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Opposite sorting and transcytosis of the polymeric immunoglobulin receptor in transfected endothelial and epithelial cells

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SUMMARY

We have transfected a polarised endothelial cell line, ECV 304, and an epithelial cell line, MDCK, with a well characterised epithelial protein, the rat polymeric immunoglobulin receptor (pIgR), in order to study the protein sorting and transcytosis in endothelial cells. The expressed protein was normally processed and the steady state distribution between apical and basolateral surfaces was similar in both cell types. MDCK cells, however, showed a marked polarity in the delivery of newly synthesised pIgR to the cell surface, and in the release of secretory component. 88% of newly synthesised pIgR in MDCK cells was first delivered to the basolateral surface and 99% of secretory component was released from the apical surface. In contrast the basolateral targeting signal of pIgR was only partially recognised in endothelial cells, with 63% of the newly synthesised pIgR being first delivered to the basolateral surface. At steady state only 43% of the pIgR was found on the basolateral membrane. The direction of dimeric IgA transcytosis in endothelial cells was from apical to basolateral surfaces, opposite to that in MDCK cells. These data suggest that endothelial cells poorly recognise the targeting signals of proteins from epithelial cells, and that the direction of transcytosis is linked to the biological role of the cells.

Key words: Endothelial cell, Polarity, Polymeric Ig receptor, Sorting, Transcytosis

INTRODUCTION

In polarised continuous endothelium, the plasma membrane is separated by tight junctions into lumenal (apical, Ap) and ablumenal (basolateral, Bl) domains. The apical domain faces the bloodstream and the basolateral domain is surrounded by subendothelial tissues and the interstitial space. A major function of endothelial cells is to facilitate the movement of materials between these surfaces, supplying nutrients from the blood to cells bathed by the interstitial fluid (Simionescu et al., 1981; Simionescu and Simionescu, 1991). Despite a large body of research on the polarity and protein transport of epithelial cells (for a review, see Drubin and Nelson, 1996), surprisingly little is known about the polarised membrane protein sorting and transcellular transport processes in endothelial cells.

Evidence for a clathrin coat-mediated pathway for internalisation and transcytosis of hormone receptors has been demonstrated in the continuous endothelium of testicular microvasculature (Ghinea et al., 1994). The major direction of transcytosis, however, is necessarily from apical to basolateral surfaces since the pathway delivers chorionic gonadotropin to the underlying Leydig cells. A similar pathway has been proposed for transcytosis of transferrin across blood-brain barrier endothelial cells. It has been shown by immunocytochemistry that the transferrin receptor, which has a basolateral location in epithelial cells, has an apical distribution in these endothelial cells (Jefferies et al., 1984; Roberts et al., 1993) and delivers holo transferrin across the blood-brain barrier (Broadwell et al., 1996). This implies a lower degree of acidification of endosomes in these cells since, in epithelial cells, iron dissociates from receptor-bound transferrin in early endosomes and is loaded onto cytoplasmic ferritin (Dautry-Varsat et al., 1983; Descamps et al., 1996). Further evidence for unusual mechanisms of sorting in endothelial cells comes from the observation that tissue factor, an apically sorted protein of endothelial cells, was targeted to the basolateral surface of MDCK cells (Camerer et al., 1996). Thus existing data argue for non-epithelial mechanisms of sorting in endothelial cells.

In order to establish a protein sorting and transcytosis system in endothelial cells, we have transfected a well-characterised epithelial membrane protein, the polymeric immunoglobulin receptor (pIgR), into a human endothelial cell line. The pIgR in epithelial cells is targeted to the basolateral surface of the cell by three amino acid residues found in the first 14 residues of the cytoplasmic domain. Deletion of this signal causes direct transport of the receptor to the apical domain (Casanova et al., 1991; Aroeti et al., 1993; Aroeti and Mostov, 1994). Not surprisingly, this region of the cytoplasmic domain is highly conserved between species (Banting et al., 1989; Piskurich et al., 1995). Following ligand binding (Mostov and Deitcher, 1986; Breitfeld et al., 1989) or phosphorylation of the receptor

cytoplasmic domain at a specific serine residue (Casanova et al., 1990; Hirt et al., 1993; Aroeti and Mostov, 1994), it has been proposed that the basolateral targeting signal is masked (Chapin et al., 1996), allowing the receptor to be transported in a microtubule-dependent manner to the apical domain without passage through lysosomes (Apodaca et al., 1994; Barroso and Sztul, 1994; Song et al., 1994). At the apical membrane the receptor is proteolytically cleaved and the ligand is released together with a portion of the receptor known as secretory component (SC) (Mostov and Blobel, 1982).

The pIgR has been transfected into several polarised cell lines of epithelial and neuronal origin, namely MDCK cells of kidney origin (Mostov and Deitcher, 1986), rabbit mammary epithelial cells (Schaerer et al., 1990), neuroendocrine PC12 cells (Bonzelinus et al., 1994) and rat hippocampal neurons (de Hoop et al., 1995). In all the epithelial cells studied the receptor is targeted to the pool of basolateral recycling receptors and can bind ligand and be transcytosed to the apical domain of the cell. In neurons, a similar asymmetry of initial delivery and ligand-stimulated movement has been demonstrated (Huber et al., 1993), arguing that dendrites and cell bodies of hippocampal neurons are equivalent to the basolateral membrane, and the axon is equivalent to the apical domain in epithelial cells. Thus this receptor contains signals recognised by a variety of cell types.

We have transfected a cDNA encoding the complete rat pIgR open reading frame into an endothelial cell line that can sort secretory proteins between the apical and basolateral surfaces. We report that the basolateral targeting signal on the pIgR is not efficiently recognised in these cells and that transcytosis of dimeric IgA transcytosis occurs predominantly from apical to basolateral surfaces of the monolayer. We conclude that endothelial cells have different sorting mechanisms compared to epithelial cells.

MATERIALS AND METHODS

Cell culture

ECV304 cells, type II MDCK cells and mouse IgA-producing hybridoma cell line 2F.11.15 (American Type Culture Collection, ATCC) were cultured in M199, DMEM and RPMI, respectively and the media were supplemented with 10% FCS. Unless otherwise mentioned all experiments were performed on cell monolayers grown on Transwell filters (Corning Costar, Cambridge, MA) with 0.4 μm pores and 4.5 cm^2 surface area (Takahashi et al., 1996). ECV304 cells were seeded at a density of 4×10^5 cells/filter and grown for 6-7 days. MDCK cells were seeded at 1.8×10^6 cells/filter and grown for 3-4 days.

Antibodies

Rabbit anti-rat pIgR C-terminal peptide antibody and secretory component (SC) peptide antibody were raised in our laboratory against the peptide sequences CQVHDGPQEA, corresponding to the carboxy terminus of the receptor, and QSPIFGPQDVSSIEGC, corresponding to the amino terminus of the receptor. The cysteines of both peptides were added to facilitate conjugation to diphtheria toxin (Chiron Pty Ltd, Australia). In addition the glutamine at the amino terminus of the second peptide was cyclised. Goat anti-rabbit IgG antibody conjugated to rhodamine was from Boehringer Mannheim. Donkey anti-rabbit IgG conjugated to horse radish peroxidase was from Amersham.

Construction of rat pIgR cDNA

A 960 bp cDNA fragment, corresponding to the 5′ end of the rat pIgR cDNA sequence, was amplified by PCR from a rat liver cDNA library. The oligonucleotide (Biotech, Australia) corresponding to the 5′ end of the pIgR sequence (5′-CGCAGTCGACTACAAGAAGTGAAC CAACATGCCGC) terminated in an *Sal*I fragment and the 3′ oligonucleotide (5′-CGTAAG CGGCCATTGTCATCCC) was positioned downstream from an endogenous *Bam*HI site, allowing the PCR product to be cut with *Sal*I and *Bam*HI and inserted into the mammalian expression plasmic, pAX-neo, which has a *neo* gene under the control of a thymidine kinase promoter and a multiple cloning site downstream of a β actin promoter (Gunning et al., 1987). A full-length open reading frame was reconstituted by cloning the 3′ *Bam*HI fragment from the previously described cDNA clone (Banting et al., 1989) into the unique *Bam*HI site of this plasmid and selecting for clones in the correct orientation by restriction mapping. The sequence of the 5′ fragment was checked by DNA sequencing.

Transfection of cell lines

ECV304 and MDCK cells were transfected with the pIgR cDNA-pAX-neo plasmid by using $CaPO_4$ precipitation and lipofectAMINE (Gibco BRL) methods. For selection of permanent transfected cell lines, cells were grown in medium with 400 μg/ml (for MDCK cells) and 600 μg/ml (for ECV304 cell) of G418 (Gibco BRL) for 10-12 days, and G418-resistant clones were isolated for further characterisation.

Immunofluorescence microscopy

Indirect immunofluorescence staining of cells grown on glass coverslips and fixed with methanol was as described (Bos et al., 1993). The rabbit anti-rat pIgR cytoplasmic domain antiserum was diluted 1:100. Rhodamine-conjugated goat anti-rabbit IgG was used at 1:250 dilution. The cells were examined by epifluorescence with appropriate barrier filters in an Olympus BH2 microscope.

Metabolic labelling, pulse chase and immunoprecipitation

Transfected cells were grown in 35 mm tissue culture dishes for pulse-chase experiments. The cells were pulsed with 300 μCi/ml of Tran^{35}S-label (ICN) for 15 minutes after starving in DMEM without L-methionine and L-cysteine for 30 minutes and then the cells were chased in culture medium with 10× L-methionine (300 mg/l) for different time points. For ^{32}P-labelling, the cells were incubated with 250 μCi/ml of ^{32}P-orthophosphate (Amersham) for 2 hours (Sefton, 1991). The cells were lysed after the chase time in lysis buffer containing with 0.5% NP-40, 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 4 mM $MgCl_2$ and protease inhibitors. The lysis buffer also contained 20 mM NaF and 2 mM sodium vanadate for ^{32}P-labelled samples. The lysates were immunoprecipitated with rabbit anti-rat pIgR tail antibodies. Protein A Sepharose beads (Pharmacia) were used for isolation of the pIgR-IgG complexes. The proteins were separated on 10% polyacrylamide gels and the radioactivity incorporated into protein bands was quantified using a phosphorimager (BAS1500, Fuji Film Co.).

Polarity of secretion

For SC measurement the transfected cells were washed with DMEM twice and replaced with DMEM containing 0.6% BSA and 20 mM Hepes, 1 ml in the apical and 1 ml in the basolateral chambers. There was no significant effect of hydrostatic pressure in the direction Ap to Bl with these volumes because equal amounts of media were collected from Ap and Bl chambers after 24 hours of incubation. The media were centrifuged at 3000 rpm for 5 minutes to remove any debris and total proteins in the medium were then isolated by mixing with 15 μl StrataClean resin (Stratagene) at room temperature for 20 minutes. The proteins were eluted from the beads by boiling in 50 μl of SDS-sample buffer at 95°C for 5 minutes. The proteins were separated by SDS-PAGE and then transferred from the gel to a

nitrocellulose membrane. The SC was immunoblotted by rabbit anti-rat SC antibody and detected by enhanced chemiluminescence.

For total secretory protein measurement the cells were metabolically labelled with 300 μCi/ml of Tran^{35}S-label for 2 hours and then the apical and basolateral media were collected and isolated with StrataClean resin as above. The secretory proteins were separated by SDS-PAGE and detected by a phosphorimager.

Biotinylation

For measuring the steady state expression of pIgR in transfected cells, the apical or basolateral membrane proteins of the cell monolayers were biotinylated by adding sulfo-NHS-biotin to a final concentration of 0.5 mg/ml in PBS$^+$ (containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$), pH 7.5, for 20 minutes, twice, on ice (Sargiacomo et al., 1989). The cells were then lysed in 0.5% NP-40 and immunoprecipitated overnight at 4°C with 10 μl of anti-pIgR serum. The total immunoprecipitate was run on a 10% SDS-polyacrylamide gel and then transferred to a nitrocellulose membrane. Biotinylated pIgR was quantified by western blot using ^{125}I-streptavidin detection (Amersham, UK) and phosphorimaging.

Selective surface protein trypsinisation

For measuring the delivery of newly synthesised pIgR from the TGN to the cell surface, filter-grown cells were pulsed with 500 μCi/ml Tran^{35}S-label for 10 minutes. After the pulse, the cells were incubated in DMEM supplemented with 0.1% BSA, 20 mM Hepes and 10× L-methionine at 18°C for 90 minutes (Casanova et al., 1991). The culture medium was then replaced with L-1-tosylamide-2-phenylethyl chloromethyl ketone (TPCK)-treated trypsin (Sigma) on either the apical or basolateral side for 60 minutes at 37°C. Soybean trypsin inhibitor, 200 μg/ml, was added to the opposite side of the cells in the same medium during the trypsin digestion. The concentrations of trypsin used were 100 μg/ml for ECV304 cells and 25 μg/ml for MDCK cells. The cells were then lysed and the lysates immunoprecipitated with anti-SC serum.

Mouse dIgA isolation and iodination

Mouse dIgA was isolated from mouse ascites produced by mouse 2F.11.15 hybridoma cells. Immunoglobulins were concentrated from the ascites by ammonium sulfate precipitation and dimeric IgA was separated by gel filtration through a Superose 6 column (Pharmacia). dIgA was verified by immunoblotting with goat anti-mouse IgA antibodies conjugated with biotin (Sigma) in reducing and nonreducing conditions. dIgA was iodinated using iodo-beads (Pierce) (Fraker and Speck, 1978). The specific activity was 1-3×10^5 cpm/μg protein.

Measurement of ^{125}I-dIgA transcytosis

Filter-grown cells were washed 3 times with cold DMEM containing 0.6% BSA and 20 mM Hepes. Then ^{125}I-labelled dIgA (1×10^6 cpm/ml, 10 μg) was added to the medium in the apical or basolateral chambers and the cells were placed on ice for 2 hours to allow the ligand to bind. The filters were then washed 6 times on ice with the above medium. Pre-warmed DMEM at 37°C with 0.6% BSA, 20 mM Hepes was then added, 1 ml each per apical and basolateral chambers. The cells with bound ^{125}I-labelled dIgA were incubated at 37°C for a further 2 hours . The apical and basolateral media were collected and counted in a gamma-counter after 15% TCA precipitation (Breitfeld et al., 1989). Untransfected cells were included in the experiments and the counts from these cells were subtracted from those measured in transfected cells. The rate of transcytosis of ^{125}I-labelled dIgA was then calculated as pg/mg cell protein.

Electrical resistance and ^{14}C-inulin permeability

The transendothelial cell electrical resistance was measured by using a Millicell ERS (Millipore) meter after cells had been grown on filters for 3-12 days. The electrical resistance of filters without cells was subtracted from the resistance of cell monolayers. ^{14}C-inulin permeability was examined at day 7. The ^{14}C-inulin was added to 1 ml of medium in the Transwell inserts and medium was collected from the outside chamber and counted after 1 hour of incubation.

RESULTS

The polarity of ECV304 cells grown on Transwell filters

ECV304 cells were used to measure the polarity of endothelial cells, as these cells form continuous monolayers on Transwell filters and have previously been used to demonstrate polarised infection with viral particles (Jakob, 1993). The formation of continuous junctions between cells was assessed in our experiments by immunofluorescence labelling of cells using an antibody against cadherin, and by measurements of monolayer permeability and electrical resistance. In initial experiments we established that by seeding at high density (4×10^5 cells per filter), a continuous band of cadherin was expressed at the junction between cells in the monolayer, and that no holes were present between cells (data not shown). This was reflected in measurements of monolayer electrical resistance, which peaked at 130±11 ohm.cm^2 (s.d., n=10) after 7 days in culture. The permeability of the monolayer to ^{14}C-inulin was 0.6% in the first hour at 37°C and 0.5% at 4°C, conditions used for selective biotinylation of the monolayer. While somewhat more leaky than MDCK cells (400 ohm.cm^2 and <0.1% inulin permeability in the first hour), the monolayers formed by ECV304 cells were sufficiently impermeable to detect polarised secretion of proteins and to perform measurements of polarised sorting (see below).

In order to verify that the ECV304 cells were capable of polarised sorting of endogenous proteins, we examined the secretion of metabolically labelled proteins into the medium bathing the apical and basolateral membranes of cells grown on filters (Fig. 1). Polarised secretion of several proteins was observed, as indicated by the arrows and arrowheads. Similar results were obtained in two different cloned cell lineages derived from the ECV304 cells and in cells supplemented with 50% newborn calf serum or endothelial cell growth factor. Six proteins that were well separated on the SDS-PAGE gels were quantitated in three experiments (Table 1). This confirmed that some proteins could be sorted with over 80% secreted from either the apical or basolateral surface of the cell (proteins 2

Table 1. Polarised protein secretion in ECV304 cells

Protein number	Molecular mass (kDa)	Polarity (% sorted to apical surface) (mean±s.d., n=3)
1	169	44.71±5.15
2	71	82.23±4.76
3	65	43.72±5.87
4	57	14.00±3.14
5	52	29.57±1.42
6	48	50.35±0.21

ECV304 cells were grown on Transwell filters and labelled with Trans^{35}S-label. The secretory proteins were separated as shown in Fig. 1 and the radioactivity incorporated into six proteins was quantified by a phosphorimager. Polarity is expressed as the percentage of recovered radioactivity in the apical medium.

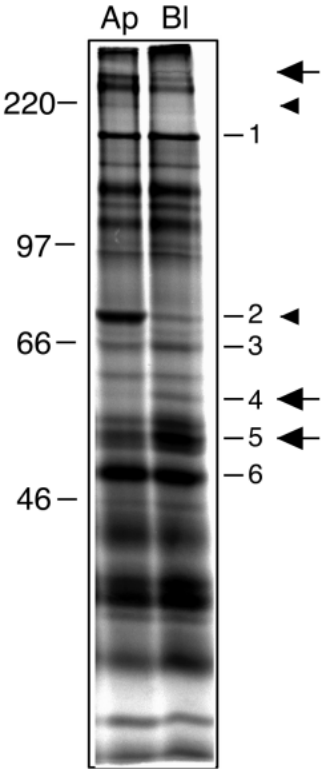


Fig. 1. Metabolically labelled proteins secreted into the apical (Ap) or basolateral (Bl) medium of ECV304 cells grown on Transwell filters. Arrowheads identify predominantly apically secreted proteins, arrows identify predominantly basolaterally secreted proteins. Six proteins (numbered 1-6) were selected for quantitation (see Table 1). Numbers on the left indicate mobilities of marker proteins in kDa.

and 4), while others were equally sorted to both membrane surfaces (proteins 1, 3 and 6). Interestingly, the polarity of secretion of protein 2 increased as the ECV304 cells were maintained for longer periods in a monolayer while the polarity of protein 4 remained constant. Thus ECV304 cells can efficiently sort proteins in the TGN and have distinct apical and basolateral secretory pathways.

Transfection of ECV304 cells with a full-length rat pIgR cDNA

A cDNA encoding the entire open reading frame of rat pIgR was constructed by PCR amplification and transfected into both MDCK cells (as control) and ECV304 cells. G418-resistant cell lines were screened by indirect immune fluorescence labelling of the rat pIgR, and cell lines were selected having different levels of pIgR expression. The levels of expression were quantitated by metabolic labelling and immunoprecipitation of the pIgR protein (Table 2). To avoid any problems caused by overexpression, the cell line ECV.T17 was chosen for further study. This cell line expressed approximately 25% of the pIgR per cell compared with MDCK.T23.

No change in the electrical resistance or ^{14}C-inulin permeability of the transfected cell lines was observed as a result of transfection of the pIgR (data not shown).

Expression and polarity of the pIgR in EVC304 cells

The intracellular localisation of the transfected pIgR was determined by indirect immunofluorescence labelling. The pIgR staining was found to have a punctate appearance and was distributed mainly in the perinuclear region of the cell. The pattern of localisation was very similar in both ECV304 cells and MDCK cells transfected with rat pIgR (Fig. 2).

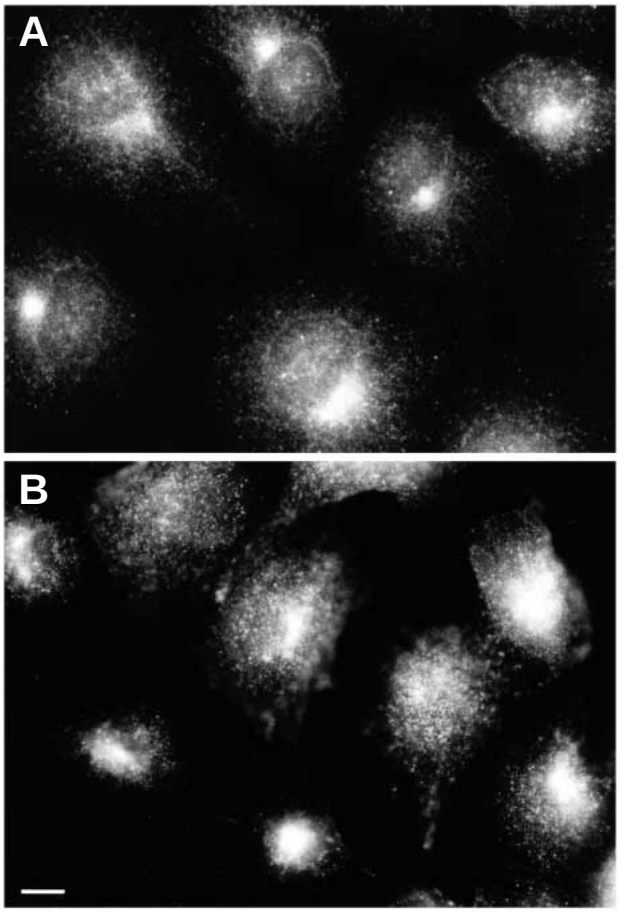


Fig. 2. Indirect immunofluorescence staining for pIgR in ECV304 and MDCK cells. Transfected ECV304 cells (A) and MDCK cells (B) were grown on glass coverslips and labelled using an antiserum against the rat pIgR cytoplasmic domain. Bar, 10 μm.

To identify whether the transfected endothelial cells were able to process the rat pIgR normally, we pulse-labelled ECV.T17 with Tran^{35}S-label and then chased for times up to 90 minutes. Immediately after the pulse only immature pIgR could be immunoprecipitated, giving a band at 116 kDa on SDS gels (arrowhead, Fig. 3A). This matured to a 120 kDa band with a $t_{1/2}$ of about 30 minutes. After 60 minutes of chase

Table 2. pIgR expression in transfected ECV304 cells

Transfected ECV cell line	pIgR expression level (% of pIgR expression in MDCK.T23)
T4	31
T7	49
T17	25
T31	76
T33	184
T35	56

The relative level of expression of pIgR in each ECV cell line was measured by labelling for 2 hours with 250 μCi/ml of Tran^{35}S-label and then immunoprecipitating the pIgR. The intensity of labelled pIgR from each cell line was quantitated and expressed relative to the MDCK.T23 cell line (100%). Similar numbers of cells were used for each immunoprecipitate.

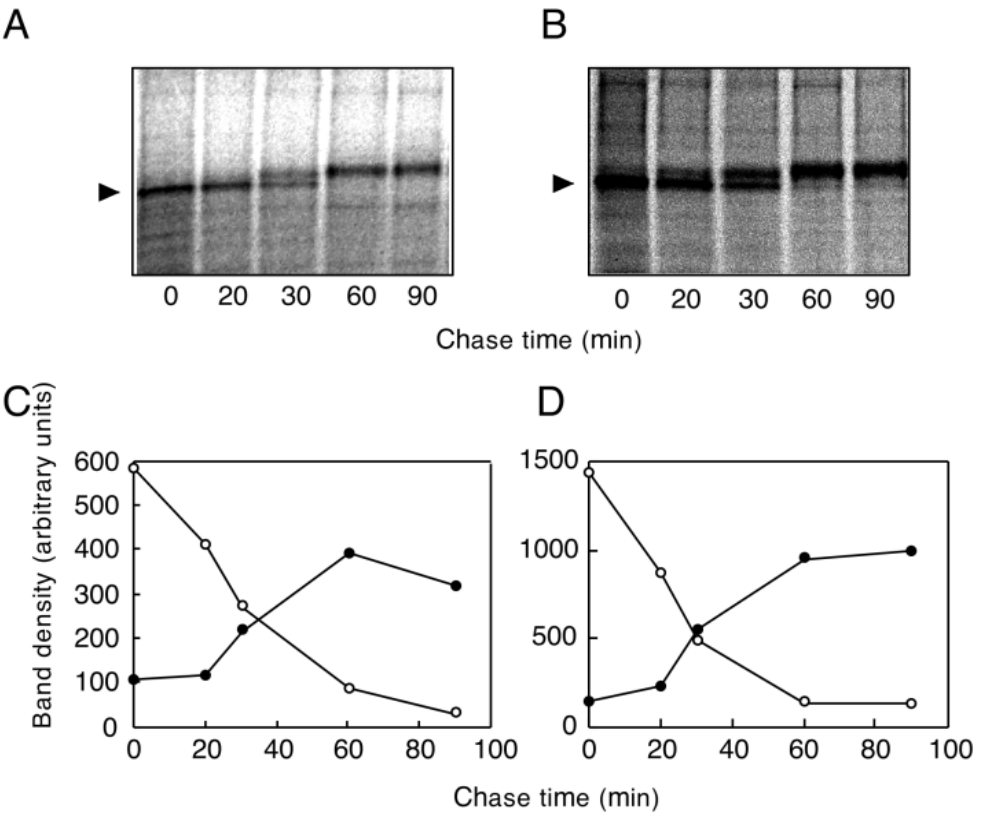


Fig. 3. pIgR biosynthesis in transfected ECV (A) and MDCK cells (B). The cells were pulse-labelled for 15 minutes with Tran^{35}S-label and chased for the indicated times. The pIgR was then immunoprecipitated and separated by SDS-PAGE. Arrowheads identify the immature pIgR protein. Quantification by phosphorimage analysis of the immature pIgR (116 kDa, filled circles) and mature pIgR (120 kDa, open circles) is shown for ECV and MDCK cells in panels (C) and (D), respectively.

the immature band had almost entirely disappeared. The kinetics of pIgR maturation were similar in both ECV.T17 and MDCK.T23 cells (Fig. 3C,D) and were similar to other reports using the rabbit pIgR (Mostov and Deitcher, 1986; Breitfeld et al., 1990), indicating that normal synthesis and maturation of the rat pIgR receptor occurs in both cell types.

To examine the surface polarity of pIgR at steady state in transfected endothelial cells, we selectively biotinylated either the basolateral or the apical domain of cells grown as a monolayer on filters. The biotinylated pIgR was detected on a western blot using ^{125}I-streptavidin (Fig. 4). In ECV.T17 cells, slightly more pIgR was expressed at the apical surface at steady state than on the basolateral surface (57% Ap and 43% Bl). Surprisingly, a similar result was obtained in the transfected MDCK cells (55% Ap and 45% Bl), in contradiction to previous reports (Casanova et al., 1991). This could be due to a slower cleavage of the rat receptor at the apical surface, allowing more receptor to accumulate at this surface after transcytosis. When both apical and basolateral surfaces were biotinylated, the detected pIgR served as a measure of total surface pIgR. In both cell lines the total amount of biotinylated pIgR was comparable to the sum of biotinylated pIgR on the apical and basolateral surfaces, indicating that leakage of the sulpho-NHS-biotin across the monolayer was small for both cell lines.

Although approximately equal amounts of pIgR were found on apical and basolateral surface of ECV.T17 cells at steady state it was possible that the initial delivery of newly synthesised pIgR molecules was polarised to one surface followed by a redistribution to a more random state. We therefore metabolically labelled ECV.T17 cells for 10 minutes and then left the cells at 18°C for 90 minutes in order to accumulate the newly synthesised pIgR in a post-TGN compartment. The cells were then incubated for 90 minutes at 37°C in medium containing TPCK-trypsin for cleavage of the surface proteins. Trypsin inhibitor was added to the opposite side of the cell monolayer to remove any possibility of trypsin leakage through the monolayer. In Fig. 5 it can be seen that about 63% of newly synthesised pIgR was delivered to the basolateral surface in ECV.T17 cells. In contrast, 88% of newly synthesised pIgR was delivered to the basolateral surface of MDCK cells, in agreement with previous reports (Breitfeld et al., 1990; Casanova et al., 1991).

Transcytosis of dimeric IgA

In order to test whether the pIgR in ECV304 cells was functional we incubated monolayers of ECV.T17 with iodinated dIgA. After a 2-hour incubation at 4°C with the labelled dIgA the cells were washed and warmed to 37°C in medium containing 0.6% BSA for a further 2 hours. The apical and basolateral media were TCA precipitated and counted. Transcytosis due to the pIgR was then calculated by subtracting the radioactivity found in wells of control monolayers of ECV304 cells that had not been transfected by the pIgR. In Fig. 6 it can be seen that the majority of pIgR-dependent transcytosis of ^{125}I-labelled dIgA in MDCK cells occurs from basolateral to apical surfaces as expected (Mostov and Deitcher, 1986; Breitfeld et al., 1989). In contrast, the opposite result was obtained with ECV.T17 cells, where the pIgR-dependent transcytosis was threefold greater in the apical to basolateral direction. The absolute values of transcytosis were similar to previous reports (Tamer et al., 1995). When the cells

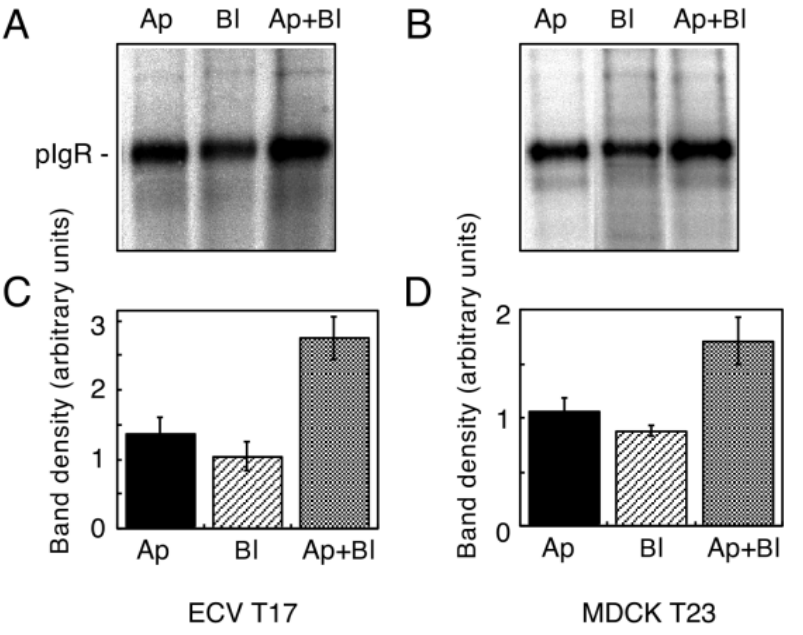
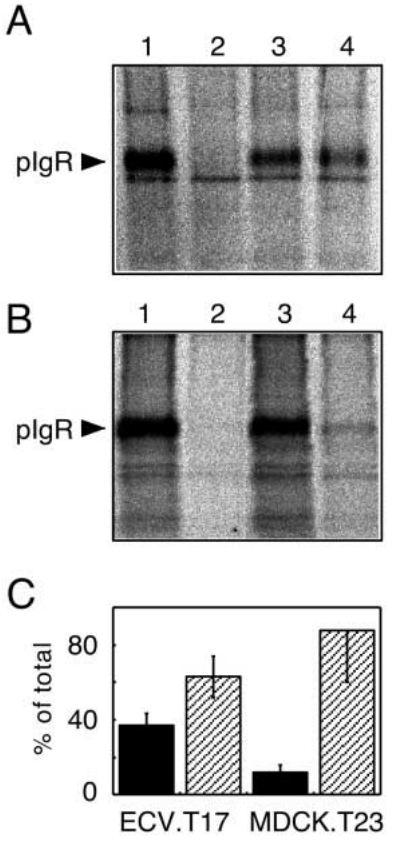


Fig. 4. Steady-state polarity of pIgR expression in transfected cell lines. The apical (Ap) and basolateral (Bl) surfaces of the transfected cells were biotinylated, either separately or together, followed by immunoprecipitation of the pIgR. The degree of biotinylation was determined by SDS-PAGE, transfer to nitrocellulose and binding of ^{125}I-streptavidin. Phosphorimages are shown for (A) ECV.T17, and (B) MDCK.T23 cells. The quantified results are shown for ECV.T17 and MDCK.T23 cells in (C) and (D), respectively (*n*=3, mean±s.d.).

were preincubated with nocodazole (33 μM) the apical to basolateral transport of ligand was 40% inhibited in ECV.T17 cells and no effect was found on Bl to Ap transcytosis (data not shown), arguing that transport was by an intracellular microtubule-dependent process.



Since it has been shown that phosphorylation of serine 664 of the pIgR is one mechanism for stimulating basolateral to apical transcytosis in epithelial cells we labelled ECV.T17 cells with ^{32}P-orthophosphate and immunoprecipitated the pIgR (Fig. 7). When labelled with Tran^{35}S-label for 2 hours, both ECV.T17 and MDCK.T23 cell lines showed a major band at 120 kDa corresponding to mature pIgR, with a less intense band of immature precursor at 116 kDa (Fig. 7, lane 1). After ^{32}P-orthophosphate labelling a single band at the mobility of the mature pIgR was observed for both cell lines (Fig. 7, lane 2).

Release of secretory component

The pIgR is normally proteolytically cleaved at the apical surface of hepatocytes allowing the dimeric IgA ligand, covalently attached to a part of the receptor, to be released. The half-life of pIgR in rat hepatocytes has been measured as 1.1 hours in vivo (Scott and Hubbard, 1992). MDCK cells have also been shown to cleave the rabbit pIgR and allow release of secretory component (Mostov and Deitcher, 1986). In MDCK.T23 cells we found that approximately 29% of the metabolically labelled rat pIgR was degraded after 8 hours of chase. In contrast, only 12% of rat pIgR was degraded in

Fig. 5. Polarised delivery of newly synthesised pIgR in transfected cell lines. ECV.T17 (A) or MDCK.T23 cells (B) were pulse-labelled for 15 minutes in Tran^{35}S-label at 37°C. Following a chase of 90 minutes at 18°C, the cells were incubated in medium containing trypsin on one side of the monolayer and trypsin inhibitor on the other. After 90 minutes in trypsin medium the cells were washed, lysed and immunoprecipitated with anti pIgR antiserum. Lane 1: no trypsin added; lane 2: trypsin added to both Ap and Bl surfaces; lane 3: trypsin added to Ap surface only; lane 4 trypsin added to Bl surface only. (C) A quantification of the data shown in A and B, assuming that the difference between lanes 1 and 3 represents Ap pIgR (solid bars) and the difference between lanes 1 and 4 represents Bl pIgR (hatched bars). Data are expressed as the percentage of total surface pIgR (Ap+Bl)±s.e.m. for 3 measurements.

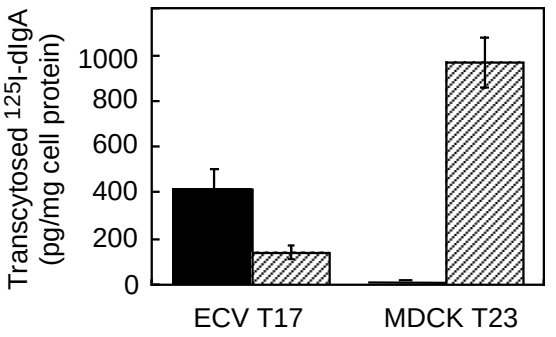


Fig. 6. dIgA is predominantly transcytosed from Ap to Bl in ECV304 cells. ^{125}I-dIgA was allowed to bind to one surface of the monolayers of ECV.T17 and MDCK.T23 for 2 hours on ice. The cells were then washed in DMEM containing 0.6% BSA and incubated in the same medium at 37°C for a further 2 hours. Medium from the opposite side of the monolayer was collected and counted. Data represent the mean±s.e.m. for 6 measurements. There was no detectable transport from apical to basolateral surfaces in MDCK.T23 cells. In ECV.T17 cells the transcytosed ^{125}I-labelled dIgA from apical to basolateral medium was significantly higher than the amount from basolateral to apical medium (n=6, P=0.021). Solid bars, Ap to Bl transcytosis; hatched bars, Bl to Ap transcytosis.

ECV.T17 cells in an 8 hour period. This could be due to a slower rate of transcytosis or lack of proteolytic enzymes. We therefore raised an antiserum against the amino terminus of the rat pIgR and looked for the release of secretory component into the tissue culture medium in both transfected cell lines. This antibody detects the full-length receptor (Fig. 8, lane 1) and any cleaved forms of the receptor retaining the amino terminus (the cell lysate used in lane 1 had been stored frozen for some time and contains a number of degraded forms of the pIgR that are not seen in fresh cell lysates). When medium was collected from apical and basolateral sides of cells grown on filters, secretory component was only observed on the apical surface of the MDCK.T23 cells (Fig. 8, lanes 4 and 5). In contrast a band of similar size was produced on both apical (66%) and basolateral (34%) sides of ECV.T17 cells (Fig 8, lanes 2 and 3). The cleavage of rat pIgR was evidently not identical in MDCK and ECV cells. Both cell lines produced bands of 80 kDa and 97 kDa, but the upper band was much more intense in MDCK cells. In ECV cells additional bands of approx. 55 kDa were found, suggesting that different proteases might be responsible for cleaving the surface receptor in ECV cells. All these bands must contain the amino-terminal epitope of the pIgR and represent cleaved portions of the extracellular domain released into the medium. No SC was detected by immunoblotting of media or cell lysates from untransfected ECV cells (Fig. 8, lanes 6 and 7). Thus in the ECV.T17 cells fragments of the pIgR are generated on apical and basolateral surfaces in approximately the same proportion as the receptor steady-state distribution.

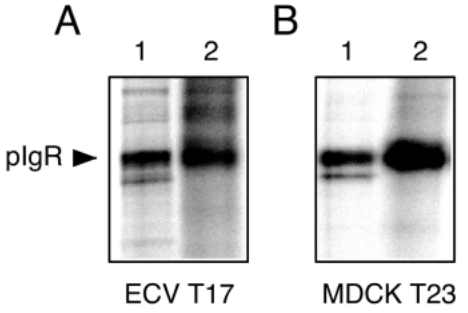


Fig. 7. Phosphorylation of pIgR in ECV.T17 and MDCK.T23. The cells were labelled for 2 hours with Tran^{35}S-label or ^{32}P-orthophosphate, respectively. The labelled pIgR was then immunoprecipitated and analysed by SDS-PAGE. (A) ECV.T17 cells, (B) MDCK.T23 cells. In the ^{35}S-labelled cells (lane 1), both the 116 and 120 kDa forms of pIgR were detected, but only the 120 kDa form of pIgR (arrowhead) was identified in ^{32}P-labelled cells (lane 2).

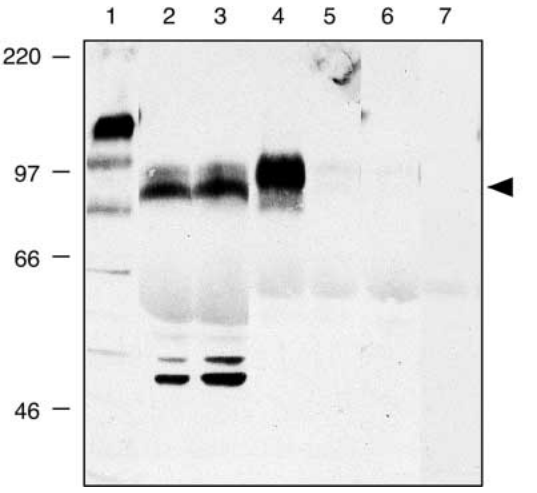


Fig. 8. Release of SC into culture medium in transfected cell lines. Cells were grown in normal medium containing 0.6% BSA and 20 mM Hepes for 24 hours. At the end of this time, the medium was collected and concentrated on StrataClean resin before loading on an SDS gel and immunoblotting with an antibody raised against the amino-terminal amino acids of the pIgR. Lane 1: ECV.T17 cell lysate; lane 2: apical medium of ECV.T17 cells; lane 3: basolateral medium of ECV.T17 cells; lane 4: apical medium of MDCK.T23 cells; lane 5: basolateral medium from MDCK.T23 cells; lanes 6 and 7: apical medium from untransfected MDCK and ECV304 cells, respectively. Twice as much medium was loaded from ECV.T17 cells than for MDCK.T23 cells. The arrowhead identifies an 80 kDa secretory component. Identical results were obtained in two independent experiments, each with duplicate samples.

DISCUSSION

The ECV304 cell line is a spontaneously transformed human endothelial cell line derived from human umbilical vein (Takahashi et al., 1990). It expresses a variety of endothelial marker proteins including VCAM-1, GMP-140 and angiotensin converting enzyme. The cells form a monolayer with a significant electrical resistance when grown on Transwell filters, indicating the formation of tight intercellular junctions (Jakob, 1993). The integrity of the monolayer was established by the continuous cadherin expression at the cell border, and by the low leakage of small molecules like NHS-

biotin, trypsin and trypsin inhibitor across the monolayer under our experimental conditions. Furthermore, in Fig. 1 it is clear that endogenously secreted proteins remain on one side of the bilayer for several hours after secretion, indicative of an intact monolayer.

We have transfected ECV304 cells with rat pIgR cDNA and have measured for the first time the sorting of a transfected epithelial cell protein in an endothelial cell line. Despite the fact that the sorting signals on the pIgR have been shown to be recognised in several different epithelial cell lines and neuronal cells (Mostov and Deitcher, 1986; Schaerer et al., 1990; Bonzelinus et al., 1994; de Hoop et al., 1995), we have found that the targeting signals on the pIgR are poorly recognised by ECV304 cells. Our data cannot be explained by overexpression of the transfected pIgR as the cell line chosen for study was a low expresser, and synthesised lower levels than the pIgR-transfected MDCK control cell line.

There have been several reports of membrane proteins that are polarised in endothelial cells in vivo (Muller and Gimbrone, 1986; Horvat and Palade, 1993; Jacobson et al., 1996). We have also found that several endogenous secretory proteins of ECV304 cells are secreted with high polarity, indicating that these cells do maintain polarity in tissue culture and can sort proteins in the TGN. Newly synthesised pIgR, however, appeared in approximately equal amounts on each cell surface and was shed as secretory component in similar amounts into medium bathing each side. In contrast, when the same cDNA was transfected into MDCK cells the receptor was delivered in a highly polarised way to the basolateral surface, while secretory component was virtually only shed from the apical surface, as previously described. Thus the difference observed was not due to the use of rat pIgR, rather than the rabbit pIgR that was used in previous studies.

We examined the ability of the TGN in ECV cells to recognise epithelial signals for basolateral targeting. We maintained the cells at 18°C during the labelling to trap the newly synthesised receptor in post-TGN vesicles and then warmed up the cells in medium containing trypsin as described by Casanova (1991). This experiment measures the efficiency of sorting of the pIgR in the TGN and is not influenced by the relative areas of apical and basolateral membranes. In contrast to MDCK cells, where 88% of the newly synthesised pIgR was delivered to the basolateral surface, indicating a sorting efficiency of 7:1, only 63% of newly synthesised pIgR in ECV cells was delivered to the basolateral surface, a sorting efficiency of 2:1. The same result was obtained whether the cells were left for 60 minutes or 90 minutes in trypsin, indicating that the result is not the consequence of a slower delivery in the ECV cells. Furthermore when trypsin was added on both sides of the cells, only 3% of the pIgR remained undegraded, indicating that pIgR was not left at intracellular locations.

We do not know the ratio of basolateral to apical membrane surface areas for ECV304 cells; however, the low aspect ratio of the cells would suggest that the ratio must be close to unity. For MDCK cells, which are much taller, the ratio is 7.3:1 (Bl:Ap) (von Bonsdorff et al., 1985). It is likely that the apical membrane of ECV304 cells grown on filters is slightly larger in surface area than the basolateral membrane since the apical membrane is distended over the nucleus while the basolateral membrane is maintained flat on the filter surface. Thus the redistribution after initial insertion to a steady-state distribution in favour of the apical domain (1.3:1, Ap:Bl) could be the result of random redistribution of the receptor.

In the absence of ligand, phosphorylation of serine 664 of the rabbit pIgR receptor has been reported to stimulate transcytosis of the receptor by masking of the basolateral targeting signal (Hirt et al., 1993; Aroeti and Mostov, 1994). Interestingly, we were able to detect phosphorylation of pIgR in ECV.T17 cells as well as in MDCK.T23 cells, although we do not know at present whether this is on the equivalent of serine 664 in the rat receptor. If it is, then there must be a defect in the recognition of apical targeting signals in these cells also, since little basolateral to apical transcytosis was detected.

The polarity of the pIgR in epithelial cells can also be demonstrated by the polarised release of secretory component into the medium bathing the cells. We confirmed this in MDCK cells expressing the rat pIgR but found no evidence for polarised release of SC in ECV304 cells. Both the rate of turnover of the pIgR, and the rate of SC production per mg of cell protein, were lower in ECV cells than in MDCK.T23 cells. This might reflect a limitation in the amount of proteolytic enzymes delivered to the apical surface of ECV304 cells capable of cleaving the pIgR.

Endothelial cells have a highly developed apical surface that must maintain a non-coagulant property and transfer nutrients to the underlying cells. Thus the apical surface of endothelial cells might be expected to be enriched in receptors like the basolateral surface of epithelial cells. This is reflected in the previous reports of basolateral epithelial cell receptors, like the transferrin receptor (Jefferies et al., 1984) and LDL receptor (Dehouck et al., 1997), which were found on the apical surface of brain microvascular endothelial cells and were involved in transcytosis of their ligands to the underlying cells. In our experiments, pIgR-transfected ECV304 cells, derived from a large vein endothelium, were able to transport dIgA more rapidly from the apical to the basolateral surface of ECV.T17 cells, suggesting that all endothelial cells might have a specialised apical to basolateral transcytosis pathway.

The pIgR has a well-defined basolateral targeting signal comprising the amino acids HRxxV (where x stands for any amino acid) situated in the membrane-proximal part of the cytoplasmic domain (Aroeti and Mostov, 1994). It is clear that this signal is not well recognised by the sorting machinery in the TGN of ECV304 cells, since delivery of the pIgR to the cell surface was much less polarised than in MDCK cells. Subsequent sorting in endosomes does not occur either, since the steady-state polarity of pIgR was even less polarised in favour of the basolateral surface. This could be due to the lack of recognition of the targeting signal by cytoplasmic proteins that normally bind to these sequences, or to masking of the basolateral targeting sequence on the receptor. For example, phosphorylation has been postulated to mask the basolateral targeting signal on the pIgR, although this does not normally occur until after the pIgR has reached the basolateral membrane in epithelial cells (Aroeti and Mostov, 1994). Apical targeting of the pIgR after binding ligand also requires masking of the dominant basolateral targeting signal, possibly by an interaction of calmodulin with the cytoplasmic domain (Chapin et al., 1996). N-glycan chains on the lumenal domain of the receptor may then drive apical transport by interaction with lectin homologues such as VIP36, which is associated

with apically sorted lipid rafts (Fiedler et al., 1994; Scheiffele et al., 1995; Simons and Ikonen, 1997). Thus the abberrant targeting and transcytosis of pIgR in endothelial cells could have a number of origins.

In addition to these considerations it is possible that targeting of receptors to intracellular locations also depends on the flux of vesicular traffic pathways to each location, especially if sorting is not 100% efficient. Increased apical secretion in endothelial cells compared with MDCK cells might decrease the ability to sort pIgR into basolaterally targeted vesicles, while increased transcytosis from apical to basolateral surfaces could determine the overall direction of transcellular transport. In view of the growing number of reported anomalies of sorting in endothelial cells, it will be interesting to determine in more detail, the mechanism by which sorting of endogenous proteins is achieved.

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