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Sorting of the Human Folate Receptor in MDCK Cells

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The human folate receptor (hFR) is a glycosylphosphatidylinositol (GPI) linked plasma membrane protein that mediates delivery of folates into cells. We studied the sorting of the hFR using transfection of the hFR cDNA into MDCK cells. MDCK cells are polarized epithelial cells that preferentially sort GPI-linked proteins to their apical membrane. Unlike other GPI-tailed proteins, we found that in MDCK cells, hFR is functional on both the apical and basolateral surfaces. We verified that the same hFR cDNA that transfected into CHO cells produces the hFR protein that is GPI-linked. We also measured the hFR expression on the plasma membrane of type III paroxysmal nocturnal hemoglobinuria (PNH) human erythrocytes. PNH is a disease that is characterized by the inability of cells to express membrane proteins requiring a GPI anchor. Despite this defect, and different from other GPI-tailed proteins, we found similar levels of hFR in normal and type III PNH human erythrocytes. The results suggest the hypothesis that there may be multiple mechanisms for targeting hFR to the plasma membrane.

Keywords: CHO, Folate receptor, MDCK, PNH, Sorting

Introduction

The human folate receptor (hFR) is involved in the cellular uptake of physiologic concentrations of folate and thus is essential for the cell's survival (Figini *et al*., 2003; Ratnam *et al*., 2003). The hFR cDNA sequence predicts a protein core of 28 kDa with several N-linked oligosaccharide chains, a twenty-five residue leader peptide, and a hydrophobic, α helical 31 residue carboxyl terminus (Elwood, 1989; Lacey *et al*., 1989; Sadasivan and Rothenberg, 1989). Potentially, the hydrophobic carboxyl terminus could serve as either a short transmembrane anchor or as the signal for cleavage and co-translational addition of a glycosylphosphatidylinositol (GPI) tail in the endoplasmic reticulum (Kim *et al*, 2002). Several lines of evidence support the attachment of a GPI-tail on the hFR (Chung *et al*., 1995). The hFR that is isolated from the KB cell line (human nasopharyngeal epidermoid carcinoma cells) contains fatty acids and inositol (Luhrs *et al*., 1987). The hFR expressed on the membranes of MA104 monkey kidney cells is released from the plasma membrane by phosphatidylinositol phospholipase C (PI-PLC) treatment (Lacey *et al*., 1989; Sinn *et al*., 2003). We transfected the hFR cDNA into MDCK cells, which are polarized epithelial cells that sort GPI-linked proteins to the apical membrane (Lisanti *et al*., 1988; Brown *et al*., 1992). We expected to find hFR sorted to the apical surface but in transfected MDCK cells; we found that hFR is functional on both the apical and basolateral surfaces of MDCK cells. This suggests that hFR does not behave as a typical GPI-tail protein. However, the same cDNA that was transfected into CHO cells produced the hFR protein that is released with PI-PLC. In addition, we measured the hFR expression on type III erythrocytes from patients with paroxysmal nocturnal hemoglobinuria (PNH). PNH is a disease that is characterized by the inability of red blood cells to express proteins that require GPI tails on plasma membranes (Mahoney *et al*., 1992; Hwa, 2001; Krauss, 2003). Unexpectedly, we found that type III PNH erythrocytes express equivalent (or greater) levels of hFR on their plasma membranes when compared to normal erythrocytes. These results show that the hFR may be sorted to the plasma membrane by different mechanisms in CHO, MDCK and PNH cells.

Materials and Methods

Materials The [^{125}I]-labeled pteroylglutamic acid [folic acid (histamine derivative of folic acid) was purchased form New England Nuclear (Boston, USA). The [^{3}H]5-MTHF (DL-N-5-

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methyltetrahydrofolate, specific activity, 50 Ci/mmol) was purchased from Moravek Biochemicals (Brea, USA). Radiopurity of these ligands was > 98% based on a HPLC analysis. Agarose was from Bethesda Research Laboratories (Gaithersburg, USA). All of the restriction enzymes and DNA modifying enzymes were purchased from Promega (Madison, USA). All other reagents were of reagent grade or higher and were purchased from the Sigma Chemical Co. (St. Louis, USA), or Fisher Scientific (Pittsburgh, USA).

Plasmid construction The pRc/CMV vector (Invitrogen, San Diego, USA) contains a cytomegalovirus (CMV) promoter and a neomycin resistance gene. The cDNA encoding hFR was isolated from a placental cDNA library and subcloned into the *EcoRI* multiple cloning site of pGEM4Z (Promega, Madison, USA). The insert-purified hFR cDNA (containing *EcoRI* ends) was blunt-ended using a Klenow large fragment (Kang *et al*., 2001; Kim *et al*., 2001). The *Hind* III-linearized pRc/CMV expression vector was blunt-ended, phosphatased with calf intestine alkaline phosphatase, and the vector was ligated with a blunt-ended cDNA insert using T4 DNA ligase. Competent *E. coli* (JM109) was transformed with the ligation mixture. Recombinant plasmids were isolated by the Qiagen maxiprep kit (Chatsworth, USA). The sense orientation of the folate receptor cDNA in the pRc/CMV vector was verified by restriction enzyme digests and subsequently by DNA sequencing. We designated the plasmid construct containing the pRc/CMV vector and the human KB cell folate receptor cDNA as pRc/FR.

Tissue culture MDCK cells (Madin-Darby canine kidney epithelial cell) and CHO cells (Chinese hamster ovary cell) were cultured in minimal essential media (9/10 DMEM) with a final folic acid concentration of approximately 200 nM (Chung *et al*., 1993). Cells were grown in monolayers in 10 cm tissue culture dishes (Oxnard, USA) containing 10 ml media at 37°C under a humidified atmosphere of 5% CO_2, and were subcultured weekly. To harvest the cells, the medium was decanted, and 1-2 ml of 2.5% trypsin versene solution was added to the dishes. The cells were incubated at room temperature for 2 min, the excess solution was decanted, and the cells were immediately resuspended in media. Standard replete minimal essential media (RMEM) containing 2 μM folic acid with Earle's salts and L-glutamine, deplete minimal essential media without added folic acid (DMEM) with Earle's salts and L-glutamine, trypsin versene solution (2.5%), penicillin/streptomycin/fungizone (PSF) solution (100X), fetal calf serum (FCS) and geneticin sulfate (antibiotic G418) were purchased through Gibco Laboratories (Grand Island, USA).

Calcium phosphate transfection For stable transfection of the MDCK and CHO cells (10 μg of pRc/FR), we used the calcium phosphate method, as suggested by the protocol supplied in the Stratagene Eukaryotic Transfection kit. Stable transfectants were selected in 500 μg/ml G418 and were maintained in 250 μg/ml G418 (Chen and Okayama, 1987; So *et al*., 2001; Lu *et al*., 2002). The stable transfectants MDCK and CHO cells were designated as MDCK FR and CHO FR.

MDCK cells grown on transwells Wild-type and transfected MDCK cells were seeded at high density in transwells and used for experiments within a week of plating (Lisanti *et al*., 1988). Tight-junction formation and cell polarity were assessed by testing monolayers for diffusion of [^{3}H]inulin (NEN, Chadds Ford, USA) from apical to the basolateral compartment (Caplan *et al*., 1986). Filter-grown MDCK cell monolayers showing < 1% permeability were used in the studies.

Western blot Stable transfectants of MDCK cells and CHO cells (2×10^6 cells/35 mm well) were plated overnight. Cells were washed twice with PBS and scraped into 1 ml PBS and 20 mM EDTA, pH 7.4. The cells were pelleted in a microcentrifuge and solubilized with PBS and 1% TX-100 for 2 h at room temperature. The samples were assayed for protein, and equal amounts of protein (25 μg) were acetone precipitated. Acetone precipitations were electrophoresed on a 12.5% SDS-PAGE, and transferred to nitrocellulose filter paper. Western blots for hFR were incubated with a 1 : 1000 dilution of rabbit antiserum to hFR, reacted with a 1 : 1000 dilution of HRP goat anti-rabbit IgG, and analyzed by the ECL system, as previously described (Lee *et al*., 2001; Ju *et al*., 2001; Kim *et al*., 2002; Nakagawa *et al*., 2002).

Immunoprecipitation of hFR Stable transfectants were plated in 35 mm wells (Falcon) at 37°C, 5% CO_2. Plating densities were adjusted so that the cells were 75% confluent when we carried out the immunoprecipitation experiments. Cells were washed once with 2 ml media containing no methionine or cysteine, and incubated in 2 ml of the same media or 1 h at 37°C, 5% CO_2. Newly-synthesized proteins were radio-labeled by pulsing the cells in 1 ml of the same media containing 100 μCi ^{35}S-methionine and 100 μCi ^{35}S-cysteine for 16 h at 37°C, 5% CO_2. At the end of the radiolabeling period, the cells were harvested and hFR immunoprecipitated (Chung *et al*., 1993).

Cells surface folic acid binding assay Cell surface binding assays using [^{125}I]-labeled pteroylglutamic acid were carried out as described (Chung *et al*., 1993). MDCK cells were incubated in 35 mm polycarbonate transwells in 9/10 DMEM containing 10% FCS, PSF, and 350 μg/ml G418. After dissociating surface-bound folates from cell surface folate receptors with 10 mM Na-acetate, pH 4.5, containing 150 mM NaCl (acid saline), the cells were rinsed with cold PBS, and incubated with 100,000 cpm [^{125}I]-labeled pteroylglutamic acid on the apical or basolateral compartment of the filter chamber for 10 min at 4°C. The filters were rinsed with cold PBS, excised and counted in a gamma counter at approximately 70% efficiency. During the time course of the experiment, no detectable leakage of [^{125}I]-labeled pteroylglutamic acid to the opposite side was observed. This confirmed that the cells were in a monolayer with tight junctions. The data reflect specific binding of radioligand, since in the presence of 500-fold excess of non-radioactive folic acid, the amount of cell surface binding was less than 1% of that seen in the controls.

PI-PLC (phosphatidylinositol-specific phospholipase C) releasability studies MDCK cells were incubated in 35 mm polycarbonate transwells in 9/10 DMEM containing 10% FCS, PSF, and 250 μg/ml G418. CHO cells were grown in plastic 35 mm tissue culture wells. Cell monolayers were rinsed once with 3 ml ice cold acid saline to remove surface bound folates, and neutralized by

rinsing once with 3 ml ice cold PBS. The cells were incubated in media with or without PI-PLC (100 mU/ml/well) for 60 min at 37°C. To measure the cell surface hFR levels in PI-PLC treated and untreated cells, cell surface folic acid binding assays were carried out as previously described.

FITC-folic acid staining MDCK cells were incubated in 35 mm collagen coated transwells in 9/10 DMEM containing 10% FCS, PSF, and 250 μg/ml G418 for one week. After dissociating surface-bound folates from cell surface folate receptors with acid saline, the cells were rinsed with cold PBS, and incubated with 100 nM FITC-folic acid (FITC-FA) on the apical or basolateral compartment of the filter chamber 10 min at 4°C. The filters were rinsed with cold PBS, mounted on glass slides, and covered with a drop of molecular probes and a glass coverslip.

[^{3}H]5-MTHF internalization assay MDCK cells were grown in 35 mm polycarbonate transwells for 1 week in deplete minimal essential media without added folic acid (DMEM) containing 10% (v/v) FCS, PSF, and 250 μg/ml G418, which resulted in a final folic acid concentration of approximately 1-10 nM. Next, [^{3}H]5-MTHF internalization assays using DL-N-5-methyltetrahydrofolate ([3',5',7,9-^{3}H]5-MTHF) were carried out as previously described (Elwood *et al*., 1986). The filter grown MDCK cell monolayers were washed with acid saline and PBS, and incubated with 500 nM [^{3}H]5-MTHF on the upper (apical) or the lower (basolateral) compartment of the filter chamber for 5 min at 4°C, then rinsed with cold PBS. The cells were then warmed to 37°C for various times. After washing three times with PBS, the filters were counted in a scintillation counter at approximately 50% efficiency. For the second set of experiments, the preincubation at 4°C was omitted. During the time course of the experiment, no detectable leakage of [^{3}H]5-MTHF to the opposite side was observed, confirming that the cells were in a monolayer with tight junctions. The data reflect the specific uptake of radioligand, since in the presence of 500-fold excess of nonradioactive folic acid, the amount of internalization was less than 1% of that seen in the controls.

PNH cells Paroxysmal nocturnal hemoglobinuria (PNH) type III erythrocytes were isolated from whole blood (Chow *et al*., 1986; Ross *et al*., 1991) and verified as type III PNH erythrocytes by lysis with cobra venom. The total red cell folate concentration was assayed from the packed cell pellet (Waxman and Schreiber, 1980; Kane *et al*., 1986). The erythrocyte membrane ghosts were collected from the packed cell pellet. The erythrocyte membrane pellet was brought up in 1 ml of the lysis buffer (5 mM sodium phosphate, pH 7.5, with 2 mM EDTA). The sample was assayed for [^{125}I] labeled folic acid binding in the absence and presence of a 1,000 fold molar excess of folic acid. Following determination of specific binding, the samples were placed in 1% Triton X-100 for 24 h at 4°C prior to analysis for protein (Kim *et al*., 2002). To determine the relative amount of specific and nonspecific binding, 50 fmol (0.1 μCi) of [^{125}I] labeled folic acid were added to an aliquot of membrane preparations. The sample was vigorously agitated at 4°C for 90 min. Following this incubation, 2 ml of lysis buffer was added and the sample was vortexed again. The sample was then centrifuged at $30{,}000 \times g$ for 20 min at 4°C. The supernatant was removed and the sample was again resuspended in 3 ml of cold lysis buffer and centrifuged again. After a total of three such washes, with counting of the supernatant after each wash, the counts were within two times the background of the gamma counter. The particulate pellet was then counted in a Gamma counter (Beckman Instruments, Richmond, USA) at 80% efficiency. Specific binding in all cases constituted > 90% of the binding observed. Following the analysis by the particulate binding assay, the samples were made 1% (v/v) in Triton X-100 and used for determination of the protein. In all cases, the binding was corrected for mg of the protein. Another aliquot from the identical membrane preparation was utilized to determine the endogenous folate that was present (Waxman and Schreiber, 1980) in the samples. Finally, to verify the expression of the folate receptor on the membrane of normal erythrocyte and type III PNH erythrocyte, the concentrations of the membrane protein were determined and equal amounts of protein (100 μg) were acetone precipitated. Acetone precipitations were electrophoresed on 8% SDS-PAGE, and transferred to nitrocellulose filter paper. Western blots for hFR were incubated with a 1 : 1000 dilution of rabbit antiserum to hFR, reacted with a 1 : 1000 dilution of HRP goat anti-rabbit IgG, and analyzed by the ECL system (Kim *et al*., 2002).

Results

Stable transfection of MDCK cells We transfected the hFR cDNA into a well-characterized subline of MDCK cells that sorts GPI proteins to the apical surface. Figure 1 shows Western blot (A) and immunoprecipitation (B) of hFR protein from the highest expressing stable transfectant (clone FR3) compared to wild-type MDCK cells, which contain no detectable levels of FR. Following transfection, we predicted that hFR would be expressed in the same way on the apical surface as other GPI-linked proteins. This was not the case. We found an atypical distribution, where 50-58% of the hFR appear on the apical membrane and 42-50% on the basolateral side of four independent transfectants that were analyzed by cell surface [^{125}I]labeled pteroylglutamic acid binding assays (Table 1). The second observation that we made is that less than 30% of the transfected hFR is released with PI-PLC from either the apical or the basolateral sides in all four independent transfectants (Table 1). To compare the amount and PI-PLC releasability of hFR that is expressed in MDCK FR to that of CHO cells, CHO cells that were transfected with the same pRc/FR were analyzed by Western blot and digested with PI-PLC. We observed that the stably transfected CHO cells produced high levels of hFR protein that is released (> 95%) by PI-PLC (Fig. 2A, Table 1), as previously described (Chung *et al*., 1995). We confirmed our results of the cell surface [^{125}I]labeled pteroylglutamic acid binding assays by cell surface FITC-folic acid (FITC-FA) binding assays. FITC-FA staining showed a positive signal on both the apical and the basolateral surfaces of the transfected MDCK cells (clone FR3, Fig. 3A, B). The FITC-FA staining was competed with cold folic acid (Fig. 3C, D), and FITC-FA staining was seen in the control MDCK cells (Fig. 3E, F). We also measured the specific internalization of the circulating form of folate (5-

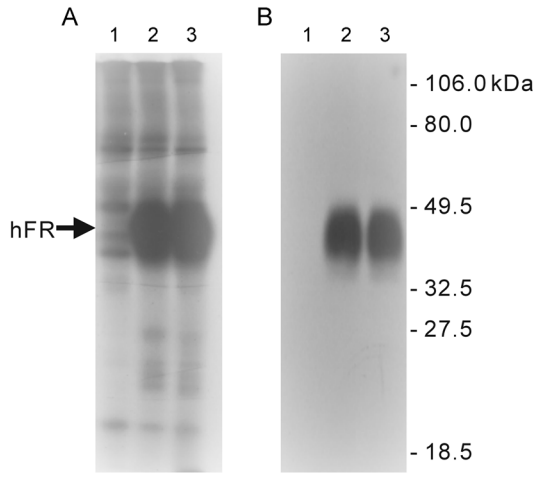

Fig. 1. Western blot and immunoprecipitation of hFR protein. Western blots (A) for hFR solubilized from wild-MDCK cells (lane 1), stably transfected clone MDCK FR3 cells (lane 2), and KB cells (lane 3) were incubated with a 1 : 1000 dilution of rabbit antiserum to hFR, reacted with a 1 : 1000 dilution of HRP goat anti-rabbit IgG, and analyzed by the ECL system (Materials and Methods). For immunoprecipitation (B), wild MDCK cells (lane 1) and stably transfected MDCK FR3 cells (lane 2) and KB cells (lane 3) were incubated in 35 mm plates in 9/10 DMEM containing 10% FCS, PSF, and 250 μg/ml G418. Newly synthesized proteins were radiolabeled with ^{35}S-methionine and ^{35}S-systeine, and immunoprecipitated using rabbit ant-hFR antiserum (Materials and Methods).

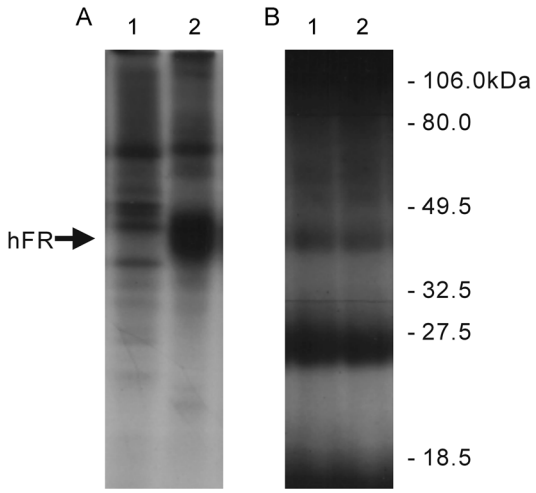

Fig. 2. Western blot of hFR protein in CHO and erythrocyte. Western blots for hFR solubilized from wild-CHO cells (lane A1), stably transfected clone CHO FR cells (lane A2), type III PNH erythrocytes (lane B1) and normal erythrocytes (lane B2) were incubated with a 1 : 1000 dilution of rabbit antiserum to hFR, reacted with a 1 : 1000 dilution of HRP goat anti-rabbit IgG, and analyzed by the ECL system (Materials and Methods).

mehyltetrahydrofolate, 5-MTHF). Stable transfectants showed specific [^{3}H]5-MTHF uptake from both the apical and basolateral surfaces (Fig. 4). Figure 4A shows the results of an experiment where the cells were first incubated with ligand for 5 min on ice, excess ligand rinsed with PBS, and then warmed to 37°C for the various times that are indicated. In such an experiment, more ligand is initially bound and then internalized by the apical surface (Fig. 4A). In contrast, Figure 4B shows the results of an experiment where the cells were continually incubated with ligand at 37°C for various times. In this case, a little difference of ligand levels is internalized from

Table 1. Cell surface binding of [^{125}I]folic acid in MDCK stable clones

Cells	Surface	^{125}I-folic acid	+cold	% PI-PLC
		Bound (cpm)	FA (cpm)	released
MDCK FR3	A	52081	<100	17%
MDCK FR5	A	48716	<100	15%
MDCK FR8	A	49680	<100	18%
MDCK FR16	A	37892	<100	115
MDCK FR3	B	38453	<100	18%
MDCK FR5	B	38453	<100	24%
MDCK FR8	B	35254	<100	30%
MDCK FR16	B	37249	<100	29%
MDCK PLAP	A			95%
CHO FR	A			95%

A, Apical surface; B, Basolateral surface; MDCK PLAP, MDCK cells stably transfected with human placental alkaline phosphatase; CHO FR, CHO cells stable transfected with hFR cDNA.

MDCK cells and CHO FR cells (10^6 cells) stably transfected with hFR were plated in 35 mm plates. After dissociating surface-bound folates from cell surface folate receptors with acid saline (described in "Materials and Methods"), the cells were incubated with radio-labeled folic acid for 10 min at 10°C with or without excess cold ligand. Aliquots of solubilized cells were counted and the results expressed as cpm per 10^6 cells per 30 min. Cell surface folate binding released with PI-PLC was determined as described in "Materials and Methods".

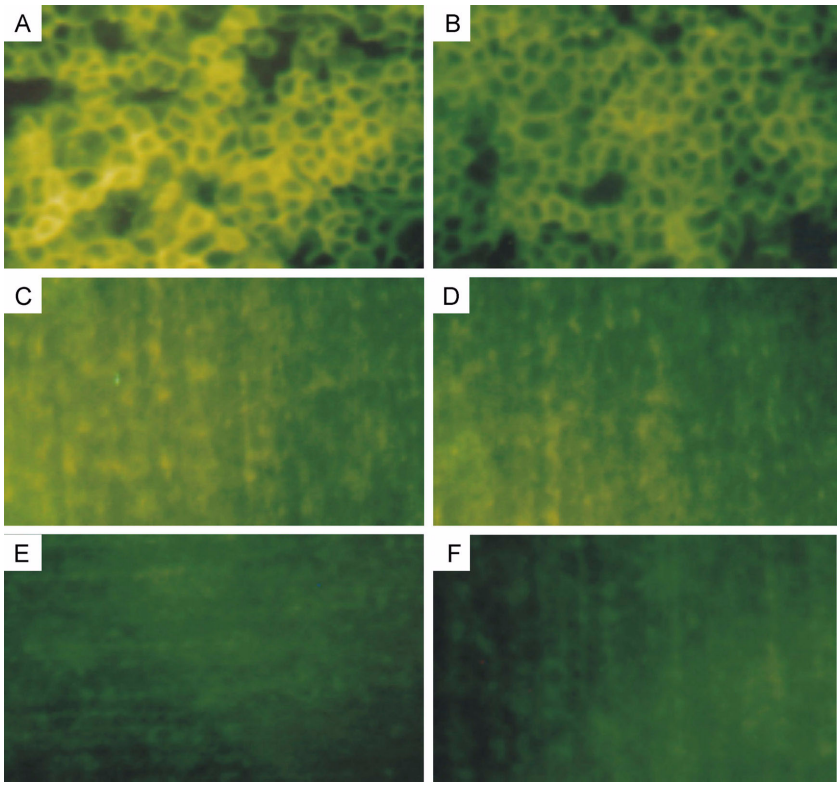

Fig. 3. Determination of cell surface FITC-folic acid binding. MDCK stable clone FR3 was incubated in 35 mm collagen-coated transwells in DMEM containing 10% FCS, PSF, and 250 μg/ml G418. After dissociating surface-bound folates from cell surface folate receptors with acid saline (Materials and Methods). The cells were incubated on the apical or basolateral sides with FITC-folic acid for 10 minutes at 4°C with or without excess cold ligand. (A) Apical surface of clone FR3 incubated without excess cold ligand. (B) Basolateral surface of clone FR3 incubated without excess cold ligand. (C) Apical surface of clone FR3 incubated with excess cold ligand. (D) Basolateral surface of clone FR3 incubated with excess cold ligand. (E) Apical surface of control MDCK. (F) Basolateral surface of control MDCK.

both the apical and basolateral surfaces.

PNH Cells We also examined a human model to address the question of membrane attachment. Paroxysmal nocturnal hemoglobinuria (PNH) is a disease involving the hematopoietic stem cell. The progeny of PNH stem cells are unable to express GPI-linked proteins on their plasma membranes. Recently, it was discovered that the defect in PNH is caused by a somatic mutation of the phosphatidylinositolglycan class A (PIG-A) gene that is involved in the synthesis of N-acetylglucosaminyl phosphatidyl inositol, the first intermediate in the pathway for GPI synthesis (Takeda *et al*., 1993). To verify the expression of hFR in normal erythrocytes and PNH erythrocytes, we carried out a Western blot analysis and [^{125}I]-folic acid binding assay. We compared the hFR expression on the plasma membrane of normal erythrocytes to that expressed on the plasma membrane of type III PNH erythrocytes. As can be seen from Fig. 2B, a similar level of hFRs were expressed on the membrane of normal erythrocytes and PNH erythrocytes. Table 2 shows that normal erythrocyte (the control group) contain approximately 0.5 fmol of folate/mg of erythrocyte membrane protein, which agrees with previous studies of normal human erythrocyte membranes (Antony *et al*., 1987). Type III PNH erythrocytes from three different patients contained values for specific folate binding of 1.1 to 1.5 fmol/mg. Although the overall increase in binding of [^{125}I] labeled folic acid in type III PNH erythrocytes may be accounted for by lower levels of residual endogenous folate in the PNH cells when compared to normal erythrocyte preparations (Table 2), these results clearly demonstrate the surface expression of hFR by type III PNH erythrocytes, cells that are unable to express other GPI-linked proteins.

Discussion

We looked at the sorting of the hFR by stably transfecting hFR cDNA into MDCK cells. In contrast to other GPI-linked proteins that are exclusively sorted to the apical membrane in MDCK cells, our results suggest a different mechanism of membrane expression for the hFR. We observed a near equal distribution of hFR between the apical and basolateral membranes in transfected MDCK cells based on three

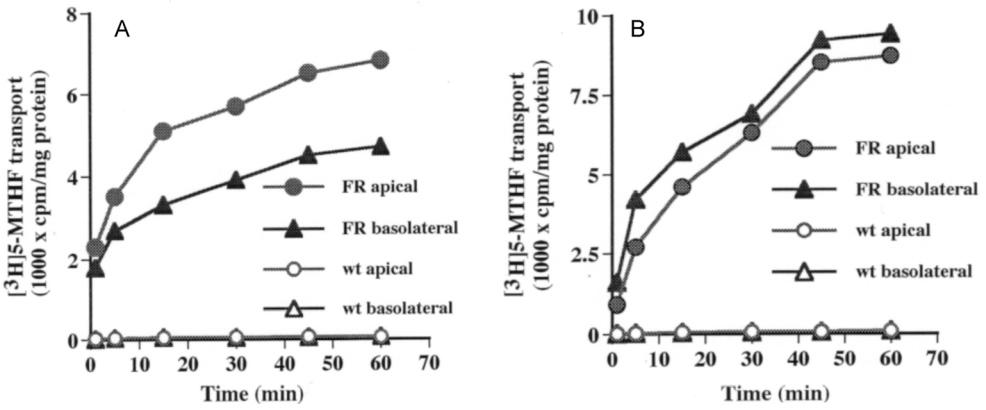

Fig. 4. 5-MTHF transport study. Figure 4A shows 5-MTHF transport in control MDCK cells and MDCK cells stably transfected with hFR (clone FR3). Cells were incubated in 35 mm polycarbonate transwells in DMEM containing 10% FCS, PSF, and 250 μg/ml G418 for one week. [^{3}H]5-MTHF internalization assays were carried out as described in "Materials and Methods" using DL-N-5-methyltertrahydrofolate ([3',5',7,9-^{3}H]5-MTHF). After dissociating surface-bound folates from cell surface folate receptors with acid saline, the cells were incubated with [^{3}H]5-MTHF for 5 min at 4°C and rinsed with cold PBS. The cells were then warmed to 37°C for the various times indicated. Fig. 4B shows 5-MTHF transport in control MDCK cells and MDCK cells stably transfected with hFR (clone FR3). Cells were incubated in 35 mm polycarbonate transwells in DMEM containing 10% FCS, PSF, and 250 μg/ml G418 for one week. After dissociating surface-bound folates from cell surface folate receptors with acid saline, the cells were continuously incubated with [^{3}H]5-MTHF at 37°C for the various times indicated.

different assays: cell surface [^{125}I]labeled pteroylglutamic acid binding, cell surface FITC-FA binding, and [^{3}H]5-MTHF transport studies. The [^{3}H]5-MTHF transport studies suggest that more hFR may be available to the ligand at the apical surface but that the recycling of the hFR may be faster at the basolateral side than at the apical side in stable transfectants of MDCK cells. When the cells were first incubated with ligand at 4°C and then warmed to 37°C, more ligand was initially bound and then internalized by the apical surface (Fig. 4A). However, when the cells were continually incubated with ligand at 37°C, little difference of the ligand levels was internalized from both the apical and basolateral surfaces (Fig. 4B). We found that less than 30% of the hFR was released with PI-PLC in stable transfectants of MDCK cells. However, we observed that the stable transfectant cells that transfected the same hFR cDNA into CHO cells produced the hFR protein (Fig. 2A) that is released (> 95%) by PI-PLC (Table 1) using the same PI-PLC preparation and under identical conditions. We also verified that the human alkaline phosphatase (a GPI-tailed protein) that is stably transfected into the same MDCK subline was released (> 95%) by PI-PLC. These controls show that the PI-PLC is active. We do not know if the lack of PI-PLC release in MDCK cells means that the GPI-tail on the hFR is chemically modified. Others have observed that the sensitivity of alkaline phosphatase to PI-PLC release is variable in different cell lines. This resistance has been attributed to the acylation of the inositol ring in the GPI anchor on alkaline phosphatase (Wong and Low, 1992). We presumed that hFR would not be expressed in PNH erythrocytes because hFR is a GPI tailed protein, but hFR is expressed in normal erythrocytes and PNH erythrocytes. The results from our studies (Table 2) with type III PNH erythrocytes indicate that in a naturally occurring human disease, the circulating erythrocytes from these individuals contain normal to slightly increased quantities of the hFR. These erythrocytes contain no other well characterized GPI anchored proteins (Luhrs and Slomiany, 1989; Sloand *et al.*, 2003). It appears that in the normal and PNH erythrocytes, the hFR protein is anchored in the erythrocyte membrane by mechanisms other than a GPI anchor. Thus, the properties of these cell lines and the results of the PI-PLC release of hFR support our hypothesis that a single hFR cDNA encodes for both a GPI-linked protein and a non-GPI linked protein. An interesting possible interpretation of the MDCK data is that the hFR on the basolateral surface is protein that is on its way to the apical surface by transcytosis. In hepatocytes, it has been shown that proteins, including GPI-tailed proteins that are destined for the apical surface, are

Table 2. Folate binding in normal and type III PNH erythrocytes

Cells	Folate binding	Endogenous folate
	(fmol bound/mg)	(pmol/ml)
Normal erythrocyte	0.470.09	884
PNH 1	1.10.07	349
PNH 2	1.50.08	247
PNH 3	0.90.10	374

The determinations of membrane folic acid binding studies using [^{125}I]-folic acid, and the endogenous concentration of folate in normal erythrocytes and 3 different type III PNH erythrocytes (described in Materials and Methods). The values were corrected for packed cell volume to estimated folate per ml of packed erythrocytes.

first routed to the basolateral surface and subsequently transcytosed to the apical surface (Schell *et al*., 1992). The targeting of the hFR to the apical surface may involve sorting first to the basolateral surfaces, followed by a slow redistribution to the apical surface by transcytosis.

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