Apical and Basolateral Transferrin Receptors in Polarized BeWo Cells Recycle Through Separate Endosomes

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Abstract. Contrary to most other epithelia, trophoblasts in the human placenta, which form the physical barrier between the fetal and the maternal blood circulation, express high numbers of transferrin receptors on their apical cell surface. This study describes the establishment of a polarized trophoblast-like cell line BeWo, which exhibit a high expression of transferrin receptors on the apex of the cells. Cultured on permeable filter supports, BeWo cells formed a polarized monolayer with microvilli on their apical cell surface. Across the monolayer a transepithelial resistance developed of $\sim 600 \ \Omega \cdot \text{cm}^2$ within 4 d. Depletion of Ca$^{2+}$ from the medium decreased the resistance to background levels, showing its dependence on the integrity of tight junctions. Within the same period of time the secretion of proteins became polarized. In addition, the compositions of integral membrane proteins at the apical and basolateral plasma membrane domains were distinct as determined by domain-selective iodination. Similar to placental trophoblasts, binding of $^{125}$I-labeled transferrin to BeWo monolayers revealed that the transferrin receptor was expressed at both plasma membrane domains. Apical and basolateral transferrin receptors were found in a 1:2 surface ratio and exhibited identical dissociation constants and molecular weights. After uptake, transferrin recycled predominantly to the domain of administration, indicating separate recycling pathways from the apical and basolateral domain. This was confirmed by using diaminobenzidine cytochemistry, a technique by which colocalization of endocytosed $^{125}$I-labeled and HRP-conjugated transferrin can be monitored. No mixing of the two types of ligands was observed, when both ligands were simultaneously internalized for 10 or 60 min from opposite domains, demonstrating that BeWo cells possess separate populations of apical and basolateral early endosomes. In conclusion, the trophoblast-like BeWo cell line can serve as a unique model to compare the apical and basolateral endocytic pathways of a single ligand, transferrin, in polarized epithelial cells.

The plasma membrane of epithelial cells has differentiated into an apical and a basolateral domain, which are morphologically and biochemically distinct. The establishment and maintenance of this cell surface polarity is achieved by several mechanisms. These include proper cell-substrate attachment, intensive cell--cell contact, where tight junctions prevent lateral diffusion of proteins and lipids from one domain to the other, and targeting of newly synthesized proteins to the appropriate domain (Rodriguez-Boulan and Nelson, 1989; Simons and Wandinger-Ness, 1990). Transcytosis from both domains may contribute to the formation of plasma membrane domains (Hubbard, 1989), but may also compromise the maintenance of cell surface polarity. However, efficient sorting of recycling proteins from proteins which are transcytosed (Mostov and Simister, 1985; Hubbard, 1989; Rodman et al., 1990), as well as separate apical and basolateral early endosomes (Oliver, 1982; Von Bonsdorff et al., 1985; Bomisel et al., 1989; Parton et al., 1989), prevent intracellular mixing of proteins from different domains.

Typically, in most epithelia the apical cell surface is opposed to the external milieu, while the basolateral cell surface faces the internal milieu, i.e., the blood supply (Simons and Fuller, 1985). In contrast, in hemochorial placenta the microvilli at the apical cell surface of trophoblasts is in direct contact with the maternal bloodstream, whereas the basal cell surface faces the fetal bloodstream. The trophoblast layer is a polarized transporting epithelium, forming a continuous interface between both circulations. Therefore, it is the major site of interchange of many blood-borne substances, such as hormones, metabolites, blood gases and nutrients, between the maternal and fetal bloodstreams (Truman and Ford, 1984). Moreover, trophoblasts produce a wide range of pregnancy-related proteins, peptide hormones, and steroid hormones.

Vast amounts of iron, essential for the developing fetus, are transported across the trophoblast. Transferrin (TF) in the

1. Abbreviations used in this paper: TF, transferrin; TfR, transferrin receptor; TGN, trans-Golgi network.
maternal circulation is the major source of this iron (for review see van Dijk, 1988). The trophoblast displays a high number of transferrin receptors (TfR) on the apical (i.e., the maternal side) cell surface (Enns et al., 1981; Seligman et al., 1979). Since iron, but not maternal Tf, passes the placenta (Gitlin et al., 1964; Contractor and Eaton, 1986), it seems likely that Tf in trophoblasts follows a diacytotic pathway as found for other cell types (van der Ende et al., 1987; Douglas and King, 1990). This means that Tf binds to TfRs on the cell surface, followed by internalization and transport to a mildly acidic endosomal compartment. Here, iron dissociates from Tf and the resulting apoTf-TfR complex is rapidly recycled to the cell surface, where apoTf is released into the medium (Dauty-Varsat et al., 1983; Ciechanover et al., 1983; Klausner et al., 1983). Iron is probably released from the trophoblast into the fetal circulation complexed to an as yet unidentified low molecular weight compound, where it then becomes associated with fetal Tf (Contractor and Eaton, 1986; van der Ende et al., 1987, 1989; Douglas and King, 1990).

In other epithelial cell types, such as found in kidney and intestine, the TfR is considered to be a marker for the basolateral cell surface (Simons and Fuller, 1985). Studies with the polarized MDCK cell line, grown on permeable filter supports, have shown that uptake and recycling of Tf is restricted to the basolateral cell surface (Fuller and Simons, 1986; Podbilewicz and Mellman, 1990). Similar results have been obtained with the human enterocyte-like cell lines Caco-2 and HT29 (Hughson and Hopkins, 1990; Godefroy et al., 1990).

The BeWo cell line has been derived from a human choriocarcinoma (Pattillo and Gey, 1968), and displays many morphological and biochemical properties common to placentental trophoblasts (van der Ende et al., 1987, 1990; Wice et al., 1990). We have previously described the transferrin-mediated uptake and release of iron by BeWo cells (van der Ende et al., 1987, 1989). In this report we used BeWo cells cultured on permeable filters, which allows direct independent access to the apical and basolateral domain. We found that filter-grown BeWo cells form a polarized monolayer, and contrary to other epithelial cells, express TfR on both the basolateral as well as the apical domain, similarly to the situation in placental trophoblasts in vivo. In addition, we provide evidence that Tf internalized from opposite domains enters two separate populations of early endosomes.

Materials and Methods

Materials

Human Tf and HRP were purchased from Sigma Chemical Co. (St. Louis, MO). N-succinimidyl-3,2-pyridyldithio propionate (SPDP) was purchased from Pharmacia (Uppsala, Sweden), and 3,3'-DAB tetrahydro-chloride was obtained from Boehringer (Mannheim, Germany). Proteinase K was purchased from Boehringer (Mannheim, Germany). mAb 66-lg-10 against human transferrin receptor (van de Rijn et al., 1983) was kindly provided by Dr. J. Hilkens (The Netherlands Cancer Institute, Amsterdam).

Cell Culture

The b24 clone of the human choriocarcinoma-derived cell line BeWo (Pattillo and Gey, 1968) was grown in MEM, supplemented with 10% FCS (MEM/FCS), penicillin, and streptomycin as described previously (van der Ende et al., 1987). Cells from confluent 75-cm² flasks (Costar Corp., Cambridge, MA) were removed with trypsin/EDTA and 1.5-2 x 10⁵ cells/cm² were seeded onto 0.4-μm pore size (6.5 or 24.5 mm diameter) polycarbonate filters (Transwell chambers; Costar Corp). Unless otherwise indicated, filters were used for study after 4 d of growth. Medium was changed on days 1 and 3 after seeding.

EM

BeWo cells on Transwells were fixed in diluted Karnovsky fluid containing 2% paraformaldehyde, 2.5% glutaraldehyde, 0.25 mM CaCl₂, and 0.5 mM MgCl₂ in 80 mM sodium cacodylate buffer (pH 7.4). The filters were cut from their holders, postfixed in 2% OsO₄, dehydrated through a series of ethanol solutions, and embedded in Epon. Thin sections were stained with uranyl acetate and lead citrate and examined in a Jeol 1200EX transmission electron microscope (Tokyo, Japan).

Trans epithelial Resistance Measurement

Electrical resistance across BeWo cells grown on filters (24.5 mm diameter) was measured using the Millipelle-ERS epithelial voltometer and electrodes (Millipore Corp., Bedford, MA). Measurements were carried out at room temperature in PBS and were corrected for resistance of filters in the absence of cells. To open the tight junctions, the filter-grown cells were incubated in 10 mM EDTA in growth medium for 30 min at 37°C prior to resistance measurements.

Metabolic Labeling and Polaronized Secretion

For analysis of protein secretion after various days of culture, BeWo cells were grown in 6.5-mm filters. Monolayers were rinsed twice with PBS containing Ca²⁺ and Mg²⁺ (PBS-CM) at room temperature, and pulsed for 20 min by adding 200 μl labeling medium in the basal compartment at 37°C in an incubator. During the pulse the apical compartment received no medium. The labeling medium consisted of methionine-free MEM containing 5% FCS, dialyzed against PBS (dFCS), and 100 μCi/ml 125I-methionine (Amer sham International, Amersham, UK). After the pulse was chased in 200 and 400 μl MEM/dFCS, containing 10 times normal concentrations of methionine, in the apical and basolateral compartment, respectively. After 3 h of chase the media were collected and 15 μl apical and 30 μl basal media were analyzed by SDS-PAGE (Laemmli, 1970), using 5-15% gradient gels.

Cell Surface Iodination

Cell surface iodination was performed on BeWo cells grown on 24.5-mm filters using the lacto-peroxidase procedure (Hubbard and Cohn, 1972). Filters were washed four times with ice-cold PBS-CM and kept on ice. Labeling medium (PBS-CM plus 25 μg/ml lactoperoxidase, 12.5 μg/ml glucose-oxidase, 250 μg/ml glucose and 0.15-0.25 mCi/ml Na-125I (Amer sham International) was added either to the apical side (1 ml) or the basal side (2 ml). After 30 min, filters were washed twice with MEM containing 20 mM Hepes, pH 7.3 (MEMH), and twice with PBS-CM. Integral surface proteins were selectively extracted by the TX-114 method (Boudier, 1981). Briefly, filters were cut out and lysed in 1 ml of 1% TX-114 in 20 mM Tris, 150 mM NaCl pH 8 (TBS) plus 1 mM PMFS, 4 mM EDTA, 1 mg/ml BSA, 1 mg/ml soybean trypsin inhibitor (SITI), leupeptin, and pepstatin at 20 μg/ml. The lysate was vortexed and incubated for 1 h at 4°C on a rocker. Nuclei and debris were removed by centrifugation (12,000 g, 15 min, 4°C) in a microfuge and the supernatant was centrifuged once more.

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Where indicated, 125I-labeled cells were incubated with 100 μg/ml proteinase K in PBS-CM, added either to the apical side (1 ml) or the basolateral side (2 ml) and kept on ice for 60 min under gentle agitation. Control filters were identically treated in the absence of proteinase K. Next, filters were washed four times with PBS-CM containing 1 mM PMFS, cut from the holder, and lysed in 1 ml of 1% TX-114 in TBS containing 4 mM EDTA, 1 mM PMFS, and 1 mg/ml SITI and BSA. The lysate was cleared from nuclei as described above and TIR was immunoprecipitated using mAb 66-lg-10. Immunoprecipitations were performed as described previously (van der Ende et al., 1990).
Preparation of Ligands

Human Tf was iron saturated using the procedure of van der Heul et al. (1978) and iodinated using IODO-BEADS (Pierce Chemical Co., Rockford, IL). Specific activities of 2-4 × 10⁶ cpm/µg Tf were obtained.

Tf was conjugated to HRP by using SPDP following the procedure of Stoorvogel et al. (1988). Free HRP and free Tf were separated from Tf-HRP conjugates by gel filtration, and the eluted fractions were analyzed by SDS-PAGE. The fractions, containing mainly 1:1 conjugates of Tf and HRP were pooled and used in experiments. The Tf-HRP conjugate was shown to bind PAGE. The fractions, containing mainly 1:1 conjugate of Tf and HRP were conjugates by gel filtration, and the eluted fractions were analyzed by SDS-PAGE. Specific activities of 2-4 x 10⁶ cpm/µg Tf were obtained.

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TF Binding and Recycling Assays

BeWo cells, grown in 6.5-mm filters, were washed three times with prewarmed MEMH containing 0.2% BSA (MEMH/BSA) and incubated for 30 min at 37°C in MEMH/BSA to deplete cells from serum Tf. For binding experiments, filters were rapidly cooled by two washes with ice-cold MEMH/BSA and incubated for 60 min on ice with 125I-Tf (0.25-10 µg/ml) in MEMH/BSA either at the apical side (150 µl), the basal side (400 µl), or at both sides. Nonspecific binding was determined by adding a 50× excess of unlabeled ligand. Compartments receiving no labeled ligand were filled with MEMH/BSA supplemented with 20 µg/ml unlabeled Tf. Finally, the filters were washed three times with MEMH/BSA, cut out, and counted in a gamma-counter.

For recycling experiments, BeWo cells were grown on 24.5-mm filters, were washed three times with prewarmed MEMH containing 0.2% BSA (MEMH/BSA) and incubated for 30 min at 37°C in MEMH/BSA to deplete cells from serum Tf. For binding experiments, filters were rapidly cooled by two washes with ice-cold MEMH/BSA and incubated for 60 min on ice with 125I-Tf (0.25-10 µg/ml) in MEMH/BSA either at the apical side (150 µl), the basal side (400 µl), or at both sides. Nonspecific binding was determined by adding a 50× excess of unlabeled ligand. Compartments receiving no labeled ligand were filled with MEMH/BSA supplemented with 20 µg/ml unlabeled Tf. Finally, the filters were washed three times with MEMH/BSA, cut out, and counted in a gamma-counter.

DAB-Cytochemistry

For uptake of 125I-Tf and Tf-HRP followed by DAB-cytochemistry, we used a modified procedure of the method of Stoorvogel et al. (1991). In this procedure the internalized 125I-Tf, present in Tf-HRP-containing vesicles, is cross-linked upon addition of both DAB and H2O2 and thus rendered nonextractable (Ajioka and Kaplan, 1987; Stoorvogel et al., 1988). BeWo cells, grown on 6.5-mm filters were depleted from serum Tf as described above. Filter cultures were washed with ice-cold MEMH/BSA, and subsequently 125I-Tf (0.5 µg/ml) was added either to the apical side (150 µl) or the basal side (400 µl) at 4°C for 60 min. After three washes with MEMH/BSA, filters were incubated with Tf-HRP (2.5-50 µg/ml) in prewarmed MEMH/BSA for 5 min at 37°C, present either at the apical or the basal side. For continuous uptake of ligands, cells were incubated for 10 min at 37°C with 125I-Tf (0.5 µg/ml) present in either the apical or basal compartment. Tf-HRP (10 µg/ml) was simultaneously present in the same or opposite compartment. Uptake was stopped by immersing the filters in ice-cold MEMH/BSA, followed by an acid-neutral wash to remove surface-bound ligand. Next, the filters were incubated in PBS containing DAB (150 µg/ml) and H2O2 (0.03 %) for 60 min on ice (Stoorvogel et al., 1988). Control filters were incubated in the absence of H2O2. Filters were then cut out and boiled in 300 µl Laemmli sample buffer (Laemmli, 1970) and analyzed by SDS-PAGE using 10% slab gels. 125I-Tf was detected by autoradiography and bands were quantified by densitometry. The amount of 125I-Tf recovered from the sample incubated in the presence of H2O2 was expressed as a percentage of the control (the sample lacking H2O2) (Stoorvogel et al., 1989).

Results

Characterization of Filter-grown BeWo Cells

When BeWo cells were seeded onto Transwell polycarbonate filters and cultured for 4-5 d, they displayed characteristics typical for polarized cells (Fig. 1). The morphology appeared similar to that of freshly isolated trophoblasts from human placenta (Klinman et al., 1986; Nelson et al., 1986). They formed polarized monolayers with irregular microvilli at the apical plasma membrane, whereas the basal plasma membrane was attached to the filter (Fig. 1 A). Large nuclei were located in the center of the cell. Golgi stacks were found randomly distributed in the perinuclear cytoplasm, whereas numerous profiles of RER and multiple free ribosomes were located throughout the cytoplasm. The cells often contained large deposits of glycogen (Fig. 1 A). The lateral cell surfaces were closely opposed, highly interdigitated, and connected by typical junctional complexes (Fig. 1 B).

We next examined whether BeWo monolayers develop transepithelial resistance, a property of tight epithelia. Transepithelial resistance was first recorded after 2 d of culturing and reached a plateau value of ~600 Ω-cm² within 4 d culturing (Fig. 2). When Ca²⁺ was chelated by EDTA, the electrical resistance decreased to background levels (Fig. 2), indicating its dependence on the integrity of tight junctions.
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Figure 2. Development of transepithelial resistance during culturing of BeWo cells grown on filters. (○—○) Cells were seeded on filters and electrical resistance was measured as described in Materials and Methods. Data points are the mean of four different experiments with duplicate measurements. (●—●) Transepithelial resistance measured after 30 min incubation in the presence of 10 mM EDTA at 37°C. Error bar, SD. (Gumbiner, 1987). After removal of the EDTA the transepithelial resistance recovered within 6 h (data not shown). The transepithelial resistance of BeWo cells appeared to be intermediate compared to those measured for filter-grown MDCK I (4267 ± 132 Ω·cm², n = 4) and MDCK II cells (204 ± 43 Ω·cm², n = 5). The transcriptional resistances measured for MDCK I and MDCK II cell are consistent with previously published data (Fuller et al., 1984). BeWo cells are known to synthesize placental steroid and peptide hormones (Pattilo and Gey, 1968), the secretion of which may occur in a polarized fashion. To investigate the secretion pattern from BeWo monolayers after different days

Figure 3. (A) Development of polarized secretion of proteins by BeWo monolayers. Cells cultured on filters for 3 d (lanes 1 and 2) or 5 d (lanes 3 and 4) were labeled for 20 min with 35S-methionine and subsequently chased for 3 h. Aliquots of the apical (a) or basal (b) media were subjected to SDS-PAGE. (B) Asymmetrical distribution of membrane proteins on filter-grown BeWo cells. Cells were labeled with 125I either at the apical (a) or at the basal (b) side. Protein extracted with TX-114 were subjected to SDS-PAGE. Small arrow heads: apically enriched proteins; large arrow heads: basolaterally enriched proteins. (M) Molecular mass markers (kD).

Figure 4. Transferrin binding to BeWo cells grown on filters analyzed by Scatchard plots. Cells were incubated at 4°C with increasing concentrations of 125I-transferrin administered to either the apical (○—○), the basal (●—●), or to both sides (△—△).
of culturing, cells were pulse labeled for 20 min by including 

Figure 5. Analysis by SDS-PAGE of surface transferrin receptors

Results show that BeWo cells, in contrast with other epithelial
cells, express TfRs on both plasma membrane domains, that
are identical in molecular weight and binding affinity.

Received Receptor-mediated Endocytosis of Transferrin
from Apical and Basolateral Domains in Polarized

The presence of TfRs on both the apical and basolateral
plasma membrane domain enabled us to follow a single cycle
of receptor-mediated endocytosis at both sides of polarized
BeWo cells using identical ligands (Tf). 125I-Tf was bound to
the apical cell surface at 4°C, excess of 125I-Tf was removed,
after which the cells were incubated at 37°C and the release
of recycled ligand into the media was monitored. Within
5-10 min, ~65% of the prebound 125I-Tf on the apical
domain was internalized and subsequently released into the
media with a half life of 15 min (Fig. 6A). Similar results
were obtained using plastic-grown BeWo cells (van der Ende
et al., 1987). The same uptake and release pattern was ob-
served from the basolateral domain (Fig. 6B). More important-
lly, in a single round of endocytosis most of the prebound
ligand (60-70%) was recycled back to the domain of ad-
ministration, whereas ~20% was transported (transcytosed)
from one compartment to the other within 60 min (Fig. 6,
A and B). The results were the same, when an iron chelator
(DTPA) was added to the medium to prevent reuptake of
transferrin by precluding rebinding of iron to apotransferrin
which returned to the cell surface (data not shown). Moreover,
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Polarized BeWo Cells Have Separate Sets of Apical
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We used DAB-cytochemistry to study whether the recycling
pathways of Tf from the apical and basolateral domain inter-
sect. The procedure was based on the observation that HRP-
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las and King, 1990). To determine if any leakage of labeled
Tf through the monolayer had occurred during the 125I-Tf
incubation, resulting in the overestimation of the number of
binding sites, labeled ligand was added to both sides simulta-
neously. The number of binding sites (3.3 x 10^4/cm^2)
was then close to the sum of binding sites found for the apical
and basolateral domain separately, while the K_d obtained
was in the same range (1.7 x 10^-8 M) as found for both do-
 mains (Fig. 4). This indicates that little, if any, leakage of
ligand through the monolayer occurred.

To gain insight in the molecular nature of the TfR on both
the apical and basal plasma membrane, BeWo monolayers
were iodinated at either cell surface. Resistance to protein-
ase K administered to the compartment opposite to the la-
beled plasma membrane domain demonstrated the selectiv-
ity of the iodination (Fig. 5). As shown in Fig. 5, the TfR
immunoprecipitated from both domains revealed identical
molecular masses of 90 kD. TfRs isolated from human
placental homogenates have the same molecular weight (Sel-
igman et al., 1979; Anderson et al., 1986). Together, our
results show that BeWo cells, in contrast with other epithelial
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Figure 6. Single cycle of \(^{125}\text{I}-\text{transferrin}\) in BeWo monolayers. Cells were incubated for 1 h at 4°C with \(^{125}\text{I}-\text{transferrin}\) added to either the apical (A) or basal (B) compartment. After removing the excess of labeled transferrin, the cells were incubated at 37°C for the indicated periods of time. After the incubation, media from the apical and basal compartments were collected separately and the intracellular and surface-bound \(^{125}\text{I}-\text{transferrin}\) was determined as acid/neutral wash resistant and sensitive, respectively.

be trapped by DAB, resulting in a reduced signal (recovery) of \(^{125}\text{I}-\text{Tf}\) at the appropriate mobility by SDS-PAGE analysis (Ajioka and Kaplan, 1987; Stoovogel et al., 1988, 1989). A trace amount of \(^{125}\text{I}-\text{Tf}\), prebound to the apical domain, was internalized for 5 min at 37°C to determine whether this was accessible to Tf-HRP internalized from either the same or opposite domain. The concentration of Tf-HRP (2.5–50 μg/ml) was varied to achieve optimal crosslinking of colocalized \(^{125}\text{I}-\text{Tf}\). As shown in Fig. 7, prebound \(^{125}\text{I}-\text{Tf}\) internalized from the apical domain could be cross-linked when Tf-HRP was taken up from the same domain (Fig. 7 A). Maximal crosslinking (recovery of 40%) occurred at a minimal concentration of 10 μg/ml Tf-HRP (Fig. 7 C). Internalized \(^{125}\text{I}-\text{Tf}\) was fully recovered when excess unconjugated Tf was present in addition to Tf-HRP (Fig. 7, A and C), indicating that Tf-mediated uptake of Tf-HRP was required for crosslinking of \(^{125}\text{I}-\text{Tf}\). Moreover, apically internalized \(^{125}\text{I}-\text{Tf}\) was also not crosslinked when Tf-HRP was administered from the basolateral domain (Fig. 7, B and C). These data indicate that Tf entered separate populations of endosomes when applied to opposite domains. To exclude the possibility that prebound \(^{125}\text{I}-\text{Tf}\) and Tf-HRP enter common endosomes in a staged fashion, we labeled the entire recycling pathway with ligand at 37°C. \(^{125}\text{I}-\text{Tf}\) (0.5 μg/ml) was continuously internalized for 10 min from the apical or basal domain, and Tf-HRP (10 μg/ml) was simultaneously added to either the same or opposite domain. After 10 min of uptake, \(^{125}\text{I}-\text{Tf}\) could be cross-linked only when Tf-HRP was administered to the same domain (recovery of 25%). In contrast, \(^{125}\text{I}-\text{Tf}\) was fully recovered, when Tf-HRP was administered to the opposite domain (Fig. 8 A). The results were similar when the incubation time was extended to 60 min, although there is a small amount of crosslinking of \(^{125}\text{I}-\text{Tf}\) when administered to a compartment opposite from that of Tf-HRP (recovery of >90%). These data show that the endocytic pathways of Tf-Tf complex from both domains involve recycling through separate sets of endosomal compartments.

Discussion

Trophoblast-like BeWo cells grown on filters form a polarized monolayer within 4 d after seeding, based on morphological, physiological, and biochemical criteria. (a) An apical microvillous domain was formed, separated from the basolateral domain by junctional complexes. (b) They developed a transepithelial resistance, comparable to those of other polarized cell systems, which was dependent on the presence of functional tight junctions. (c) BeWo cells developed asymmetry, which was demonstrated by two biochemical approaches. First, we showed that BeWo cells secrete different sets of proteins into the apical and basal media. Polarized secretion was also found in a variety of other model systems such as MDCK cells (Caplan and Matlin, 1989), Caco-2 cells (Rindler and Traber, 1988; Hughson et al., 1989), rat ureter epithelial cells (Glasser et al., 1988), and endothelial cells (Sporn et al., 1989; Unemori et al., 1990). The polarized secretion from BeWo cells started as soon as the electrical resistance across the monolayer reached its plateau value. Moreover, polarized BeWo cells secreted several proteins, which were not found in the medium of nonpolarized cells. Thus, filter-grown BeWo cells undergo a biochemical differentiation, resulting in a polarized phenotype. In this respect, our results resemble the observations of Carson et al. (1988) who found an increased apical secretion of keratin sulphate proteoglycans from monolayers of rat uterine epithelial cells in parallel with the development of cell polarity. Secondly, asymmetric integral membrane protein composition of the apical and basolateral surface was demonstrated using a domain-selective iodination procedure in combination with TX-114 detergent extraction. Such a structural surface polarity is a common property of epithelia, which function in vectorial transport (Simons and Fuller, 1985). These data are consistent with Vanderpuye et al. (1987, 1988), who reported structural differences between apical and basal plasma membranes isolated from
After incubation, cells were incubated with DAB, with or without H_2O_2 as indicated. After DAB cytochemistry, cells were processed for SDS-PAGE as described in Materials and Methods. (C) Quantification of the data; Tf-HRP added to the basolateral domain (○—○) or added to the apical domain (△—△) with or without excess unlabeled Tf (△—△).
human placental trophoblasts. Interestingly, by examining the total composition of surface proteins in MDCK cells, Sargiacomo et al. (1989) reported that the integral apical proteins were clustered into four major sets ranging from $M$, 30 to 100 kD, while the basal proteins grouped around $M$, 30-70 kD. We did not observe such typical clusters on either domain in BeWo cells. This difference probably reflects a cell type specific phenomenon.

To our knowledge, our results demonstrate for the first time a nonpolarized expression of the TfR in polarized cells cultured in vitro. In other polarized cell lines the TfR is predominantly expressed on the basolateral cell surface, the side active in uptake of nutrients from the blood supply (Fuller and Simons, 1986; Godefroy et al., 1988; Hughson and Hopkins, 1990). Our finding of substantial TfR activity at the apical surface of BeWo cells is consistent with its proposed function in the hemochorial placenta, since binding of maternal Tf to apical TfRs on trophoblasts is essential for transplacental iron transport from mother to fetus (van Dijk, 1988). Observations made by Vanderpyue et al. (1986) suggesting the presence of TfR at the apical as well as the basolateral plasma membrane on placental trophoblasts are also in agreement with our findings. The reason for the apical localization of the transferrin receptor is unclear. Peptide map analysis of the human placental and red blood cell transferrin receptor revealed no structural differences (Enns and Sussman, 1981). Moreover, only one gene coding for the transferrin receptor localized to chromosome 3 (3q26.2-qter) has been found (McClelland et al., 1984; Rabin et al., 1985). Whether the placental and apical located transferrin receptor is derived from an alternative splicing pathway or by cell type specific posttranslational modification is not known.

It remains to be clarified, whether basal TfRs in some way participate in the transplacental transport of iron, or serve other function(s). One attractive option is that basal TfRs play a role in regulating the intracellular iron pool in trophoblasts. In trophoblasts as well as in BeWo cells, intracellular iron is stored in a form bound by ferritin (van der Ende et al., 1987; Douglas and King, 1990). Cellular iron modulates the synthesis of both ferritin and TfR by a translational control on the corresponding mRNAs (Theil, 1990); e.g., iron in excess increases ferritin synthesis for iron storage and decreases TfR synthesis, resulting in a decreased iron uptake. Conversely, at low iron levels receptor synthesis and iron uptake are increased, whereas ferritin synthesis and iron storage decrease. Thus, basal TfRs in trophoblasts may act as a fetal feedback control of placental iron uptake and
storage by monitoring the iron saturation level of fetal transferrin. Possibly, the BeWo cell system could serve as a useful model for studying the mechanisms underlying transplacental iron transport.

In filter-grown BeWo cells, internalization of Tf occurs from both apical and basolateral domain, as was shown from the single cycle experiments (Fig. 6). Importantly, most of the internalized Tf recycled to the domain of uptake, suggesting that the recycling pathways of TfR from both domains are separate. This was supported further biochemically using a modified procedure of the DAB-cytochemistry technique, which allows colocalization of radioactive labeled ligands with ligand conjugated to HRP (Courtoy et al., 1984; Stoorvogel et al., 1988, 1989). We showed that Tf-HRP and [125I]Tf, internalized from opposite domains, do not mix in a common endosome, demonstrating separate populations of apical and basolateral endosomes. By using endocytic fluid-phase markers, MDCK cells have been shown to possess distinct apical and basolateral early endosomes, which are exclusively involved in endocytosis initiated at the corresponding cell surface domain. However, in these cells the endocytic pathways from the apical and basolateral domain converge after 15 min of uptake of fluid-phase markers, presumably in late endosomes (Bomsel et al., 1989; Parton et al., 1989; van Deurs et al., 1990). In contrast, Hughson and Hopkins (1990) reported that in Caco-2 cells HRP, applied to the apical surface, rapidly (within 5 min) reached endosomes containing anti-TfR antibody conjugated to gold, which was basolaterally internalized for 60 min. However it is possible, that the antibody-gold marker reached apical early endosomes after long incubations. Moreover, in this study meeting of basolaterally added Tf-HRP and apically added con A-gold was also only observed after 15 min. In BeWo cells a minor fraction (<10%) of the labeled transferrin was crosslinked by Tf-HRP after 60 min uptake from opposite domains. This might be caused by mixing in the trans-Golgi-network (TGN) (Stoorvogel et al., 1988) or alternatively in late endosomes (Stoorvogel et al., 1991). However, it was recently shown that late endosomes are reached by Tf with the same kinetics as early endosomes (Stoorvogel et al., 1991). The resialylation of the TIR occurs with a half time of 5-6 h, suggesting either slow recycling through the TGN or more likely only a fraction of the internalization TIR follows the route through the TGN (Snider and Rogers, 1985). Moreover, Stoorvogel et al. (1988) observed that recycling of Tf, and thus TIR, through the TGN occurs with rates comparable to that through early endosomes. Since we do not observe meeting of both ligands after 10 min of uptake from opposite domains, we believe that the small amount of crosslinking found after prolonged incubations is caused by reuptake of leaked or transcytosed TF-HRP. Because the DAB-cytochemistry depends on enzymatic activity of the HRP, very small amounts of Tf-HRP are sufficient to observe crosslinking (Stoorvogel et al., 1989).

In conclusion, we have developed a novel polarized cell system with trophoblast-like BeWo cells, which illustrates an important difference in surface expression of TfR as compared to other polarized cell lines. It would be of particular interest to determine whether this is caused by a different sorting mechanism of newly synthesized TfR prior to cell surface delivery or due to sorting in the endocytic pathway. In addition, the localization of the TIR on apical and basolateral plasma membrane domains in BeWo cells allows us to study recycling pathways of a single ligand at both cell surfaces.

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