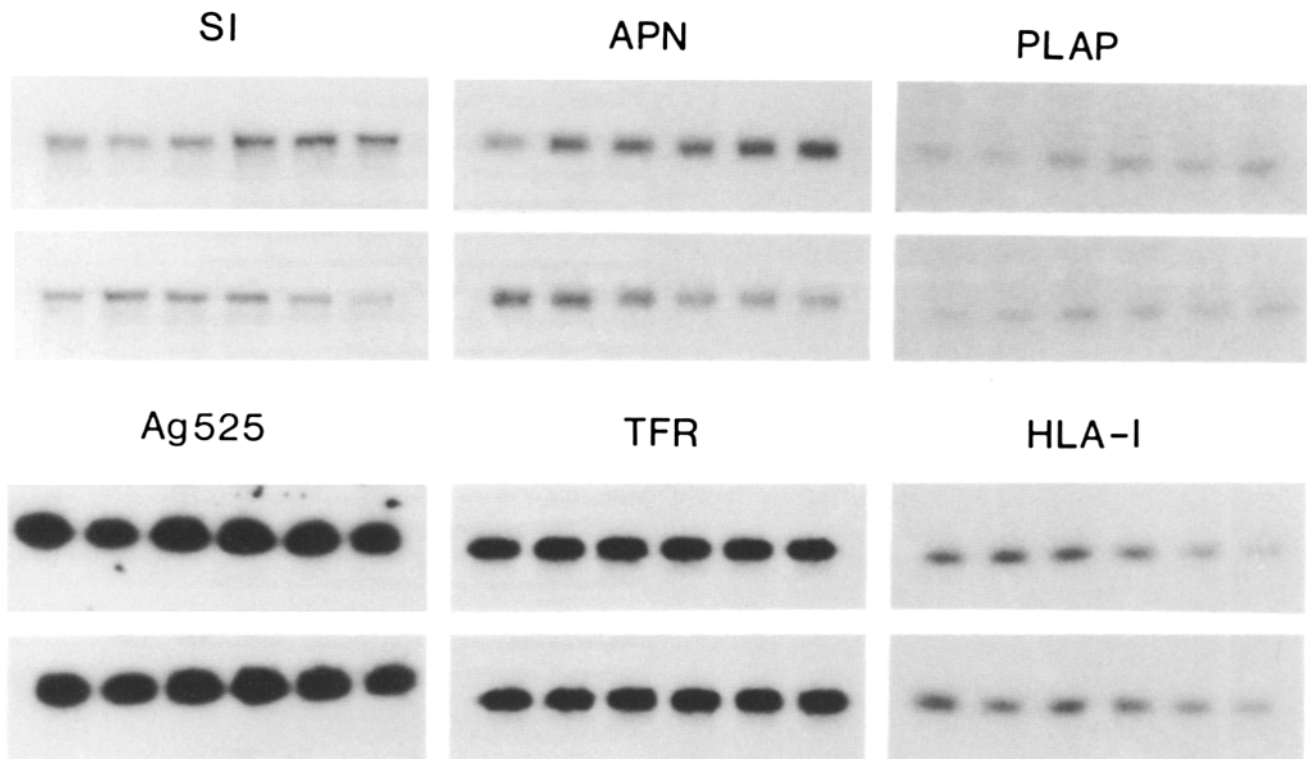
We followed the appearance of Ag 525 and HLA-I at the cell surface by a methionine/cysteine pulse and selective cell surface biotinylation at different times of the chase (21). After immunoprecipitation, the antigens were released from the beads by boiling and reprecipitated with streptavidin coupled to agarose beads (21). The resulting purified antigens were then analyzed by SDS-PAGE and fluorography (Fig. 3 B). The intracellular processing of Ag 525 and HLA-I was followed by pulse chase, immunoprecipitation, and endoglycosidase H digestion (Fig. 3 A). Densitometric analyses of the autoradiograms are shown in Fig. 3 C. Both Ag 525 and HLA-I appeared directly at the basolateral surface with very little expression on the apical surface (<3%). Ag 525 and HLA-I appeared with respective half-times of 50 and 90 min. Transport from the site of acquisition of endoglycosidase H resistance to the cell surface was rather fast (~20 min). TFR had a half-time of appearance of 50 min on the basolateral membrane with a comparable delay (15 min) and a mistargeted fraction of ~2% (data not shown).

#### Transport of Apical Markers

We studied the processing and the transport of SI, APN, and ALP in Caco-2 cells grown on filters. The maturation of these proteins was followed by the shift in molecular weight caused by the addition of complex oligosaccharides (17). As previously shown (17, 44), SI and APN were processed at very different rates (Fig. 4 A), with half-times of 150 and 70 min, respectively. ALP displayed an interesting processing pattern, with a precursor form heavily labeled by <sup>35</sup>S-cysteine and <sup>35</sup>S-methionine and a mature form exhibiting lower levels of radioactivity (Fig. 4 A). The lesser intensity of the mature form might be due to decreased antibody recognition, to increased ALP insolubility during the final stages of transport, or to degradation in the endoplasmic reticulum. Alternatively, the addition of the GPI anchor may result in a loss of methionine residues, as shown for intestinal carcinoembryonic antigen (45).

The pattern of surface expression was different for the three apical proteins (Fig. 4 B). SI appeared on the apical domain with a half-time of 170 min; only a small fraction (4%) was detected on the basolateral side as a broad, flat peak that disappeared by the end of the chase (5 h). APN was delivered to the apical membrane with a half-time of 130 min; this was preceded by a transient peak on the basolateral membrane (at 17% of the maximum apical level) between 60 and 150 min (Fig. 4, B and C). An identical pattern of surface expression of APN was observed in three independent experiments. ALP was delivered to the apical surface with a half-time of 130 min; a substantial amount (20%) was also detected on the basolateral membrane where it remained stable for at least 5 h (Fig. 4, B and C). The detection of significant basolateral pools of apical proteins during bio-

A



**Figure 5.** (A) Transcytosis of SI, APN, and ALP in Caco-2 cells. Cells were pulsed for 20 min and chased for 60 min (APN) or 100 min (SI, ALP, HLA-I, Ag 525, and TFR). At that time point the cells were labeled on the basolateral side with S-NHS-SS-biotin, a cleavable analog of biotin, at 4°C, and warmed to 37°C for 0, 30, 60, 120, 180, and 240 min. After this second chase, cells were reduced (*lower panels*) or not (*upper panels*) with glutathione on the apical side. The different proteins were purified by the double immunoprecipitation and streptavidin beads precipitation protocol described in Materials and Methods and analyzed by SDS/6–12% PAGE and fluorography. (B) The graphs show the quantification of the autoradiograms and are expressed in arbitrary units of optical density for unreduced ( $\square$ ), reduced ( $\blacklozenge$ ), and transcytosed ( $\blacksquare$ ) amounts of each protein. Transcytosed protein was calculated as the difference between unreduced and reduced samples.

genesis prompted us to determine their contribution to the final surface distribution of the enzymes.

#### **Transcytosis of Apical Proteins**

To study directly the fate of the basolateral pools of SI, APN, and ALP we devised the following protocol. Caco-2 cells grown on filters were pulsed with  $^{35}\text{S}$ -cysteine and  $^{35}\text{S}$ -methionine for 20 min, chased for either 60 min (for APN) or 100 min (for ALP and SI) at 37°C, and then biotinylated on the basolateral surface with the cleavable reagent S-NHS-SS-biotin at 4°C. Cells were rewarmed to 37°C. At the indicated times of the second chase the cells were reduced with glutathione from the apical side (50 mM, 2 × 25 min, 4°C). The proteins were then analyzed by SDS-PAGE and fluorography after immunoprecipitation and streptavidin-agarose precipitation. Results of these experiments are shown in Fig. 5. Radioactivity in the three basolateral markers used as controls—Ag 525, HLA-I, and TFR—showed no significant decrease upon apical reduction, which demonstrated that the glutathione effect was restricted to the apical side. On the

other hand, application of apical glutathione resulted in a progressive loss of radioactivity 60 min after basolateral biotinylation for APN (maximal reduction of 65%) and for SI (maximal reduction of 80%) compatible with transcytosis to the apical surface. In contrast to SI and APN, ALP showed only a slight increase of sensitivity to apical reduction with time (10%) compatible with a low level of transcytosis. To minimize sample variation, the last time point of the experiment was done in triplicate; the percentages of TFR, SI, APN, and ALP sensitive to apical reduction are shown in Table I. These results clearly demonstrated a transcytotic route for SI and APN. Although not shown directly (the levels of the two proteins on the basolateral membrane at the steady state were too low to apply the cleavable biotin/glutathione/streptavidin blotting assay for endocytosis described in next section for the apical pools), these data indicate that SI and APN must be removed from the basolateral membrane with kinetics at least as fast as those observed for transcytosis ( $t_{1/2} \sim 90$  min). Direct determinations of this value by a protease assay gave very similar results (29).

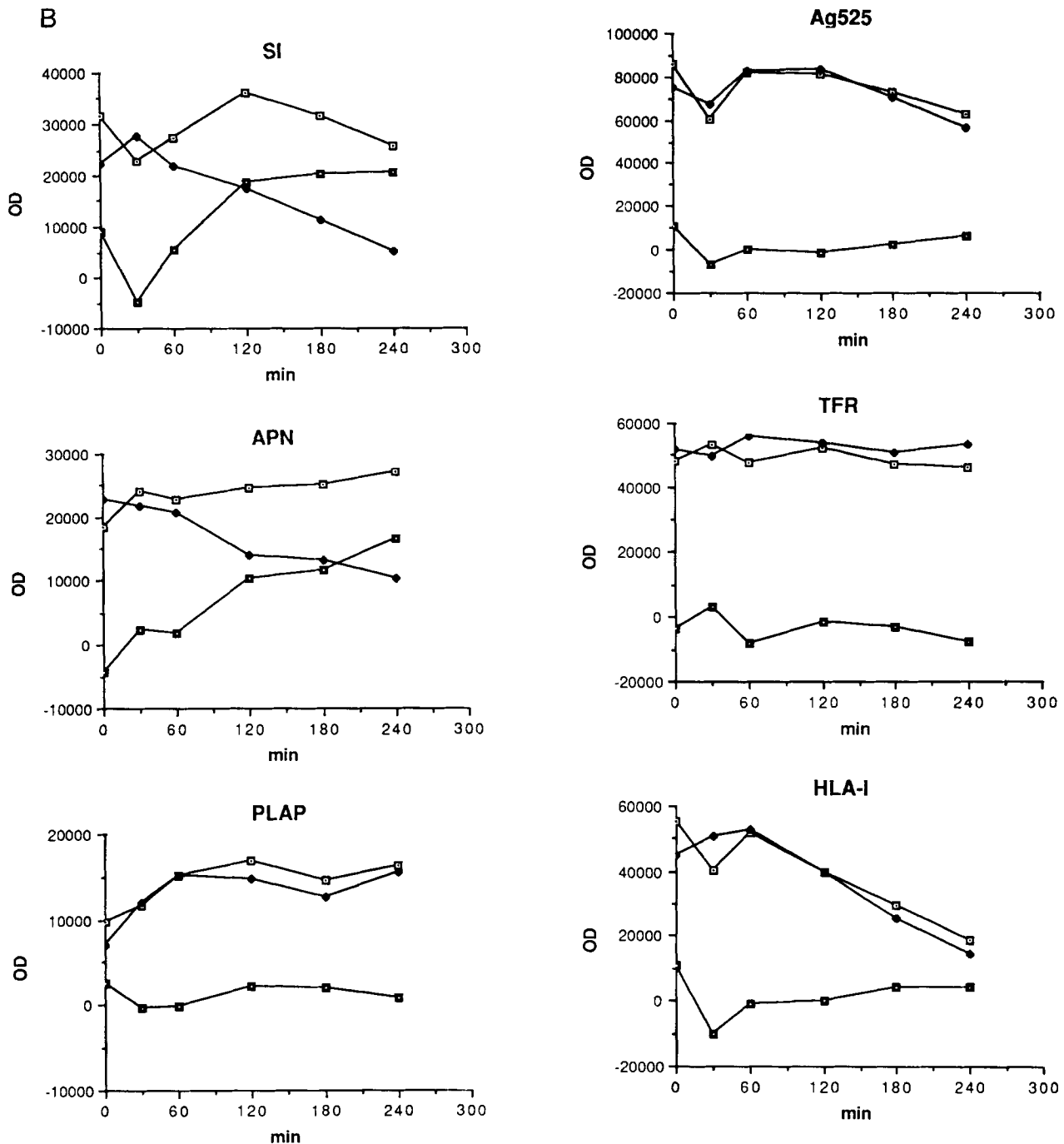


Figure 5 (continued).

### Stable Localization of Apical Proteins at the Apical Membrane

The data discussed above indicate that SI and APN are removed rather quickly from the basolateral membrane but attain a more stable localization at the apical membrane. To explore the basis for such a different behavior of these proteins in the two membranes, we studied directly their apical endocytic rates. Caco-2 cells were labeled with S-NHS-SS-biotin at 4°C and then incubated at 37°C for different times. Endocytosed antigen was detected as the biotin fraction

resistant to glutathione reduction, which was visualized by blotting with <sup>125</sup>I-streptavidin after immunoprecipitation and analysis by SDS-PAGE under nonreducing conditions (15, 21). The results showed clearly that endocytosis of SI, APN, and ALP from the apical membrane was very slow (Fig. 6 A). (Small amounts of SI [5%] and APN [10%] were resistant to glutathione reduction, but this was also observed at time 0). As a control, the same type of experiment was performed with Ag 525 on the basolateral membrane (Fig. 6 B). The levels of Ag 525 protected from reduction increased from 6% (at 60 min) to 19% (at 4 h); reduction from

**Table I. Transcytosis of the Basolateral Pools of Apical Proteins in Caco-2 Cells**

	- Glut	+ Glut
SI	100 ± 0.8	21.3 ± 5
APN	100 ± 23	36.5 ± 1.9
ALP	100 ± 15	90.2 ± 37
TFR	100 ± 8	109 ± 6

The amount of protein transcytosed in 3 h (APN and TFR) and in 4 h (SI and ALP) was calculated as described in the legend to Fig. 5. Experimental points were done in triplicate to account for sample variation. The amounts of un-reduced biotinylated protein (- Glut) recovered after the immunoprecipitation/streptavidin precipitation protocol are taken as 100%; the values obtained from samples subjected to apical reduction (+ Glut) are expressed as percentages of the - Glut samples.

the apical side had no effect on this basolateral antigen (Fig. 6 B, lanes c-f). These results suggest that the stable final localization of SI and APN in the apical surface may in part be attributed to slow endocytosis of the enzymes from this membrane.

### Most of APN Transits through the Basolateral Membrane

The kinetics of surface delivery of APN indicated that a minimum of 20% of the newly synthesized APN molecules was transported first to the basolateral membrane; our transcytosis assay indicated that most of these molecules were retargeted to the apical membrane. However, these data did not allow us to determine the extent of basolateral APN traffic; i.e., whether this was only a corrective mechanism for a small mistargeted population or the main biogenetic route of this enzyme. To measure the fraction of APN that transits through the basolateral membrane, APN antibodies were added continuously to the basal medium during a pulse-chase experiment. The suitability of this form of cell surface immunoprecipitation was determined by measuring the polarized distribution of APN and Ag 525. Caco-2 monolayers grown on Transwells were biotinylated from both sides at the same time and incubated in the presence of apical or basolateral antibodies for 2 h at 37°C. After extensive washing, the antigen-antibody complexes were extracted, precipitated, and revealed by SDS-PAGE and <sup>125</sup>I-streptavidin blotting (Fig. 7). The results obtained, 98% apical APN and 98% basolateral Ag 525, were in close agreement with those obtained by the biotin polarity assay (see above). Doubling the concentration of the antibodies did not significantly affect these results (Fig. 7, compare lane 1 with 3 and lane 2 with 4). Recovery was efficient for total surface APN (85%), but somewhat lower for Ag 525 (55%).

To measure the fraction of newly synthesized APN transiting through the basolateral membrane, Caco-2 cells were pulsed for 20 min with <sup>35</sup>S-methionine and <sup>35</sup>S-cysteine, chased for 20 min in chase medium, and exposed to apical or basolateral APN antibodies for the next 180 min (Fig. 8 A). A large fraction (60%) of the APN precipitable by apical antibodies was precipitated by antibodies added to the basal medium during the 3-h chase. A fourfold increase in the concentration of antibody (Fig. 8 A, lanes j-l) increased the percentage of basolaterally precipitable APN to 70%. In control monolayers that received identical amounts of basal antibodies for 2 h after 200 min of chase, the fraction of

basolaterally precipitable APN was <4% (Fig. 8 A, lanes g-i). These results indicate that the bulk of APN is first expressed on the basolateral membrane of Caco-2 cells and is then transcytosed to the apical membrane.

### Discussion

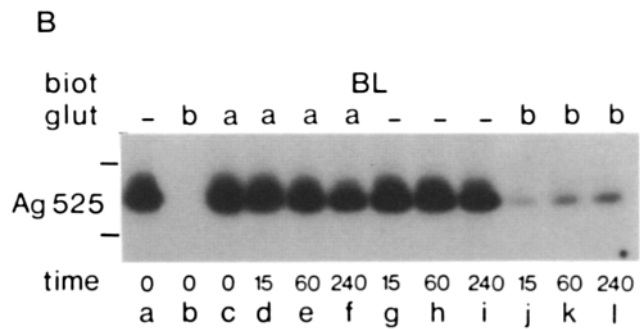
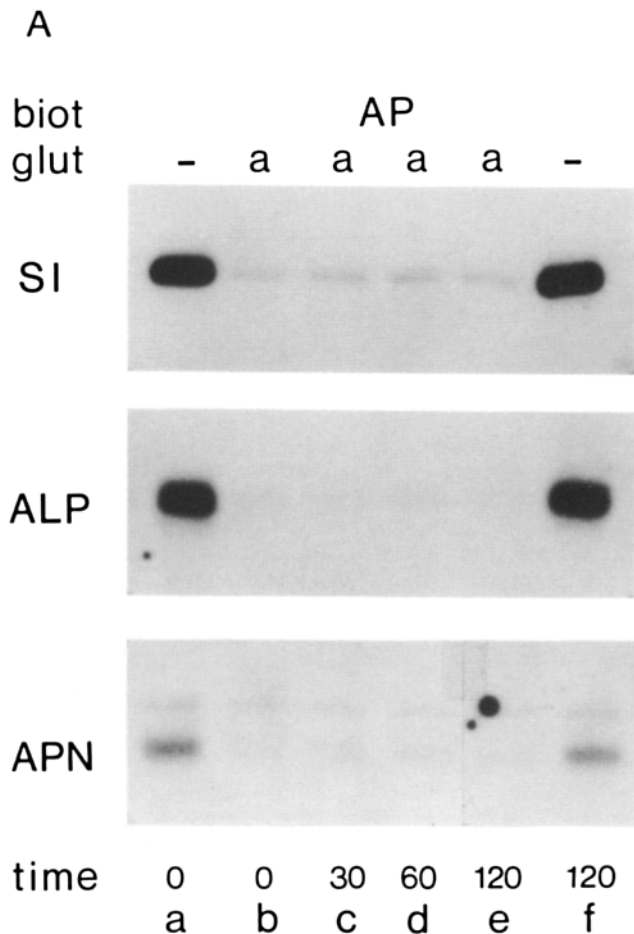
We report here the characterization of the biogenetic pathways of six endogenous plasma membrane proteins of Caco-2 cells, a polarized human carcinoma cell line. Like differentiated small intestine enterocytes, Caco-2 cells express a variety of hydrolases and a well-developed brush border (36). Under our culture conditions, they exhibit a transmonolayer electrical resistance of ~300 ohms · cm<sup>2</sup> and an asymmetric distribution of plasma membrane markers. We have characterized here the surface localization of six of these markers, three apical (APN, SI, and ALP) and three basolateral (HLA-I, TFR, and Ag 525). With a biotin assay (43) they were found to be polarized to their respective surfaces with the following ratios: 49:1, 65:1, 9:1, 1:12, 1:24, and 1:22 (Fig. 1).

APN and SI were particularly interesting to us because of their reported transcytotic pathway during biogenesis in native intestinal cells (16, 27). Using biotin targeting assays recently developed by our laboratory (21, 22, 24, 43), APN was transiently detected on the basolateral surface between 40 and 120 min (with a peak at 60 min), shortly before its appearance on the apical membrane (60 min). For SI, the basolateral expression peak was flatter (maximum of 4%) and considerably broader (90-240 min), with the first expression detected at ~90-120 min of chase. In contrast, ALP appeared to be directly targeted to the apical surface with an important fraction (20%) missorted to the basolateral membrane that behaved as a stable population (*t*<sub>1/2</sub> > 5 h). The three basolateral antigens studied here were targeted to their surface efficiently, with only a very small fraction (<3%) stably expressed at the apical domain.

The early transient basolateral expression of APN was compatible with a basolateral stage in its biogenesis. Alternatively, the basolateral pool could represent enzyme fated to be endocytosed and quickly degraded, without any precursor relationship to the apical enzyme. To distinguish between these two possibilities, we used a cleavable biotin transcytosis assay: 65% of the basolateral pool of AP (75%, if one corrects for a 10% glutathione-resistance in controls; see Fig. 6) was shown to transcytose in 3 h whereas 80% (90% after correction) of SI transcytosed in 4 h. These values compare well with the results by Matter et al. (29); they probably underestimate, however, the transcytotic capacity of the cell, since the low temperature incubation included in the assay may disrupt microtubular facilitation of this process. Only a minor fraction of the ALP was transcytosed within 4 h. Likely, this results from the mode of anchoring of this enzyme: Glycosylphosphatidylinositol (GPI)-anchored proteins interact poorly with coated pits and display low endocytic rates (7a). The slow transcytosis of this enzyme may explain why the level of basolateral ALP during biogenesis (20%) was higher than the level found at steady state (10%). Basolateral proteins were slowly (Ag 525) or quickly (TFR; see reference 18) endocytosed, but were not transcytosed, and constituted an excellent control for the APN and SI transcytosis experiments.

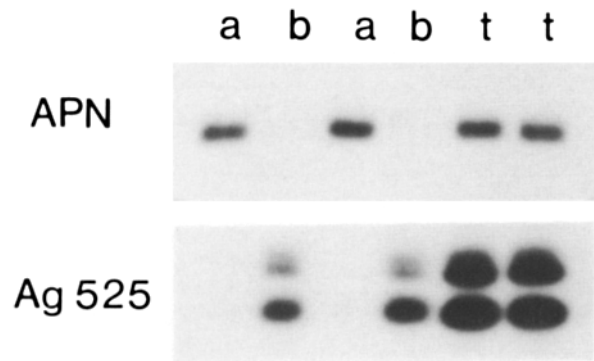
Our results clearly indicate that the basolateral pools of SI





**Figure 6.** (A) Endocytosis of SI, ALP, and APN from the apical membrane of Caco-2 cells. Monolayers were labeled with S-NHS-SS-biotin from the apical side at 4°C and reduced with glutathione (*glut*) added to the same side immediately or after incubation at 37°C for 30, 60, or 120 min. SI, APN, and ALP were immunoprecipitated and analyzed by SDS/6–12% PAGE under nonreducing conditions and revealed by <sup>125</sup>I-streptavidin blotting. Very little endocytosis was observed even after 2 h at 37°C. (B) Endocytosis of Ag 525 in the basolateral membrane of Caco-2 cells. Cells were labeled on the basolateral membrane at 4°C with S-NHS-SS-biotin and reduced with glutathione (*glut*) from the apical (*a*) or the basolateral (*b*) side, immediately or after incubation at 37°C for 15, 60, or 240 min. Ag 525 was immunoprecipitated and analyzed by SDS/6–12% PAGE under nonreducing conditions and revealed by <sup>125</sup>I-streptavidin blotting. Ag 525 was endocytosed (lanes *j–l*) but not transcytosed (lanes *d–f*).

and APN can act as precursors for the apical pool. However, the simultaneous delivery of SI to apical and basolateral membranes indicates that, in Caco-2 cells, the bulk of this enzyme is, most likely, directly targeted to the apical mem-

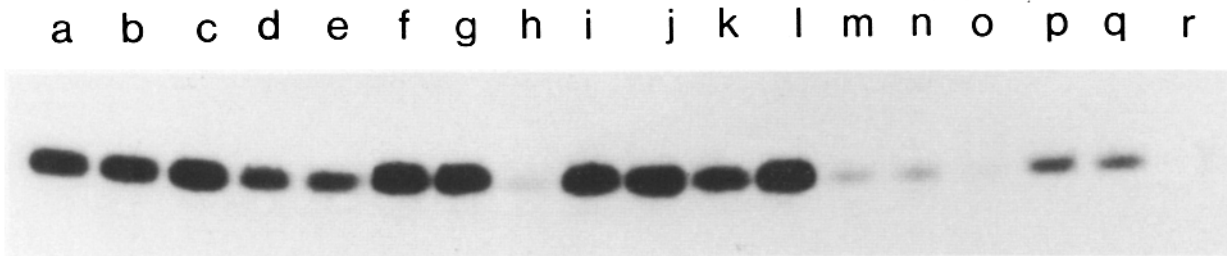


**Figure 7.** Surface immunoprecipitation of APN and Ag 525 in Caco-2 cells. Confluent monolayers grown on filters were labeled simultaneously apically and basolaterally with sulfo-NHS-biotin and incubated for 2 h at 37°C with 10 (lanes 1 and 2) or 20 μl/ml (lanes 3 and 4) of anti-APN or anti-Ag 525 added to either the apical (*a*) or the basolateral (*b*) side. After rinsing, extraction, and precipitation (see Materials and Methods), biotinylated APN and Ag 525 were revealed by SDS-PAGE (6–12%), transfer to nitrocellulose, and <sup>125</sup>I-streptavidin blotting. Control filters were directly immunoprecipitated after biotinylation to estimate the total amount of labeled antigen (*t*).

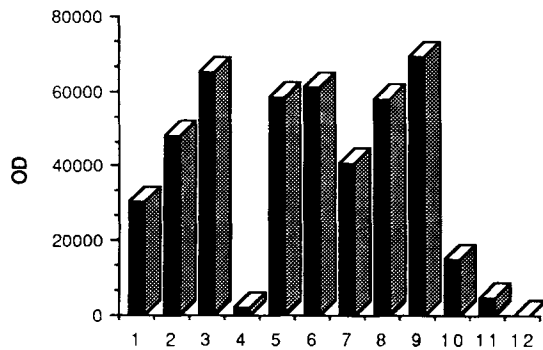
brane. The transient appearance of APN on the basolateral membrane has, on the other hand, all the characteristics of a precursor form: early appearance, transient peak, and efficient transcytosis. Continuous addition of APN antibodies to either the apical or basal medium during metabolic pulse-chase experiments (33) demonstrated that at least 60–70% of APN transported to the apical membrane was exposed to antibodies present on the basolateral surface. Since this is just a minimal estimate, given that APN accessibility may be lower from the basolateral side (suggested by differences in APN and Ag 525 surface recoveries; see Fig. 7) our results indicate that the indirect pathway is the route followed by most (if not all) of newly synthesized APN molecules in Caco-2 cells. These results are at variance with those by Matter et al. (29) who (referring to data not shown) reported that a maximum of 40% of APN followed the basolateral route and that basolateral delivery was simultaneous with apical delivery. Our results (identical in three independent experiments) agree with previous *in vivo* work in intestinal cells that used a pulse-chase/cell fractionation approach (27).

A final piece of information emerging from this report is that SI and APN have very low endocytic rates in the apical membrane, as shown by a reducible biotin assay (Fig. 6). Although technical problems prevented us from measuring endocytic rates of SI and APN from the basolateral membrane, these must be at least as fast as the transcytotic rates, which are considerably faster than the apical endocytic rates. The slow endocytosis of apical SI has been reported recently by

A



B



tation. (B) Bar graph of experiment A. Bars represent arbitrary units of optical density after scanning of the autoradiogram; for duplicate samples, the average of both values was plotted. 1 (lanes *d* and *e*): antibody (10  $\mu\text{l/ml}$ ) added basally at chase time 20 min for 180 min; 2 (lanes *a* and *b*): antibody (10  $\mu\text{l/ml}$ ) added apically at chase time 20 min for 180 min; 3 (lanes *c* and *f*): antibody (10  $\mu\text{l/ml}$ ) added to both sides at chase time 20 min for 180 min; 4 (lane *h*): antibody (10  $\mu\text{l/ml}$ ) added basally at chase time 200 min for 120 min; 5 (lane *g*) antibody (10  $\mu\text{l/ml}$ ) added apically at chase time 200 min for 120 min; 6 (lane *i*): antibody (10  $\mu\text{l/ml}$ ) added to both sides at chase time 200 min for 120 min; 7 (lane *k*): identical to 1 except for 40  $\mu\text{l/ml}$  of antibody; 8 (lane *j*): identical to 2, except for 40  $\mu\text{l/ml}$  of antibody; 9 (lane *l*) identical to 3, except for 40  $\mu\text{l/ml}$  of antibody; 10 (lanes *p* and *q*): supernatants of 1, immunoprecipitated with fresh antibody; 11 (lanes *m* and *n*): supernatants of 2, immunoprecipitated with fresh antibody; 12 (lanes *o* and *r*): supernatants of 3, immunoprecipitated with fresh antibody.

**Figure 8.** Measurement of the fraction of APN that transits through the basolateral membrane by continuous supplementation of APN antibodies during pulse-chase experiments. (A) Cells were pulsed with  $^{35}\text{S}$ -methionine/cysteine (see Materials and Methods) for 20 min, and exposed after 20 min of chase (lanes *a-f* and *j-l*) or 200 min of chase (lanes *g-i*) to 10  $\mu\text{l/ml}$  (lanes *a-i*) or 40  $\mu\text{l/ml}$  (lanes *j-l*) of anti-APN ascites added to the chase medium; incubation in the presence of antibody was for 180 min (lanes *a-f* and *j-l*) or for 120 min (lanes *g-i*). The antibodies were added apically (lanes *a*, *b*, *g*, and *j*), basolaterally (lanes *d*, *e*, *h*, and *k*) or to both sides (lanes *c*, *f*, *i*, and *l*). At the end of the incubation, the monolayers were rinsed five times, 10 min each, at 4°C, and the fraction of antibody-associated APN precipitated with protein A-Sepharose and revealed by SDS-PAGE (6–12%) /fluorography. Lanes *m-r* correspond to the supernatants of *a-f*, respectively, immunoprecipitated after addition of fresh APN antibodies to evaluate the amount of APN remaining in the cell extracts after surface immunoprecipitation.

Matter et al. (30); in a separate paper (29) this group reported comparable rates of basolateral endocytosis and transcytosis for APN, but were unable to measure basolateral endocytosis or transcytosis of SI. If confirmed by additional experiments, the differential endocytic behavior of these proteins in apical and basolateral plasma membrane domain of Caco-2 cells may possibly uncover novel regulatory mechanisms for endocytosis. In this regard, it is interesting to mention that Gorvel et al. (13) recently presented biophysical evidence (using fluorescence energy transfer) that suggests conformational differences between apical and basolateral APN.

An important question raised by the results reported here is, What are the mechanisms and the subcellular compartment(s) involved in sorting of basolateral APN to the apical membrane? Presumably, sorting may occur by selective endocytosis of apical proteins into transcytotic vesicles or by nonselective endocytosis followed by sorting of apical markers into transcytotic vesicles and bona fide basolateral proteins into recycling vesicles. The recent demonstration of the meeting of apical and basolateral endosomes in a late en-

dosomal compartment of Caco-2 cells (18) suggests such a compartment as a good candidate for the location of APN sorting.

Our results are consistent with the notion that, to a large extent, plasma membrane proteins themselves have routing information in their structure and that this information is decoded by a sorting machinery relatively conserved in different epithelial cells. The limited set of cases analyzed here and in other recent publications (6, 29, 33, 34) suggests that a given epithelial cell has both transcytotic and direct routes to the apical surface and that a given apical protein may use one or the other, or both. Displaying both transcytotic and direct routes to the apical surface, Caco-2 cells have a phenotype intermediate between MDCK cells (which use the direct route for all apical proteins studied so far; 9, 22, 24) and hepatocytes (where only an indirect route seems to be present [2]). To identify cell type-specific targeting pathways, it will be necessary to determine whether a given protein is processed similarly or differently in various epithelial cell types. The targeting assays developed here and in preceding papers (15, 21, 22, 24) should help in the search

for sorting signals and mechanisms that direct proteins along the various biogenetic pathways by facilitating the study of the routes followed by transfected proteins altered in putative sorting domains.

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