Expression and Differential Polarization of the Reduced-folate Transporter-1 and the Folate Receptor α in Mammalian Retinal Pigment Epithelium*

The differential polarized distribution of the reduced-folate transporter (RFT-1) and folate receptor α (FRα), the two proteins involved in the transport of folate, has been characterized in normal mouse retinal pigment epithelium (RPE) and in cultured human RPE cells. RPE cells mediate the vectorial transfer of nutrients from choroidal blood to neural retina. Whereas FRα is known to be present in many cell types of the neural retina, in situ hybridization analysis in the present study demonstrated that RFT-1 is present only in RPE. Laser-scanning confocal microscopy using antibodies specific for RFT-1 demonstrated an apical distribution of this protein in cultured human and intact mouse RPE, which contrasts with the basolateral distribution of FRα in these cells. The expression of RFT-1 in the RPE cell apical membrane was confirmed by functional studies with purified apical membrane vesicles from bovine RPE. These studies, done with N5-methyltetrahydrofolate (the predominant folate derivative in blood) and folate as substrates, have shown that RFT-1 functions in a Na+- and Cl−-independent manner. The transporter is specific for folate and its analogs. A transmembrane H+ gradient influences the transport function of this protein markedly; the transport mechanism is likely to be either folate/H+ co-transport or folate/OH− exchange. Based on the differential polarization of FRα and RFT-1 in RPE, we suggest that these two proteins work in a concerted manner to bring about the vectorial transfer of folate across the RPE cell layer from the choroidal blood to the neural retina. This constitutes the first report of the differential polarization of the two folate transport proteins in any polarized epithelium.

The one-carbon derivatives of the water-soluble vitamin folic acid are essential for intermediary metabolism. These derivatives are required for the synthesis of purine and pyrimidine nucleotide precursors of RNA and DNA and also for metabolism of several amino acids. Since folate and its one-carbon derivatives are lipophobic bivalent anions, they do not traverse biological membranes by simple diffusion but have to be taken up into the cells by specific transport processes (1). Two types of transport processes have been identified at the functional and molecular level for folate entry into mammalian cells (1–4). First, there is a folate receptor (FR)1 that binds folate and internalizes the bound folates via receptor-mediated endocytosis (3, 4). The entire FR protein is exposed to the exterior of the cell and is anchored to the plasma membrane via glycosylphosphatidylinositol. There are three isoforms of this receptor (α, β, and γ) among which only the α-isofrom has been shown to participate in the cellular uptake of folates in normal cells. Although the FRα has a very high affinity for non-reduced folate (Kd < 1 nM), it interacts also with reduced folates, although with much less affinity. The second transport process is mediated by the reduced folate transporter (RFT-1) (1, 2). This is a typical transporter protein with multiple membrane-spanning domains. It interacts with reduced folates much more efficiently than with non-reduced folate.

FRα is expressed almost ubiquitously in mammalian cells. Since every cell requires folate for metabolism, FRα provides the mechanism for the cellular uptake of folate from the blood. The obligatory nature of this uptake mechanism for cell survival is evident from the findings that the targeted disruption of the FRα gene is lethal and that the knockout mice do not survive beyond the early embryonic stage (5). In contrast to FRα, the expression of RFT-1 is limited to those cells that are involved in vectorial transfer of folates from one side of the cell to the other (1). This includes the absorptive cells of the kidney and placenta and the hepatocytes in the liver. Since these cells are polarized and express FRα as well as RFT-1, it is believed that these proteins may be differentially polarized in these cells to mediate the vectorial transfer of folate. To date, however, there have been no studies reported in the literature relating to the differential polarized distribution of the FRα versus RFT-1 in any of the mammalian polarized cells.

A tissue that has been largely ignored with respect to mechanisms of folate transport has been the retina, yet folate deficiencies have been implicated in the visual disorder nutritional amblyopia (6–8). This disease is characterized by reduced central vision, cecocentral scotoma, pallor of the optic disc, loss of papillomacular-bundle fibers, and optic atrophy (8). Folate de-

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1 The abbreviations used are: FR, folate receptor; RFT, reduced-folate transporter; RPE, retinal pigment epithelium; MTF, methyltetrahydrofolate; PBS, phosphate-buffered saline; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; NMDG, N-methyl-D-glucamine; Mes, 4-morpholineethanesulfonic acid.
Differential Polarization of Folate Transport Proteins in RPE

MA. Restriction enzymes and pGEM-T vector were from Promega (Madison, WI). The TRIZol reagent for the isolation of total RNA and oligo(dT)-cellulose for purification of poly(A)+ RNA were from Life Technologies, Inc. The digoxigenin-labeling kit, the alkaline phosphatase-coupled anti-digoxigenin antibody, and the nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate-chloro-form solution were from Roche Molecular Biochemicals. Tissue-Tek OCT embedding compound was from Miles Laboratory (Elkhart, IN). Inject maleimide-activated mariculture keyhole limpet hemocyanin was from Pierce. The antibody against FRα was a generous gift from Dr. M. Ratnam, Medical College of Ohio (Toledo, OH). The antibody against Na+-K+-ATPase was from American Revealed (Valle, NY). The fluorescein isothiocyanate-conjugated AffiniPure goat anti-rabbit IgG was from Jackson ImmunoResearch Laboratories (West Grove, PA).

Animals—Five- to six-week-old albino (ICR) mice were obtained from Harlan Sprague-Dawley, Indianapolis, IN. Two-month-old New Zealand rabbits were obtained from Robinson’s Bunny Farm (Clemons, NC). Animals were maintained on a 12-h light:12-h dark lighting cycle and were fed the standard purina mouse chow diet and rabbit chow diet, respectively. Care and use of the animals adhered to the principles set forth in the DHEW Publication, NIH 80-23, “The Guiding Principles in the Care and Use of Animals.”

Cell Culture—ARPE-19 cells were maintained at 37 °C in a humidified atmosphere of 5% CO2 in Dulbecco’s modified Eagle’s medium/phenol red-free Earle’s F-12, supplemented with 100 units/ml penicillin (100 units/ml) and streptomycin (100 μg/ml). Cultures were passaged by dissociation in 0.05% (w/v) trypsin in phosphate-buffered saline (PBS). After trypsinization, cells were seeded on Nunc 8-well chamber slides coated with 5 μg/cm2 mouse laminin and maintained with Dulbecco’s modified Eagle’s medium/F-12 supplemented with 1% fetal bovine serum to promote differentiation of the cells. Cells were allowed to differentiate for at least 4 weeks prior to each experiment (17, 18). BeWo cells were maintained in Dulbecco’s modified Eagle’s medium/F-12 medium, supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. For immunohistochemistry, subcultures of these cells were seeded on 8-well chamber slides and grown to confluency. After the cells were confluent, forskolin (100 μM) was added to the medium, and the growing cells were fed daily for 3 days prior to use in immunohistochemical analysis.

In Situ Hybridization—In situ hybridization was performed on mouse eyes to localize the mRNA transcripts encoding RFT-1. To prepare the probes for these studies, total RNA from mouse placenta was extracted using the TRIZol reagent. The RNA was subjected to reverse transcription-polymerase chain reaction. The upstream primer 5'CGTGTTCCTGTGGGTGTGTGTT-3' and the downstream primer 5'-TGTTGGGACGGGATCTAC-3' corresponded to nucleotide positions 426–443 and 1036–1054, respectively, in the cloned mouse RFT-1 cDNA (19). By using these primers, a 629-base pair sequence was amplified. The polymerase chain reaction product was cloned into the pGEM-T vector, and the orientation of the cDNA insert was determined by sequencing. The single-stranded antisense riboprobe specific for RFT-1 was prepared by labeling the cDNA clone with the restriction enzyme NcoI and then transcribing the cDNA in vitro using SP6 RNA polymerase. The single-strand sense (control) riboprobe was prepared using the restriction enzyme NotI for linearization and the T7 RNA polymerase for transcription. The synthesized riboprobes were labeled using a digoxigenin-labeling kit. Additional controls for the in situ hybridization included omission of the antisense riboprobe specific for the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). By using the upstream primer 5'-ACCGGATTTGCGGTTT-3' and the downstream primer 5'-TCTGGGATCAAAAATGAGGAG-3', an 1100-base pair sequence was amplified and cloned into the vector, and synthesized riboprobes were digoxigenin-labeled as described above.

Eyes from albino mice were enucleated, frozen immediately in Tissue-Tek OCT section at 10-μm thickness, and fixed in 4% paraformaldehyde. Following our published protocol (15), sections were rinsed in ice-cold PBS and treated with active 1% (w/v) proteinase K (1 μg/ml) in PBS for 4 min. The proteinase K activity was stopped by rinsing the slides in glycine (2 mM). The slides were fixed in PBS, equilibrated in 5× SSC, and were prehybridized for 2 h at 58 °C in 50% (w/v) formamide, 5× SSC, 2% (w/v) blocking reagent (provided with the Digoxigenin Nucleic Acid Detection Kit), 0.1% (w/v) N-lauroylsarcosine, and 0.02% (w/v) sodium dodecyl sulfate. Sections were hybridized with the probes (1 μg/ml) and were incubated overnight at 58 °C. They were washed twice in 2× SSC at room temperature, twice in 1× SSC at 55 °C, and twice in 1× SSC in a humidified atmosphere of 5% CO2.
Preparation of Antibodies against RFT-1—In addition to the five antibodies purified in the laboratory of Dr. Sirotnek (20), an additional antipeptide antibody was raised against the peptide sequence RPKRSLLFNNRDGRC which corresponds to residues 205–220 of human RFT-1. The peptide (2 mg) was conjugated to 2 mg of Injekt maleimide-activated marculie keyhole limpet hemocyanin and purified by overnight dialysis. Approximately 300 μg of the peptide-conjugated hemocyanin in Freund’s complete adjuvant was administered intradermally to New Zealand White rabbits (approximately 50 μl each). The initial injection was followed by two boosters. Antiserum was obtained 10 days after the second booster and purified using affinity chromatography.

Interferential Microscopy—Analysis of RFT-1 in Intact RPE and in Cultured RPE and BeWo Cells—Immunohistochemical analyses were used to localize RFT-1 in cultured human ARPE-19 cells, human trophoblast BeWo cells, and in intact eyes from mice. The cells and 10-μm thick cryosections were fixed with ice-cold methanol and acetone, respectively, and blocked with 10% normal goat serum. Samples were incubated with one of several peptide antibodies against RFT-1 (20) or with the additional affinity purified antibody against RFT-1 that was made in our laboratory. Cells were incubated with the primary antibody for 3 h at room temperature at a dilution of 1:2000; tissue sections were incubated for 3 h at room temperature at a dilution of 1:50 followed by an overnight incubation at 4 °C. In companion experiments, cells or cryosections were incubated with an antibody against FRα at a dilution of 1:50 or the antibody against Na+-K+-ATPase at a dilution of 1:50. As the FRα has been localized to the basolateral membrane of intact mouse RPE (15), it was used in the present study for comparison to RFT-1. The Na+-K+-ATPase was used as a marker for the apical membrane of the RPE (22–24) and the basolateral membrane of BeWo cells. Incubation with 0.1% normal rabbit serum or with buffer only served as negative controls. After rinsing, all samples were incubated overnight at 4 °C with a fluorescein isothiocyanate-conjugated AffiniPure goat anti-rabbit IgG at a dilution of 1:100. Cells and cryosections were optically sectioned (z series) using a Bio-Rad MRC-600 Laser Scanning Confocal Imaging System. Analysis of images used the COMOS software package (Bio-Rad). Additional analyses of the cells were performed using a Nikon Diaphot 200 Laser-scanning Confocal Imaging System (Molecular Dynamics, Sunnyvale, CA). Images were analyzed using the Image Display 3.2 software package (Silicon Graphics, Mountain View, CA).

Preparation of Bovine RPE Apical Membrane Vesicles—Membrane vesicles were prepared following our published methods (25). For each membrane preparation, 30 bovine eyes were obtained fresh from a local slaughterhouse. All steps were performed at 4 °C. The cornea of each eye was removed and the eyecup inverted. Following removal of the neural retina, the RPE was collected and placed in ice-cold buffer (2.4 mM Tris/NaOH, 60 mM mannitol, 1 mM EGTA, pH 7.2). The RPE was homogenized for 1.5 min at high speed in a Waring blender (Waring Products Corp., New York). A stock solution of 1 mM MgCl2 was added to the homogenate to create a final concentration of 30 mM. The mixture was stirred for 1.5 min, incubated for an additional 10 min, and centrifuged at 3,000 × g for 15 min. The supernatant, containing apical membranes, was collected by filtration through several layers of cheesecloth. It was then centrifuged at 46,000 × g for 35 min to pellet the membranes. The pellets were resuspended in a preloading buffer (20 mM Hepes/Tris, 300 mM mannitol, pH 8.0), and the centrifugation was repeated. The resulting pellet was resuspended in a small amount of the preloading buffer, and the protein concentration was determined to 10 mg/ml. The membranes were stored in liquid nitrogen until use.

Transport Experiments—The transport of [3',5',7,9-H]-methyltetrahydrofolate ([3',5',7,9-H]MTF) and [3',5',7,9-H]folic acid was measured in membrane vesicles at room temperature by a rapid filtration method using Millipore filters (DWP type, 0.85 μm pore size). Transport was initiated by mixing 40 μl of membrane vesicle preparation (400 μg of membrane protein) with 160 μl of uptake buffer containing 30 nM [3',5',7,9-H]MTF or 20 nM [3',5',7,9-H]folic acid. Transport was allowed to proceed for a desired time following which it was terminated by addition of 3 ml of ice-cold uptake buffer. The mixture was filtered and washed with two changes of 10 ml each of ice-cold buffer. The filter was transferred to a scintillation vial and 3 ml of liquid scintillation spectrometry was associated with it and determined by liquid scintillation spectrometry.

The composition of the uptake buffer in most experiments was 20 mM Mes/Tris, 300 mM mannitol, pH 5.0. Under these conditions, there was an inwardly directed transmembrane H+ gradient (or outwardly directed transmembrane OH− gradient) due to the pH gradient across the membrane (intravesicular pH = 8.0, extravesicular pH = 5.0). For experiments dealing with the influence of Na+, the uptake buffers consisted of 20 mM Hepes/Tris, pH 8.0, containing 150 mM NaCl, sodium gluconate, or N-methyl-d-glucamine (NMDG)-chloride. For experiments dealing with the influence of pH, the uptake buffers of varying pH values (5.0–8.0) were prepared by appropriately mixing the following two buffers: 20 mM Mes/Tris, 300 mM mannitol, pH 5.0, and 20 mM Hepes/Tris, 300 mM mannitol, pH 8.0. The substrate specificity of the transport process was investigated by assessing the influence of unlabeled folate analogs (MTF, folate, and methotrexate) and other vitamins (ascorbate, thiamine, nicotinamide, and pantothenate) on the transport of radiolabeled MTF or folate.

Folate Binding Assay—To confirm that the folate transport measured in the apical RPE membrane vesicles was not due to the presence of FRα, a ligand binding assay was performed following a protocol published by Spinnella et al. (26). The ability of the membranes to bind folate acid in the absence and presence of unlabeled folic acid was measured. The binding of [3H]folic acid to apical membranes from human placenta, known to contain FRα, was also measured as a positive control. Membranes were resuspended in 25 ml of acidic buffer (10 mM Na+ acetate/acetate, 150 mM NaCl, pH 3.5) to release any endogenous bound folate. Membranes were immediately centrifuged at 46,000 × g for 35 min. The resulting pellet was resuspended in binding buffer (200 mM NaHPO4, 10 mM NaCl, pH 7.4), and the protein concentration was determined. Binding was initiated by adding 50 μl of the membrane suspension (final concentration, 30 μg of membrane protein) to 150 μl of binding buffer containing 10 nM [3H]folic acid and, in some samples, 10 μM unlabeled folic acid. Binding was performed at 4 °C, which prevents transport but allows binding. Binding was allowed to continue for 90 min, after which the mixture was filtered through a glass fiber filter (Osmonics, Westboro, MA) using a rapid filtration technique. The filter was washed with two changes of 10 ml each of ice-cold binding buffer and was transferred to a counting vial. The radioactivity associated with the filter was measured by liquid scintillation spectrometry.

RESULTS

In situ Hybridization—We have recently analyzed the expression pattern of FRα mRNA in the retina by in situ hybridization (15). FRα mRNA is expressed in every cell type in the retina. The expression pattern included ganglion cells, cells of the inner nuclear layer (amacrine, bipolar, horizontal, and Müller cells), photoreceptor cells, and the RPE. In the present study, we analyzed the expression pattern of RFT-1 mRNA in the retina. In situ hybridization was performed on cryosections of mouse eyes (n = 6) to determine in which retinal tissues mRNA transcripts encoding RFT-1 were expressed. These studies were performed using digoxigenin-labeled riboprobes. Fig. 1A shows a hematoxylin and eosin-stained cryosection of the outer retina for comparison to the non-stained in situ hybridization data. As shown in Fig. 1B, RFT-1 mRNA was expressed only in the RPE cell layer of the retina as indicated by the dark blue staining in this cellular layer when an RFT-1-specific antisense riboprobe was used. All other retinal cell layers were negative. Hybridization of the tissue sections with the sense probe of RFT-1 showed no positive signal (Fig. 1C). Hybridization of cryosections with antisense probes specific for GAPDH showed abundant intense expression throughout the neural retina and
RPE, whereas hybridization with the sense probe for GAPDH showed no positive reaction (data not shown).

The specific expression of RFT-1 mRNA only in the RPE is in marked contrast to the widespread expression of FRα mRNA in retina (15). Thus our studies identify the RPE as the only cell type in retina that expresses both the transport mechanisms for folate, namely FRα and RFT-1. In contrast, all remaining cell types in retina express only FRα. These findings have significant relevance to the unique physiological function of the RPE in the vectorial transfer of folate from the choroidal blood into neural retina.

**Immunohistochemical Analysis**—If the co-expression of FRα and RFT-1 in the RPE is indeed relevant to the vectorial transfer of folate, the two proteins are expected to be localized differentially in the basolateral membrane of the cell versus the apical membrane. Our previously published studies have already established the expression of FRα in the basolateral membrane of the RPE in intact mouse RPE (15). The receptor protein is not detectable in the apical membrane. In the present study, we sought to localize the distribution of RFT-1 in intact mouse RPE and cultured human RPE cells using several antibodies against RFT-1. The antibodies used included anti-peptide antibodies prepared against five different regions of RFT-1 as described by Chiao et al. (20) and an additional affinity-purified antibody against residues 205–220 of human RFT-1. Fluorescent immunohistochemical methods were used to determine where the RFT-1 protein was distributed in cryosections of mouse eye. Fig. 2A shows a hematoxylin and eosin-stained cryosection of mouse retina shown for comparison to non-stained sections subjected to immunohistochemistry. Fig. 2B shows a cryosection of mouse retina labeled with an antibody directed against mouse RFT-1 generated against residues 50–64. Laser-scanning confocal microscopy revealed an intense band of fluorescence on the apical RPE surface. There was no labeling on the basal surface of the RPE. Six different antibodies were used in this immunolocalization experiment, and each of them yielded similar results. For comparison, the antibody against the FRα (Fig. 2C) was used in companion experiments, and the FRα protein was found to be localized to the basolateral surface as has been reported recently for intact mouse RPE (15). Sections incubated with normal rabbit serum showed no positive labeling (data not shown).

To determine whether the differential distribution pattern of RFT-1 and FRα observed in the apical versus basolateral membrane in intact mouse RPE was maintained in cultured human RPE cells, we performed immunohistochemical studies in the polarized, well differentiated ARPE-19 cells. These cells are a rapidly growing RPE cell line established in the laboratory of Dr. Larry Hjemelander (University of California, Davis). They form a uniform population of polarized epithelial monolayers on porous filter supports. They retain features characteristic of RPE including defined cell borders, a cobblestone appearance, noticeable pigmentation (16, 17), and the capacity to phagocytose outer segment disks (27). Fig. 3 shows the immunolocalization of RFT-1 and FRα in cultured ARPE-19 cells grown for 4 weeks on chamber slides. Optical sections of cells labeled with RFT-1 are shown in Fig. 3, A and B. Cells that were scanned vertically (xy plane) demonstrated the distribution of RFT-1 on the apical membrane in cultured RPE as evidenced by the bright band of fluorescence across the apical membranes of the cells (Fig. 3A). When these same cells were scanned horizontally (xy plane), a dome-like fluorescence pattern was observed, again consistent with an apical distribution of RFT-1 (Fig. 3B). The distribution pattern of FRα is shown in Fig. 3, C and D. The vertical optical sections (xy plane) of cells labeled with an antibody directed against FRα showed a band of fluorescence on the lateral surfaces of the cells suggestive of a basolateral distribution for the receptor (Fig. 3C). Given that the basal portion of the cells are exposed to the chamber slide, only the lateral and apical membranes are accessible to labeling. Hence, a protein with a distribution pattern on the basal as well as lateral surfaces would be detectable only on the lateral surface of these cells in this experimental approach. Horizontal optical sections (xy plane) of these cells revealed a ring-like fluorescence pattern consistent with a basolateral distribution (Fig. 3D). A protein that has been well established to distribute along the apical surface of the RPE is the Na⁺-K⁺-ATPase (22–24). Cultured ARPE-19 cells incubated with antibodies against Na⁺-K⁺-ATPase (Fig. 3, E and F) demonstrated an apical distribution of the protein as expected. The xy scan (Fig. 3E) demonstrates the apical distribution of the protein. The xy scan in Fig. 3F shows a dome-like fluorescence pattern, indicative of an apical distribution. Cells incubated with normal rabbit serum showed no immunolabeling (data not shown).

The findings of these immunohistochemical studies are important in that they represent the first report of the polarized distribution of RFT-1 and FRα in intact RPE and in a well...
differentiated cultured RPE cell line. It is noteworthy that in intact tissue the expression of RFT-1 was specific to the RPE only and not evident in other cells of the neural retina corroborating the data from the in situ hybridization experiments described above. The data suggest that only RPE, the cell type in the retina involved with vectorial transport of nutrients, expresses this transporter. The present findings showing the differential expression of FRα and RFT-1 in the basolateral membrane and the apical membrane, respectively, in RPE suggest that these two proteins function in a coordinated manner to carry out the transcellular movement of folate from the choroidal blood into the subretinal space. The FRα in the basolateral membrane is likely to be responsible for the entry of folate from the blood into the RPE, and RFT-1 is likely to mediate the exit of folate from the cell into the subretinal space. The distribution of FRα in the basolateral membrane of the RPE is in contrast to the known localization of the protein in the apical membrane of the placental syncytiotrophoblast, another polarized epithelium involved in the vectorial transfer of folate from the mother to the fetus (28). As expected from its role in the vectorial transfer of folate, the placenta expresses FRα as well as RFT-1 (29–31). However, the differential distribution of the two proteins in the syncytiotrophoblast has not been demonstrated. Since the receptor protein localizes to the apical membrane of this cell (31), we hypothesized that RFT-1 expression is localized to the basal membrane. To test this hypothesis, we performed immunohistochemical analysis of the expression of FRα and RFT-1 in BeWo cells, a human placental trophoblast cell line that is widely used as model for syncytiotrophoblast. This cell line polarizes in culture with distinct basolateral and apical membranes and has been used in studies involving vectorial transfer of nutrients (32). Fig. 4 shows the immunolocalization of RFT-1 and FRα in BeWo cells grown on chamber slides for 3 days in forskolin-treated medium. Forskolin is known to induce differentiation and polarization of BeWo cells. Optical sections of cells labeled with RFT-1 are shown in Fig. 4, A and B. Cells that were scanned vertically (zy plane) demonstrated the distribution of RFT-1 on the lateral surfaces of the cells suggestive of a basolateral distribution for the transporter (Fig. 4A). Horizontal optical sections (xy plane) of these cells revealed a punctate ring-like fluorescence pattern also consistent with a basolateral distribution (Fig. 4B). The distribution pattern of FRα in BeWo cells is shown in Fig. 4, C and D. The vertical optical sections (zy plane) of cells labeled with an antibody directed against FRα showed a band of fluorescence on the apical membrane (Fig. 4C). When these same cells were scanned horizontally (xy plane), a dome-like fluorescence pattern was observed, again consistent with an apical distribution of FRα (Fig. 4D).

There are at least three other tissues that perform vectorial transfer of folate. These are the intestine, kidney, and liver. The absorptive cells of the intestine and kidney mediate the transcellular movement of folate from the lumen into blood in which folate enters the cells across the apical membrane and exits the cells across the basolateral membrane. In the liver, hepatocytes transfer folate from the blood into bile as a part of the enterohepatic circulation of folate. This involves the entry of folate into hepatocytes across the sinusoidal membrane (basolateral membrane) and exit of folate across the canalicular membrane (apical membrane). Whereas the FRα has been localized to the brush border of proximal kidney tubule cells (33), there is no information available on the differential polarized distribution of FRα and RFT-1 in these cell types.

Functional Characteristics of RFT-1 in Bovine RPE Apical Membrane Vesicles —The immunohistochemical data in RPE clearly show that RFT-1 is present in the apical membrane and FRα is present in the basolateral membrane. This provides us an opportunity to study the functional characteristics of RFT-1 using purified RPE apical membrane vesicles without the interference of the FRα. For this purpose, we used apical membrane vesicles prepared from bovine RPE. The purity of this preparation has been established by demonstrating the enrichment of the apical enzymes Na⁺–K⁺-ATPase, alkaline phosphatase, and 5’-nucleotidase, all three of which demonstrated an approximate 12-fold enrichment (25). Bovine RPE apical membrane vesicles prepared in this manner have been used in our laboratory to study the transport of taurine (25) and γ-aminobutyric acid (34). Since our immunohistochemical data (15) clearly demonstrated the presence of FRα in RPE, albeit in the basolateral surface, we wanted to be certain that the apical membrane vesicles prepared in this study were not contaminated with the receptor. To confirm the absence of the FRα in the bovine RPE apical membrane, a ligand binding assay was performed. In this assay, the equilibrium binding (90 min incubation at 4 °C) of [3H]folate (10 nM) to the membranes was measured in the absence and presence of excess (10 μM) unlabeled folate to calculate specific binding. The human placental brush border membranes, which contain FRα (30), were used as positive control. The placental brush border membranes were prepared as described previously from our laboratory (35).

FIG. 3. Laser-scanning confocal microscopic immunolocalization of RFT-1 and FRα in cultured human ARPE-19 cells grown 4 weeks on laminin-coated chamber slides. A, C, and E are optical sections taken in a vertical plane (x and y). A and B show cells incubated with an antibody against RFT-1. C and D show cells incubated with antibody against FRα. E and F show cells incubated with antibody against Na⁺–K⁺-ATPase. Double-headed arrows labeled a and b denote apical and basolateral regions of the membrane viewed in a vertical dimension.

FIG. 4. Laser-scanning confocal microscopic immunolocalization of RFT-1 and FRα in cultured human placental BeWo cells grown 3 days on chamber slides in the presence of 100 μM forskolin. A and C are optical sections taken in a vertical plane (x and y), and Z and D are taken in a horizontal plane (x and y). A and B show cells incubated with an antibody against RFT-1. C and D show cells incubated with antibody against FRα. Double-headed arrows labeled a and b denote apical and basolateral regions of the membrane of the cells viewed in a vertical dimension.
Membranes were washed with an acidic buffer, pH 3.5, to release any endogenous folate bound to the receptor prior to its use in the ligand binding assay. As shown in Fig. 5, the placental brush border membranes possessed high levels of [3H]folate binding activity. The binding of [3H]folate, which is a measure of FR density, was 3.07 ± 0.14 fmol/µg of protein at 10 nM [3H]folate. More than 90% of this binding was specific and inhibitable by unlabeled folate. In contrast, the bovine RPE apical membranes possessed negligible [3H]folate binding that was inhibitable by unlabeled folate.

After establishing the purity of the bovine RPE apical membrane vesicles and also the absence of the FRa in the membrane preparations, we investigated the ion dependence of the transport process mediated by RFT-1 present in these membrane vesicles. The transport function was monitored by measuring the uptake of [3H]MTF. The membrane vesicles were preloaded with 20 mM Hepes/Tris buffer, pH 8.0, containing 300 mM mannitol. The uptake medium, pH 8.0, contained 150 mM NaCl, 150 mM Na gluconate, or 300 mM mannitol. These buffers were chosen to determine the possible role of a Na+ gradient and a Cl− gradient in the transport process independently. Since the intravesicular pH was the same as the extravesicular pH, there was no H+ gradient across the membrane when the influence of Na+ and Cl− was studied. As shown in Fig. 6, the transport of MTF is energized by a transmembrane pH gradient (intravesicular pH = 8.0 and extravesicular pH = 5.0) and absence (○) of a pH gradient (intravesicular pH = 8.0 and extravesicular pH = 8.0). Samples were taken for estimation of uptake at the indicated times. Results are means ± S.E. of 2–4 determinations from at least two vesicle preparations.

In these experiments, a 30-s incubation was used to measure the initial uptake rates. The extravesicular pH was altered in the presence of a transmembrane pH gradient across the membrane when the influence of Na+ and Cl− was studied. As shown in Fig. 7A, the transport of MTF was measured in the presence (●) of a transmembrane pH gradient (intravesicular pH = 8.0 and extravesicular pH = 5.0) and absence (○) of a pH gradient (intravesicular pH = 8.0 and extravesicular pH = 8.0). Samples were taken for estimation of uptake at the indicated times. Results are means ± S.E. of 2–4 determinations from at least two vesicle preparations.

In these experiments, a 30-s incubation was used to measure the initial uptake rates. The extravesicular pH was altered in the presence of a transmembrane pH gradient across the membrane when the influence of Na+ and Cl− was studied. As shown in Fig. 7A, the transport of MTF was measured in the presence (●) of a transmembrane pH gradient (intravesicular pH = 8.0 and extravesicular pH = 5.0) and absence (○) of a pH gradient (intravesicular pH = 8.0 and extravesicular pH = 8.0). Samples were taken for estimation of uptake at the indicated times. Results are means ± S.E. of 2–4 determinations from at least two vesicle preparations.
expected to reach equilibrium and be equal under both experimental conditions. But this was not the case. However, we were able to demonstrate the typical overshoot phenomenon for glutamate uptake in the presence of an inwardly directed Na\(^+\) gradient and outwardly directed K\(^+\) gradient in these membrane vesicles (data not shown). Therefore, it appears that MTF is transported into the vesicles actively in response to the initial transmembrane H\(^+\) gradient, but the transported MTF does not dissociate completely from RFT-1 inside the vesicles. A partial dissociation from RFT-1 does seem to occur as evident from the data that the H\(^+\) gradient-induced stimulation of MTF accumulation inside the vesicles was much higher at the initial periods of incubation than at equilibrium. Any possible role of FR\(_a\) in MTF binding in these membrane vesicles is ruled out because an acidic pH (i.e. pH 5.0) is expected to abolish, not stimulate, the binding of MTF to FR\(_a\).

To be certain that the transporter we were analyzing in the bovine apical membrane vesicles was indeed RFT-1, we analyzed the substrate specificity of the transport process. As shown in Fig. 8, the transport of \([^{3}\text{H}]\text{MTF}\) was dramatically reduced in the presence of unlabeled MTF, folate, and methotrexate, known substrates for RFT-1 (1). Other vitamins such as ascorbate, thiamine, niacinamide, and pantothenate did not inhibit \([^{3}\text{H}]\text{MTF}\) transport. These data provide evidence of the subtype specificity of this transporter.

As RFT-1 is known to transport folate, although at a lower affinity compared with MTF, we used the bovine apical membrane vesicles to assess the transport of \([^{3}\text{H}]\text{folate}\) by the transporter. The characteristics of folate transport in these membrane vesicles were similar to those of MTF transport. Folate transport was Na\(^+\)- and Cl\(^-\)-independent (data not shown) and was stimulated by an inwardly directed H\(^+\) gradient (Fig. 9, A and B). Again, there was no overshoot in the time course of folate transport.

The substrate specificity of this system was tested using \([^{3}\text{H}]\text{folate}\) as a substrate (Fig. 10). As with \([^{3}\text{H}]\text{MTF}\) transport, the addition of unlabeled folate analogs (MTF, folate, and methotrexate) inhibited the transport of \([^{3}\text{H}]\text{folate}\). Transport was not affected when ascorbate, thiamine, niacinamide, or pantothenate was added, demonstrating that RFT-1 recognizes only folate and its analogs as substrates.

**DISCUSSION**

To date, FR\(_a\) and RFT-1 are the only proteins that have been shown to be involved in the cellular uptake of folate. However, FR\(_a\) is expressed widely in mammalian tissues, whereas the expression of RFT-1 is very restricted. Thus far, RFT-1 mRNA
has been detected only in the intestine, kidney, liver, brain, and placenta (1). Interestingly, the intestine, kidney, liver, and placenta are known to be involved in the vectorial transfer of folate. We suspect that even in the brain, RFT-1 expression may be restricted to the endothelial cells of the blood-brain barrier and in the epithelial cells of the choroid plexus, the two regions of the brain that are capable of vectorial transfer of various solutes. Therefore, we hypothesize that RFT-1 expression is restricted strictly to those tissues involved in vectorial transfer of folate. The present studies identify RPE as a new member of this selective group of tissues expressing RFT-1. The primary function of RPE is to mediate the vectorial transfer of nutrients including folate from the choroidal circulation to the subretinal space to nourish the neural retina.

The cells involved in vectorial transfer of solutes are all polarized with two distinct domains of the plasma membrane facing the two sides of the cell between which the vectorial transfer occurs. This is true with the absorptive cells of the intestine, kidney, and placenta, hepatocytes in the liver, epithelial cells of the choroid plexus, endothelial cells of the blood-brain barrier, and the RPE. The expression of RFT-1 has been established unequivocally at least in the intestine, kidney, liver, placenta, and liver. With respect to the second protein involved in folate uptake, namely the FRα, its expression has been demonstrated beyond doubt only in the placenta, kidney, and RPE. Whether or not FRα is expressed specifically in the absorptive cells of the intestine and in hepatocytes of the liver is not known. Therefore, the mechanism of vectorial transfer of folate in these three tissues and the exact role of RFT-1 in the process remain uncertain. However, there is evidence to suggest the presence of RFT-1 in the brush border membrane of the intestinal absorptive cells (36–38) and in the sinusoidal membrane of the hepatocytes (39, 40). Interestingly, these two membranes represent the entry point in the vectorial transfer of folate in the intestine and liver. The identity of the protein responsible for the exit of folate across the basolateral membrane of the intestinal absorptive cells and the canaliculig membrane of the hepatocytes has not yet been established. However, it is very unlikely that FRα participates in the exit mechanism because this protein, known to be located entirely on the external surface of the plasma membrane attached via a glycosylphosphatidylinositol lipid anchor, is suitable only for the entry of folate into the cell. In fact, whether or not the normal intestinal absorptive cells and the hepatocytes express FRα has not been investigated. It is possible that these two cell types do not express FRα and that RFT-1 itself mediates the entry as well as the exit of folate at the two poles of the plasma membrane in these cells. Since RFT-1 is an integral membrane protein, it is capable of mediating folate transfer in either direction. The direction of folate transfer via RFT-1 is determined by the direction of transmembrane gradients for H⁺ and folate.

The situation is interestingly very different in the case of the syncytiotrophoblast, the absorptive cell of the placenta, and the RPE in the retina. These two cell types express both FRα and RFT-1. The co-expression of these proteins in the RPE has been demonstrated for the first time in the present study, whereas the co-expression in the placental syncytiotrophoblast has been known for some time from earlier studies. Then the question arises as to the differential role of these two proteins in the vectorial transfer of folate across these cell layers. The present investigation was undertaken to address this question primarily with respect to the RPE. These studies establish for the first time the differential location of FRα and RFT-1 in the RPE in normal eye tissue as well as in an in vitro cell culture model system. RPE is a polarized cell with its basolateral membrane apposing the choroidal circulation and its apical membrane lining the subretinal space. We have demonstrated in the present study that the expression of RFT-1 is restricted to the apical membrane, whereas the expression of FRα is restricted to the basolateral membrane. Since FRα is capable of mediating not only the cellular uptake of folate, its presence in the basolateral membrane is ideally suited to participate in the entry of folate from the choroidal blood into the RPE. RFT-1, being a transmembrane protein in contrast to FRα, is capable of mediating folate transfer in both directions across the membrane. The location of RFT-1 in the RPE apical membrane suggests that this protein participates in the exit of folate from the RPE into the subretinal space. Thus, FRα in the basolateral membrane and RFT-1 in the apical membrane function in a coordinated manner to carry out the vectorial transfer of folate across the RPE cell layer.

Since the RPE apical membranes that contain RFT-1 can be isolated with relative ease in the form of vesicles, this has provided us an opportunity to study the functional properties of this transport protein. These membrane preparations do not contain FRα. Thus, the analysis and interpretation of folate transport in these membrane preparations are straightforward because of the exclusive mediation of the transport process by RFT-1. The present studies show that RFT-1 functions in a Na⁺- and Cl⁻-independent manner. A transmembrane H⁺-gradient influences the transport function of this protein markedly. Since the transport function of RFT-1 is stimulated by a transmembrane pH gradient in which the extravesicular pH is lower than the intravesicular pH, this suggests that the transport mechanism is likely to be either folate/H⁺ co-transport or folate/OH⁻ exchange. These findings are similar to those observed with intestinal border membrane vesicles (36–38) and hepatocyte sinusoidal (basolateral) membrane vesicles (39, 40). Such a mechanism is likely to render the transport process electroneutral. The membrane potential is therefore not expected to influence the transport process.

This represents the first report of the differential localization of FRα and RFT-1 in any polarized cell that is capable of vectorial transfer of folate. This study also offers for the first time a mechanism for the coordinated function of the two proteins at the two poles of the RPE cell plasma membrane to carry out the transfer of folate from the choroidal blood into the neural retina. The physiological relevance of the co-expression of FRα and RFT-1 in the RPE becomes readily apparent from the results of the present studies.

In addition, the present studies also provide information relevant to the transplacental transfer of folate. Although it is known that the syncytiotrophoblast expresses FRα and RFT-1, the membrane localization has been established only for FRα (30). The receptor protein is present in the brush border (apical) membrane. The location of RFT-1 in the syncytiotrophoblast has not been identified. The placental brush border membrane faces the maternal circulation and thus represents the entry point for folate for the transplacental transfer of this vitamin from the mother to the fetus. Therefore, FRα located in this membrane is ideally suited to mediate the uptake of folate from the maternal blood into the syncytiotrophoblast. Since this cell expresses RFT-1, we hypothesize that the exit of folate across the basal membrane that faces the fetal circulation is mediated by RFT-1. This hypothesis is supported by the findings of the present investigation with BeWo cells, a model for the placental syncytiotrophoblast, which demonstrate the location of FRα in the brush border membrane and RFT-1 in the basolateral membrane. Thus, the present studies provide the first glimpse of the mechanism of vectorial transfer of folate across the placental syncytiotrophoblast. According to this
mechanism, FRα mediates the entry of folate from maternal blood into the syncytiotrophoblast across the brush border membrane of the cell and RFT-1 mediates the exit of folate from the syncytiotrophoblast into the fetal circulation across the basal membrane of the cell. It must be emphasized that additional work is needed to establish the validity of this hypothetical mechanism. The present study has demonstrated the polarized distribution of FRα and RFT-1 only using the BeWo cells, an in vitro cell culture model system. Similar studies need to be carried out with normal placenta to determine whether the polarized distribution of the two proteins observed in BeWo cells is also true in the normal placental syncytiotrophoblast.

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