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Modulation of transcytotic and direct targeting pathways in a polarized thyroid cell line

Chiara Zurzolo, André Le Bivic[1], Andrea Quaroni[2], Lucio Nitsch[3] and Enrique Rodriguez-Boulan

Department of Cell Biology and Anatomy, Cornell University Medical College, New York, NY 10021, USA, [1]Biologie de la Differentiation Cellulaire, Faculté des Sciences de Luminy, Marseille, 13288, France, [2]Section of Physiology, Division of Biological Science, Cornell University, Ithaca, NY 14853, USA and [3]CEOS, CNR and Dipartimento di Biolgia e Patologia Cellulare e Molecolare, University of Naples, Italy

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Two biosynthetic pathways exist for delivery of membrane proteins to the apical surface of epithelial cells, direct transport from the trans-Golgi network (TGN) and transcytosis from the basolateral membrane. Different epithelial cells vary in the expression of these mechanisms. Two extremes are MDCK cells, that use predominantly the direct route and hepatocytes, which deliver all apical proteins via the basolateral membrane. To determine how epithelial cells establish a particular targeting phenotype, we studied the apical delivery of endogenous dipeptidyl peptidase IV (DPPIV) at early and late stages in the development of monolayers of a highly polarized epithelial cell line derived from Fischer rat thyroid (FRT). In 1 day old monolayers, surface delivery of DPPIV from the TGN was unpolarized (50%/50%) but a large basal to apical transcytotic component resulted in a polarized apical distribution. In contrast, after 7 days of culture, delivery of DPPIV was mainly direct (85%) with no transcytosis of the missorted component. A basolateral marker, Ag 35/40 kD, on the other hand, was directly targeted (90-98%) at all times. These results indicate that the sorting machinery for apical proteins develops independently from the sorting machinery for basolateral proteins and that the sorting site relocates progressively from the basal membrane to the TGN during development of the epithelium. The transient expression of the transcytotic pathway may serve as a salvage pathway for missorted apical proteins when the polarized phenotype is being established.

Key words: dipeptidyl peptidase IV/protein sorting/thyroid epithelium

Introduction

Epithelial cells carry out vital vectorial functions (ion, metabolite and antibody transport and secretion) essential for the homeostasis of the internal medium and for immunological defense, which depend on the asymmetric distribution of plasma membrane proteins between distinct apical and basolateral domains (Rodriguez-Boulan and Nelson, 1989). Recent work has demonstrated a striking tissue-specific variability between epithelial cell types regarding the polarized distribution of plasma membrane proteins (Pathak *et al.*, 1990) and the intracellular sorting of these molecules *en route* to the cell surface. The prototype kidney epithelial cell line, MDCK, delivers both exogenous viral glycoproteins (Matlin and Simons, 1984; Misek *et al.*, 1984; Rodriguez-Boulan *et al.*, 1984; Rindler *et al.*, 1984; Brewer *et al.*, 1991), transfected GPI-anchored proteins (Lisanti *et al.*, 1990; Powell *et al.*, 1991) and endogenous proteins (Lisanti *et al.*, 1989; Le Bivic *et al.*, 1990a) vectorially to their sites of residence at apical or basolateral membranes after intracellular sorting at the Trans-Golgi Network (TGN). In contrast, liver epithelial cells appear to lack a direct pathway to the apical surface (bile canaliculi) since all proteins destined for this domain are initially delivered to the basolateral surface and then transcytosed to their final destination (Bartles *et al.*, 1987; Bartles and Hubbard, 1988). A third scenario is provided by the native intestinal cells (Hauri *et al.*, 1979) and the human intestinal Caco-2 cell line, which express an intermediate phenotype with some apical proteins using a direct route and others using an indirect (transcytotic) route (Le Bivic *et al.*, 1990b; Matter *et al.*, 1990). In all three model systems, basolateral proteins are sorted more efficiently than apical proteins at the TGN and, in general, are always delivered vectorially from the TGN to the basolateral surface (Le Bivic *et al.*, 1989, 1990a,b; Matter *et al.*, 1990).

Recent work has addressed the question of whether the variability in apical targeting pathways is due to cell/tissue-specific changes in the sorting machinery or to the comparison of different proteins in different cells. Dipeptidyl peptidase IV (DPPIV) and aminopeptidase N (APN), which follow an indirect route to the apical surface in liver (Bartles and Hubbard, 1988) and intestinal cells (Massey *et al.*, 1987), were vectorially targeted to the apical surface when transfected into MDCK cells (Wessels *et al.*, 1990; Casanova *et al.*, 1991b; Low *et al.*, 1991). These experiments indicated that the differences in the handling of apical proteins are not intrinsic to the proteins themselves but are due to cell/type-specific differences in the apical targeting machinery. Nevertheless, all of the studies mentioned above were carried out at a fixed time of culture and did not consider the possibility that the cells may dynamically change their targeting mechanisms during establishment of the polarized phenotype. In fact, epithelial cells undergo marked structural rearrangements in surface and cytoplasmic polarity, Golgi distribution and microtubule cytoskeleton arrangement as the monolayer is established. Surface polarity is established gradually with time and the steady-state distributions of apical and basolateral markers develop at different rates (Balcarova-Stander *et al.*, 1984; Herzlinger and Ojakian, 1984; Vega Salas *et al.*, 1987; Bacallao *et al.*, 1989). However, developmental changes in the targeting pathways have never been reported.

To determine the contribution of direct and indirect pathways to the establishment of a polarized epithelial

phenotype, we studied the biogenetic routes to the cell surface of DPPIV, an apical marker (Bartles *et al.*, 1985; Hartel *et al.*, 1988) and of Ag 35/40 kD, a basolateral marker (Amerongen *et al.*, 1989), at different times of culture, using biotin targeting assays recently developed in our laboratory (Le Bivic *et al.*, 1989; Hanzel *et al.*, 1992). The experiments were carried out in FRT cells, a novel polarized cell line of thyroid origin which, like the MDCK cell line, becomes highly polarized when grown on filters and accurately sorts different viral envelope glycoproteins to opposite surface domains (Nitsch *et al.*, 1985; Zurzolo *et al.*, 1991, 1992). Our results show a developmental change in the targeting profile of DPPIV in FRT cells. Whereas a large fraction of this enzyme uses the transcytotic route at early stages of polarization, this route is progressively abandoned, with time of culture, in favor of a direct apical pathway. Thus, both direct and transcytotic pathways operate in FRT cells but their relative contribution to the final distribution of DPPIV changes during development of the polarized monolayer.

Results

The Fischer rat thyroid (FRT) cell line displays a highly polarized epithelial phenotype (Nitsch *et al.*, 1985; Zurzolo *et al.*, 1991). On plastic, confluent FRT monolayers develop typical domes. On polycarbonate filters, FRT cells form tight monolayers with trans-epithelial resistances of up to $\sim 10-12\ 000$ ohms·cm^2 after 5–7 days of culture and distinct apical and basolateral surface domains. As previously shown in MDCK cells (Rodriguez-Boulan and Sabatini, 1978), influenza virus buds from the apical surface while vesicular stomatitis virus (VSV) assembles at the basolateral surface, which correlates with restricted apical or basolateral distributions of, respectively, influenza hemagglutinin and VSV G protein (Zurzolo *et al.*, 1992).

Steady-state distribution of apical and basolateral antigens

We analyzed the steady-state surface distribution of several endogenous plasma membrane markers after 7 days in culture by indirect immunofluorescence (Figure 1) and a

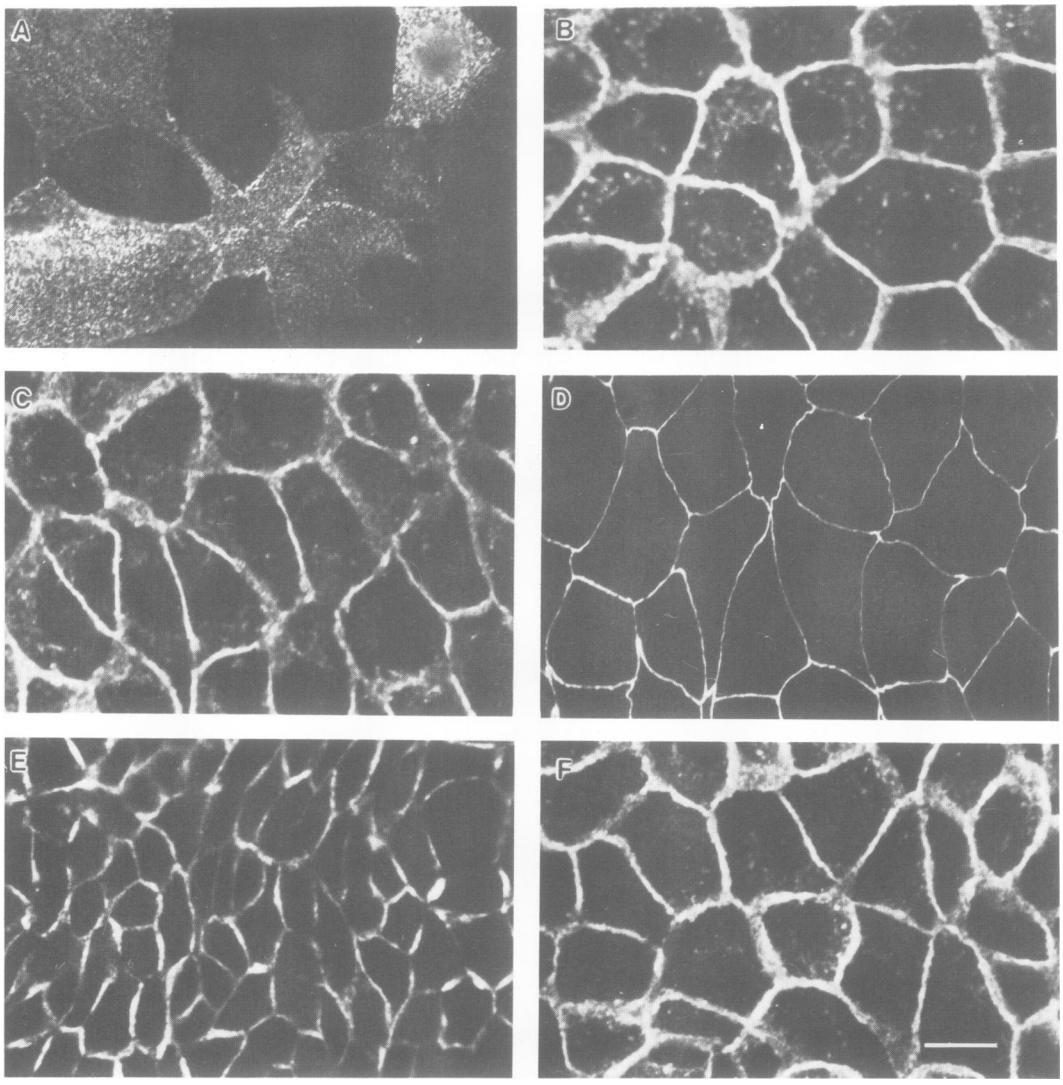

Fig. 1. Localization by indirect immunofluorescence of various endogenous membrane proteins in FRT cells grown to confluency on coverslips. Apical localization of DPPIV in non-permeablized cells (**A**), basolateral localization of Ag 35/40 kD (**B**), uvomorulin (**C**), ZO1 (**D**), α NaK-ATPase (**E**) and β NaK-ATPase (**F**), in cells permeabilized with 0.075% saponin. All basolateral antigens tested were negative at the apical membrane, whereas apical DPPIV displayed a faint basolateral staining (data not shown). Bar = 10 μm.

biotin assay (Figure 2). DPPIV displayed a typical apical distribution, as previously shown in liver and intestinal epithelia (Bartles *et al.*, 1985; Hartel *et al.*, 1988) and in the polarized intestinal cell line Caco-2 (Le Bivic *et al.*, 1990b; Matter *et al.*, 1990). Both α and β subunits of NaK-ATPase, uvomorulin (E-cadherin, L-CAM), Ag 35/40 kD (Figures 1 and 2) and transferrin receptor (data not shown), displayed typical basolateral localizations. The tight junction associated protein ZO-1 was detected by immunofluorescence as a continuous belt at the apical–lateral interphase (Figure 1D). For the biotin assay, the α and β subunits of NaK-ATPase were co-immunoprecipitated with a monoclonal antibody against β NaK-ATPase (Figure 2A); the low biotinylation level of the α subunit may probably be attributed to a smaller number of free primary amino groups exposed on the extracellular domain of the plasma membrane (Noguchi *et al.*, 1986; Sweadner, 1989). Quantitative scanning densitometry of two independent experiments indicated that ~80–90% of DPPIV was apical while the basolateral markers were all more polarized (>99%) (Figure 2B).

Development of polarized distribution of DPPIV and Ag 35/40 kD

Previous studies in MDCK cells have shown that surface polarity, as monitored by monoclonal antibodies or unilateral methionine uptake, increases progressively with time in culture (Balcarova-Stander *et al.*, 1984; Herzlinger and Ojakian, 1984; Vega Salas *et al.*, 1987). We measured the polarized distribution of an apical marker, DPPIV, and a basolateral marker, Ag 35/40 kD, at different times after plating. Confluent FRT monolayers, grown on filters for 1, 3 and 7 days, were biotinylated from each side with S-NHS-biotin. DPPIV and Ag 35/40 kD were immunoprecipitated and analyzed by SDS–PAGE, [^{125}I]streptavidin blotting and scanning densitometry of the fluorograms (Figure 3A and B). The apical polarity of DPPIV increased from 65% on the first day to ~85–90% on the seventh day. The basolateral distribution of Ag 35/40 kD was established on the first day of culture (>80% on the basolateral surface) and increased to 99% by day 7. The increasingly polarized distribution of surface antigens correlates with an increase in TER from >1000 ohms·cm^2 at day 1 of culture to a plateau of >10–12 000 ohms·cm^2 after 5–6 days in culture.

Biosynthesis of DPPIV and Ag 35/40 kD

The biosynthesis and processing of DPPIV and Ag 35/40 kD was studied in 7 day old monolayers grown on filters by pulsing with [^{35}S]methionine/[^{35}S]cysteine for 20 min followed by chasing for various times (Figure 4). DPPIV was processed within 20–40 min to a higher molecular

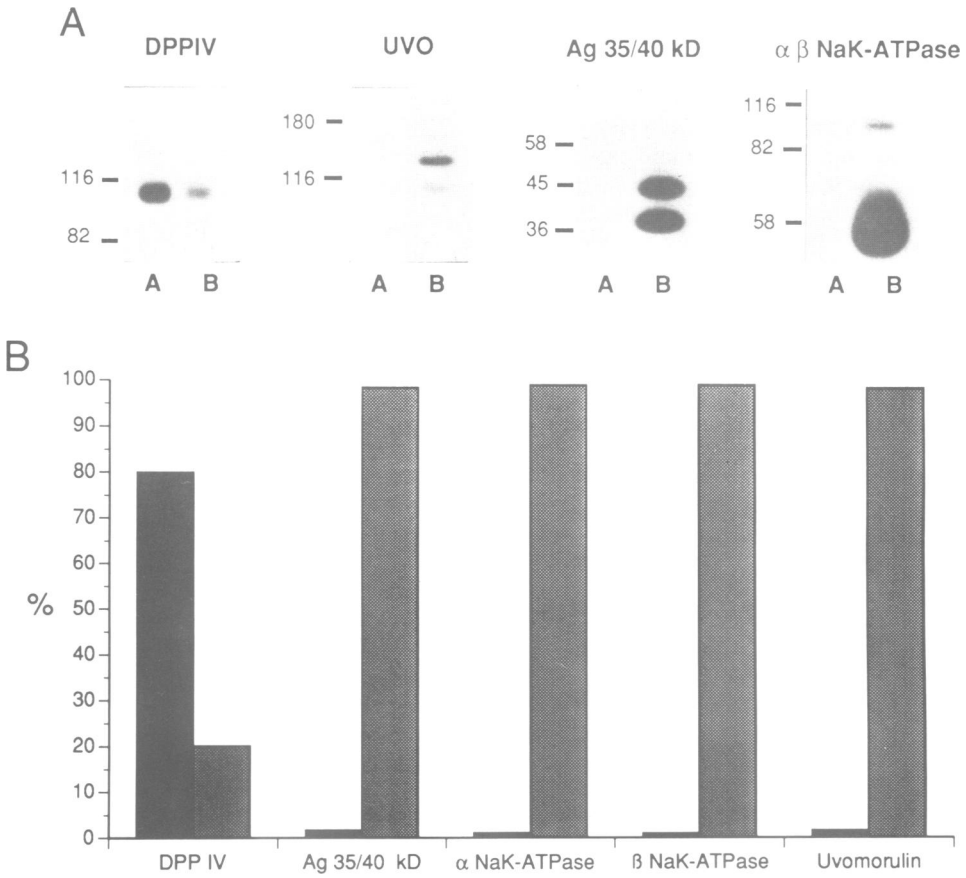

Fig. 2. (**A**) Immunoprecipitation of DPPIV, uvomorulin, Ag 35/40 kD and NaK-ATPase after surface labelling of the apical (A) or the basolateral (B) membrane of FRT cells with sulfo-NHS-biotin. The biotinylated proteins were revealed, after SDS–6–12% PAGE and transfer to nitrocellulose, by [^{125}I]streptavidin blotting. Molecular weights are in kDa. DPPIV was mainly labelled from the apical side while uvomorulin, Ag 35/40 kD and NaK-ATPase were mainly detected on the basolateral side. (**B**) Steady-state distribution between apical (black bars) and basolateral (gray bars) plasma membrane of DPPIV, uvomorulin, Ag 35/40 kD and NaK-ATPase in FRT cells after 7 days in filter culture. Fluorograms of two independent experiments were quantitated and the results are expressed as percent apical and basolateral surface expression for each protein.

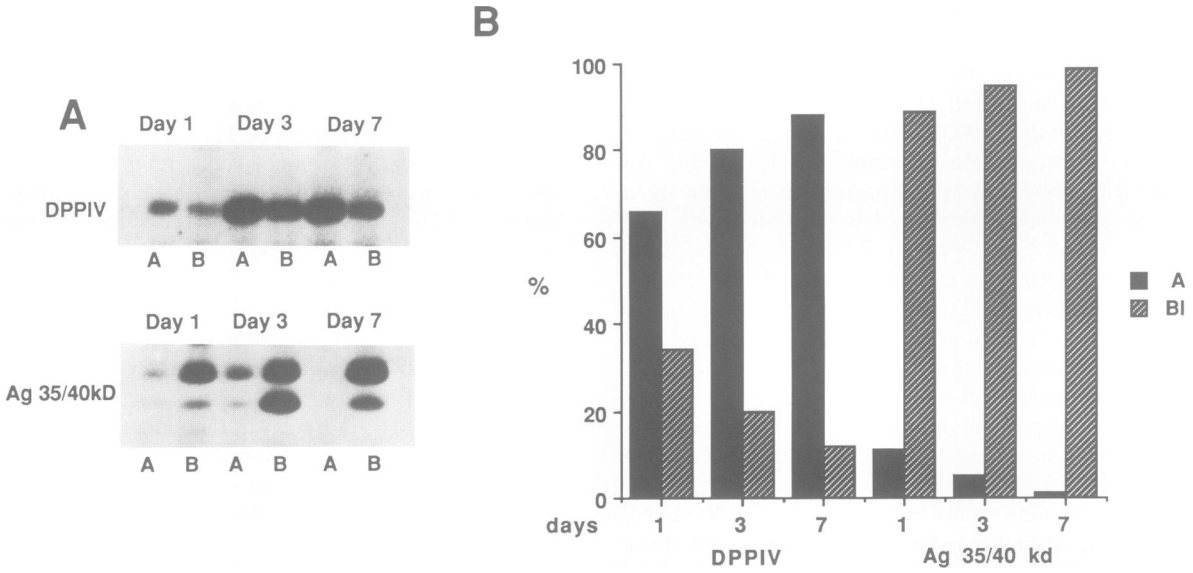

Fig. 3. (**A**) Immunoprecipitation of DPPIV and Ag 35/40 kD after surface labelling of the apical (A) or the basolateral (B) sides of FRT cells with sulfo-NHS-biotin after 1, 3 and 7 days of filter culture. Biotinylated proteins were revealed, after SDS−6−12% PAGE and transfer to nitrocellulose by [^{125}I]streptavidin blotting. (**B**) Steady-state distribution of apical (black bars) and basolateral (gray bars) of DPPIV and Ag 35/40 kD in FRT cells after 1, 2 or 7 days filter culture. Fluorograms of two independent experiments were quantitated within the linear range of the densitometer and the results were expressed as percent apical and basolateral surface expression for each protein.

weight form. Previous work has shown that the faster migrating precursor band is endoglycosidase H sensitive and the slower band (110 kDa) is endo H resistant due to the addition of complex carbohydrates in the Golgi apparatus (Bartles *et al.*, 1987; Hong *et al.*, 1989; Low *et al.*, 1991). Ag 35/40 kD migrated at all times of chase as a single band of ~40 kDa indicating no apparent modifications in glycosylation. We have no clear explanation for the double band (35/40 kD) detected at the cell surface (Figures 2A and 3A). Since it is only seen after biotinylation (see also Figures 5A and 6A), it may be due to a biotin-induced proteolytic event at the plasma membrane level.

Developmental changes in the targeting of newly synthesized DPPIV and Ag 35/40 kD

To follow the surface appearance of the newly synthesized DPPIV and Ag 35/40 kD, we used radioactive pulse−chase and surface domain selective biotinylation, at different times of the chase, of FRT monolayers grown on filters. After 7 days at confluency, the TER plateaued at ~10−12 000 ohms·cm^2 and both antigens were maximally polarized (Figure 3B). Biotinylated DPPIV and Ag 35/40 kD were immunoprecipitated together with both specific antibodies. The immunoprecipitates were resuspended in 10% SDS, reprecipitated with streptavidin−agarose beads and analyzed by SDS−PAGE fluorography (Figure 5A). DPPIV was initially detected on the apical surface after 30 min of chase and accumulated there with a half-time of ~90 min, reaching a plateau by 240 min (Figure 5B). No transient peak was observed on the basolateral membrane where a small amount of DPPIV (~15% of total surface delivery) was progressively detected with exactly the same kinetics as the apically delivered protein, also plateauing after 240 min (Figure 5B). The initial surface appearance of Ag 35/40 kD was on the basolateral surface and was also detected at 30 min of chase (Figure 5A and B). However, this protein reached a plateau much faster than DPPIV, after only 60 min of chase. Very little Ag 35/40 kD (<2%) was detected at any time of chase at the apical surface (Figure 5A and B).

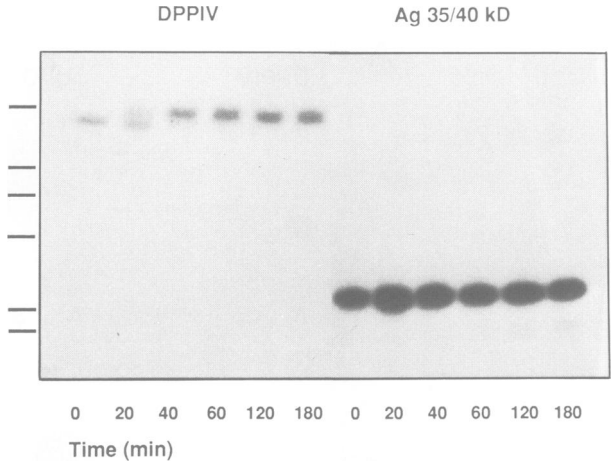

Fig. 4. Biosynthesis of DPPIV and Ag 35/40 kD in FRT cells. Cells were pulsed for 20 min and chased for the times indicated. DPPIV and Ag 35/40 kD were immunoprecipitated, analyzed by SDS−6−12% PAGE and revealed by fluorography. For DPPIV it is possible to identify a mature fully glycosylated form; Ag 35/40 kD is revealed as a unique band of ~40 kDa while at the cell surface it is always detected as a doublet. Molecular masses (top to bottom) are 116, 82, 64, 58, 36 and 26 kDa.

A very different situation was observed in FRT monolayers confluent for just 1 day. All monolayers studied had a TER of 1000−2000 ohms·cm^2. In these cells, delivery of DPPIV was equal to apical and basolateral surfaces until the 120 min time point (Figure 6A and B). Delivery to the apical surface continued to increase between 120 and 240 min whereas delivery to the basolateral surface decreased continuously after 120 min (Figure 6A and B). This indicates that a pool of DPPIV passed through the basolateral membrane and then accumulated on the apical membrane. A similar pattern was observed in two independent experiments. In contrast, delivery of Ag

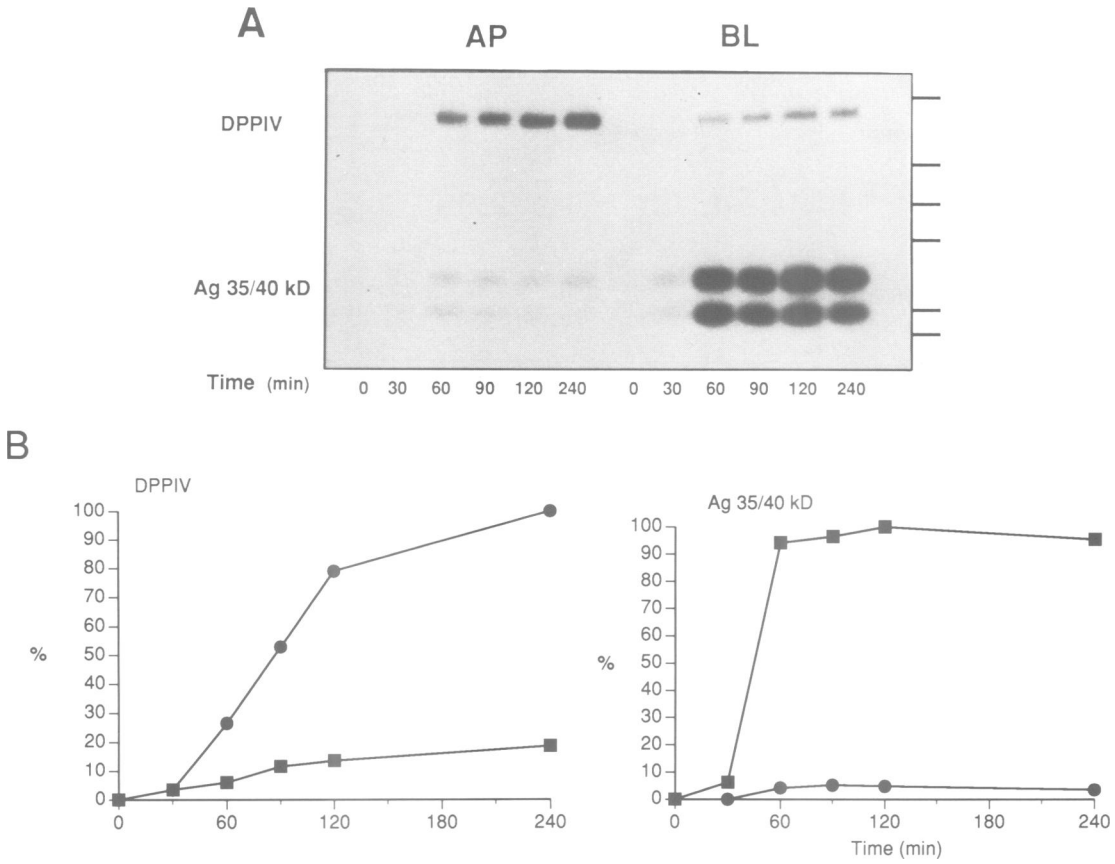

Fig. 5. (**A**) Appearance at the cell surface of newly synthesized DPPIV and Ag 35/40 kD in cells grown on filters for 7 days. The resistance of the monolayer was >10 000 ohms cm^2. Cells were pulsed for 20 min and chased for the times indicated. Newly synthesized DPPIV and Ag 35/40 kD were detected at the cell surface as described in Materials and methods. The two proteins were immunoprecipitated together by coupling both specific antibodies to the protein A–Sepharose beads, they were then analyzed by SDS–6–12% PAGE and revealed by fluorography. (**B**) 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. Apical (●) and basolateral (■) forms.

35/40 kD was highly polarized to the basolateral surface, although somewhat less than at day 7 (90% versus 98%); the decreased polarity in delivery may account for the somewhat less polarized distribution of this protein at day 1 than at day 7.

Fate of basolateral pool of DPPIV

The results of the targeting experiments suggested that DPPIV might be vectorially targeted to the apical surface at day 7 but may utilize a transcytotic route at day 1. To determine directly the fate of the basolateral pool of DPPIV, we carried out a biotin transcytosis assay. 1 day and 7 day FRT monolayers were pulse labelled with [^{35}S]methionine/cysteine and chased for 120 min. At this time, when the levels of newly synthesized DPPIV on the basolateral membrane were maximal, the monolayers were labelled with the reducible reagent sulfo-NHS-SS-biotin from the basolateral side. At various times after labelling, the fraction of biotinylated DPPIV reaching the apical surface by transcytosis was measured by reduction with glutathione added only to the apical surface (Le Bivic *et al.*, 1990b). A striking difference was observed in the levels of transcytosis of DPPIV at days 1 and 7. At day 1, large amounts of basolateral DPPIV (>50%) were transcytosed to the apical surface in 4 h. No transcytosis of DPPIV was detected in 7 day old monolayers in up to 12 h incubation times (Figure 7A and B). As a control, basolateral Ag 35/40 kD, labelled and processed simultaneously in identical fashion, did not show any evidence of early or late transcytosis. Thus, FRT cells transcytose DPPIV at early times in the formation of a polarized monolayer but abandon this targeting route in favour of direct delivery as the monolayer matures.

Discussion

To explore whether the biogenetic pathway of a plasma membrane protein changes with the state of polarization of the monolayer, we studied the surface delivery of an apical protein in monolayers cultured for different times. We carried out our studies in FRT cells, a well polarized epithelial line of thyroid origin that expresses high levels of endogenous DPPIV, a model protein whose biogenetic route has been well characterized in various epithelial cell types.

We were surprised to find that there are drastic developmental changes in the apical targeting pathway for DPPIV. At day 1, there was no apparent preferential sorting of DPPIV in the Golgi apparatus; about equal levels of the enzyme entered the apical and the basolateral routes to the cell surface and a large fraction of the newly synthesized enzyme reached the apical surface via a transcytotic route. In contrast, in fully polarized FRT monolayers (confluent for 7 days) DPPIV was targeted directly to the apical surface and transcytosis of this enzyme was undetectable. Thus, once efficient apical sorting in the TGN is established, transcytosis of DPPIV is turned off. Our data do not exclude the

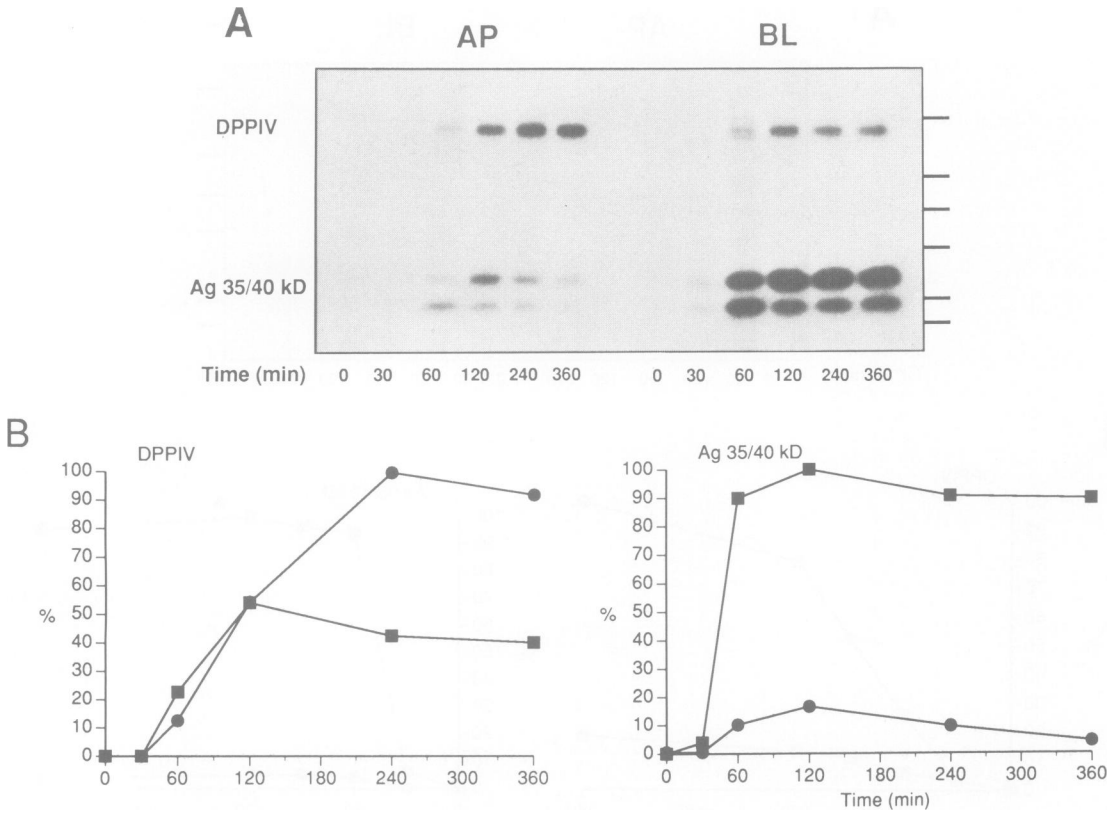

Fig. 6. (**A**) Appearance at cell surface of newly synthesized DPPIV and Ag 35/40 kD in cells grown on filters for 1 day. The resistance of the monolayer was already >1000 ohms cm^2. Cells were pulsed for 20 min and chased for the times indicated. Newly synthesized DPPIV and Ag 35/40 kD were detected at the cell surface as described in Materials and methods. Immunoprecipitated DPPIV and Ag 35/40 kD were analyzed by SDS–6–12% PAGE and revealed by fluorography. (**B**) 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. Apical (●) and basolateral (■) forms.

possibility that transcytosis of other proteins be preserved. The only data available on transcytosis of DPPIV in MDCK cells were obtained after 4 days of confluency; they demonstrated an exceedingly slow transcytotic transfer (Casanova *et al.*, 1991b), similar to our results at day 7. Thus, epithelial cells display not only tissue-specific changes but also developmental variations in their targeting pathways. According to a recent model (Simons and Wandinger-Ness, 1990), our data could be accounted for by an undeveloped apical targeting mechanism at day 1 that results in the bulk flow of DPPIV along a default basolateral route. Alternatively, a specific mechanism regulating incorporation of basolateral proteins into the basolateral route may become more efficient in excluding apical proteins. Within the last year, specific sorting signals have been identified for several basolateral proteins; their removal results in targeting to the apical surface of the mutant proteins (Casanova *et al.*, 1991a; Le Bivic *et al.*, 1991; Hanzel *et al.*, 1992). A molecular explanation of the observations reported here must await the identification of putative sorting receptors involved in sorting in the TGN and in the basolateral membrane/endosomes. We postulate that the transient transcytotic pathway described here may play a role in the initial passage from an unpolarized to a polarized stage during establishment of the epithelial monolayer. Transcytotic delivery may serve as a salvage pathway for misplaced proteins trapped in the incorrect compartment by tight junction closure.

Our results support and expand the cell/type-specific sorting hypothesis in that they demonstrate the direct apical delivery of endogenous DPPIV in an epithelial cell line of different (thyroid) origin. Over 80% of DPPIV followed the direct pathway in monolayers that had been confluent for 7 days. However, although FRT and MDCK cells share this mechanism for direct apical delivery of DPPIV, they differ in the handling of some viral glycoproteins (Zurzolo *et al.*, 1992) and endogenous and transfected GPI-anchored proteins (Zurzolo,C. and Rodriguez-Boulan,E., manuscript in preparation). Taken together, these observations strongly indicate that multiple mechanisms exist for the targeting of molecules to a given surface domain, rather than a single mechanism for each surface. This redundancy in surface delivery mechanisms may have evolved to prevent possible lethal consequences of the alteration in polarity of those proteins.

Unlike DPPIV, Ag 35/40 kD was directly targeted to the basolateral surface at all times. This indicates that apical and basolateral sorting machineries develop along independent lines and that they are distinct from each other. Another interesting observation that is reported for the first time here is that both machineries increased their efficiency as the monolayer matured, which provides an explanation for previous data (Balcarova-Stander *et al.*, 1984; Herzlinger and Ojakian, 1984; Vega Salas *et al.*, 1987), as well as for data in this report, that indicate that the surface distribution of plasma membrane markers becomes more stringently polarized with time in culture. The molecular basis of this increased sorting efficiency is unclear. It may result from improved sorting in the Golgi apparatus, or from

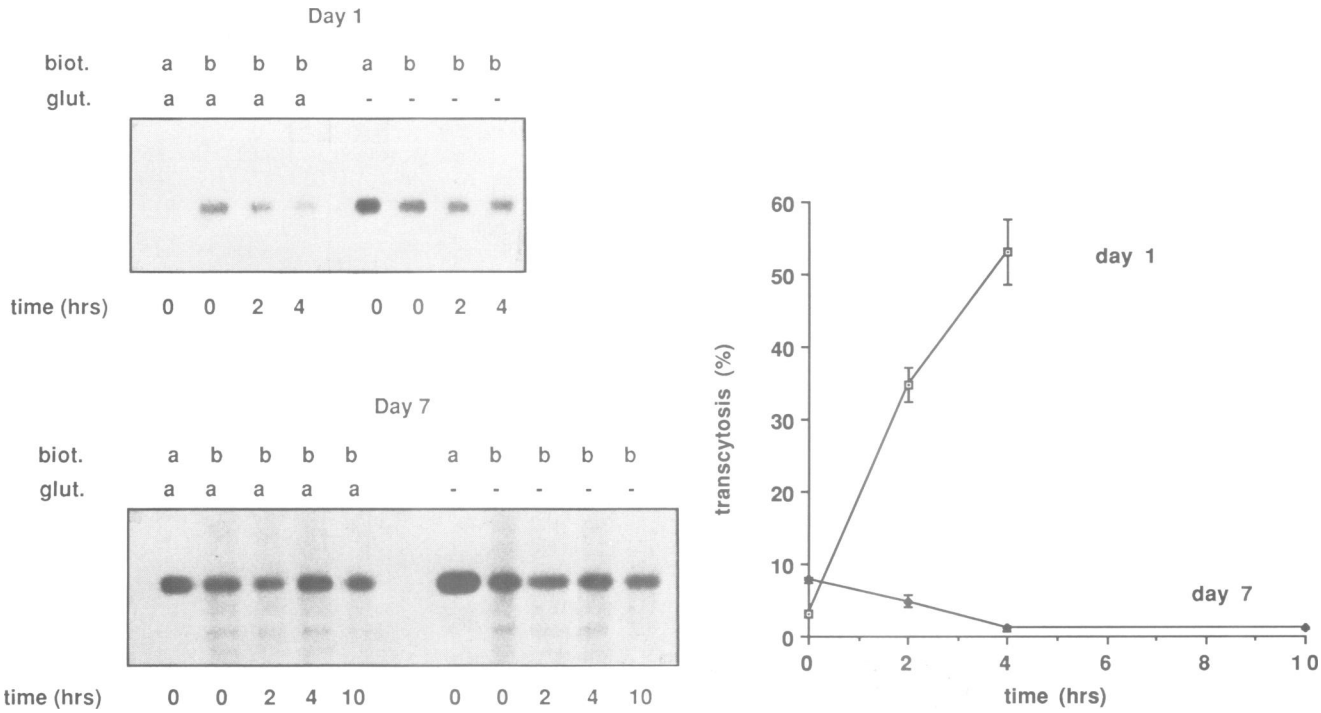

Fig. 7. Transcytosis of DPPIV in FRT cells at day 1 and at day 7. **Left:** filter-grown monolayers were pulse labelled for 20 min with [^{35}S]methionine/[^{35}S]cysteine, chased for 120 min, labelled from the basolateral (b) or the apical (a) sides with S-NHS-SS-biotin, a cleavable analogue of biotin, for 15 min at 20°C, and then warmed to 37°C for the indicated times. The cells were then reduced (+) or not (−) with glutathione, added to the apical side. DPPIV and Ag 35/40 kD (internal control, not shown), were immunoprecipitated together, resuspended and the biotinylated (surface) fraction precipitated with streptavidin−agarose and analyzed by SDS−6−12% PAGE and fluorography. As a control, 85−95% of apically biotinylated DPPIV is sensitive to glutathione reduction. At day 1, the transcytosis assay of DPPIV could not be continued beyond 4 h due to a shorter half-life (compared with day 7) of the protein (after 10 h, only ~20% of DPPIV was detectable in unreduced cells). **Right:** quantitation of the autoradiograms by scanning densitometry; the results were expressed as percentages of the basolaterally biotinylated protein in the control (glut −) cells.

improved accuracy in the targeting of post-Golgi vesicles to the cell surface. The latter may result from enhanced polarization of surface receptors for apical and basolateral carrier vesicles (since most plasma membrane proteins become more polarized in mature monolayers) or may reflect an increasingly organized microtubule network along an apical−basal axis (Bacallao *et al.*, 1989; Gilbert *et al.*, 1991).

In summary, our results indicate that the targeting phenotype of FRT cells resembles that of Caco-2 cells at day 1 of culture and that of MDCK cells after 7 days of culture. Hence, it is possible that some of the differences previously reported between different epithelial cell types on the handling of apical proteins may be explained not only by fixed differences in the expression of tissue/cell-specific sorting machineries, but also by differential rates of development of the direct and the transcytotic pathways.

Materials and methods

Reagents

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

Cells, antibodies and cell culture

FRT cells were grown in F12 Coon's modified media supplemented with 5% fetal bovine serum (FBS). For experiments, cells were grown on Transwells (Costar Inc., Cambridge, MA) and were used between 1 and 7 days after confluency. Monoclonal antibodies against rat DPPIV (CLB4/40) and β subunit of rat NaK-ATPase (IEC1/48) were previously described (Amerongen *et al.*, 1989; Marxer *et al.*, 1989; Petell *et al.*, 1990). The following antibodies were generous gifts: rabbit polyclonal antibody against uvomorulin (R.Kemler, Max Planck Institute, Freiburg, Germany); rat monoclonal antibody against ZO1 (D.Goodenough, Harvard, NY). Monoclonal antibody against human transferrin receptor was purchased from Boehringer (Mannheim, Germany). The mouse monoclonal antibody CLB1/41 (against the Ag 35/40 kD) was produced by fusion of NSI myeloma cells with spleen cells, obtained from a BALB/c mouse immunized with a partially purified surface membrane fraction prepared by the Kessler method (Kessler *et al.*, 1978) from rat small intestinal crypt cells [isolated by the method of Weiser (1973)]. All techniques used in the determination of the antigen specificity of this antibody have been described previously (Quaroni and Isselbacher, 1985). CLB1/41 specifically immunoprecipitated from Triton X-100 solubilized, ^{14}C-labelled crypt cell surface membranes, a protein of M_r 35 000 which lacked all brush border enzyme activities tested (lactase, DPPIV, aminopeptidase N, alkaline phosphatase, sucrase, maltase).

Biotinylation assays

Biotinylation of monolayers on Transwells with S-NHS-biotin was carried out twice successively for 20 min each at 4°C as described (Sargiacomo *et al.*, 1989). For transcytosis experiments, cells were labelled with S-NHS-SS-biotin for 20 min at 20°C to avoid microtubular disruption since it has been shown that intact microtubules are required for basolateral to apical transcytosis (Breitfeld *et al.*, 1990; Hunziker *et al.*, 1990; Gilbert *et al.*, 1991). 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 (Le Bivic *et al.*, 1989).

Pulse chase experiments

Cells on filters were incubated for 30 min in DMEM without methionine or cysteine and pulsed for 20 min in the same medium containing 0.8 mCi/ml of tran^{35}Slabel and 0.4 mCi/ml of [^{35}S]cysteine (NEN, Boston, MA) as described (Misek *et al.*, 1984). After washing with DMEM, cells were

chased in DMEM containing 5 × cysteine and methionine at normal concentrations and kept at 4°C in bicarbonate-free DMEM, 20 mM HEPES and 0.2% bovine serum albumin before biotinylation.

Immunoprecipitation and streptavidin precipitation

After biotinylation cells were processed for immunoprecipitation as described previously (Le Bivic *et al.*, 1989) using 1/100 dilutions of both monoclonal antibodies (ascites fluid) and polyclonal antibodies. To recover the immunoprecipitated biotinylated antigens, the beads were boiled in 10 μl of 10% SDS for 5 min, diluted with lysis buffer and centrifuged for 1 min at 15 000 *g*. Biotinylated antigens present in the supernatants were precipitated with streptavidin–agarose beads as described (Le Bivic *et al.*, 1989). Finally the beads were boiled in SDS–PAGE sample buffer and analyzed by SDS–PAGE (Laemmli, 1970). Dried gels were processed as described (Chamberlain, 1979) using preflashed films and densitometry analysis was carried out within the limits of linearity. Alternatively, immunoprecipitated antigens from biotinylated cells were directly analyzed by SDS–PAGE and blotted with [^{125}I]streptavidin after transfer to nitrocellulose (Towbin *et al.*, 1979; Sargiacomo *et al.*, 1989).

Immunofluorescence

Cells grown on coverslips at confluency were processed for immunofluorescence as described (Rodriguez-Boulan, 1983). For ZO1 staining, the cells were fixed with cold methanol (−20°C).

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