Transfection of a Glycosylated Phosphatidylinositol-anchored Folate-binding Protein Complementary DNA Provides Cells with the Ability to Survive in Low Folate Medium

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Abstract

KB cells express a folate-binding protein that is anchored to the plasma membrane by a glycosylated phosphatidylinositol (GPI) tail and these cells can grow in medium containing a very low folate concentration (1 nM). In contrast, mouse 3T3 cells do not express a membrane-associated folate-binding protein and cannot grow under similar low folate conditions. In these studies, 3T3 cells were transfected with a vector containing the cDNA that codes for the KB cell folate-binding protein. In contrast to the wild-type 3T3 cells, the transfected 3T3 cells express a level of folate-binding protein similar to KB cells, 1 and 1.4 ng/μg protein, respectively. The capacity for binding [^{3}H] folate to the surface of transfected 3T3 cells cultured in folate-deficient medium is 7.7 pmol/10^6 cells, and this is ~ 50% of the surface binding capacity of KB cells under similar culture conditions. Moreover, after treatment of the transfected 3T3 cells with phospholipase C specific for phosphatidylinositol, the binding of [^{3}H] folate to the surface of these cells is reduced by 90%, indicating that, like the KB cells, the folate-binding protein is anchored to the plasma membrane by a GPI tail. Although the doubling time of wild-type 3T3 cells markedly increases after 13 d of culture in folate-deficient medium, the doubling time of both the transfected 3T3 cells and KB cells do not change. The results of these experiments indicate that the GPI-anchored folate-binding protein provides a mechanism to maintain a level of folate that permits the folate-dependent metabolic functions necessary for cell survival under low folate conditions. (*J. Clin. Invest.* 1992. 90:840–847.) Key words: complementary DNA • folate • folate-binding protein • plasma membrane • transfection

Introduction

Two forms of human folate-binding protein (FBP),[1] a soluble and a membrane-associated form, have been identified in the cytosol and membranes of a variety of cells and in biologic fluids such as milk and plasma (1, 2). The KB cell, a cultured cell line derived from a human epidermoid carcinoma, has a high level of expression of a membrane FBP, a small fraction of which is released as a soluble form into the culture medium (3–5). We have recently demonstrated that the membrane FBP in KB cells is a member of a unique group of proteins that is anchored to the plasma membrane by a glycosylated phosphatidylinositol (GPI) tail, rather than by a transmembrane hydrophobic peptide (6). A similar GPI-linked FBP has been identified by Lacey and co-workers in MA104 cells, a monkey kidney epithelial cell line (7).

Several other cell lines, such as L1210 (8–10) and CCRF-CEM (11) leukemia cells, have been described in which a high-affinity FBP, undetectable in the parental cell line, is induced after selection of cells in low folate medium. However, unlike these cell lines, KB cells survive without selection in medium containing a low concentration of folate (1 nM), i.e., 2,000-fold less than contained in normal, folate-replete medium (2.3 μM) (3–5). Although the GPI-anchored FBP has a higher affinity for oxidized than reduced folates, it has been demonstrated that this membrane FBP in KB cells mediates the uptake of the physiologic folate, 5-methyltetrahydrofolate (5-methyl THF) (12, 13). For these reasons, it has been postulated that the GPI-anchored membrane FBP of KB cells provides a mechanism for the uptake of folate when the concentration of folate in the medium is very low and this results in the survival of KB cells under conditions of relative folate depletion.

However, because the intracellular concentration of folate in KB cells cultured under such folate deficient conditions is very low, it is also possible that the ability to survive in folate-deficient medium may be the consequence of an epiphenomenon in this carcinoma-derived cell line which is independent of the membrane-associated FBP or which is an additional adaptation in concert with the expression of the FBP.

To address this question, we have transfected the cDNA encoding the FBP in KB cells (14) into mouse 3T3 cells, a cell line that does not express a membrane FBP and cannot survive under folate-deficient conditions. This has provided the opportunity to study the growth characteristics under conditions of folate depletion and folate excess in the transfected and wild-type cells without requiring the selection of cells by slow conditioning in culture medium containing low folate which could lead to other adaptations in intracellular folate metabolism.

Methods

Materials

The KB cell line, derived from an epidermoid carcinoma, and the murine 3T3 cell line were purchased from the American Type Culture

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1. *Abbreviations used in this paper:* CAT, chloramphenicol acetyltransferase; FBC, folate binding capacity, FBP, folate-binding protein; FD and FR, folate deficient and folate replete; GPI, glycosylated phosphatidylinositol; PCR, polymerase chain reaction; PI-PLC, phospholipase C specific for phosphatidylinositol; THF, tetrahydrofolate.

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Collection (Rockville, MD). Dulbecco's minimal essential medium (DME), specially prepared without folic acid and methionine was obtained from Gibco Laboratories (Grand Island, NY), as well as hygromycin and trypsin for tissue culture. Fetal calf serum (FCS), penicillin, streptomycin, L-glutamine, Hanks' balanced salt solution (HBSS) without calcium and magnesium were obtained from Sigma Chemical Co. (St. Louis, MO). Ultrapure Triton X-100 was obtained from International Biotechnologies Inc. (New Haven, CT). Dithiothreitol was purchased from Calbiochem-Behring Corp. (La Jolla, CA). Prestained and unstained molecular weight markers were obtained from Integrated Separation Systems (Hyde Park, MA). Autofluor autoradiography enhancer was obtained from National Diagnostics (Manville, NJ). Protein A-Trisacryl and bicinchoninic acid protein assay reagent were obtained from Pierce Chemical Co. (Rockford, IL). (6S)-N^5-methyl THF was obtained from SAPEC S.A. (Lugano, Switzerland).

Trans-[^{35}S] (80% [^{35}S]methionine, 20% [^{35}S]cysteine) (sp act > 1,000 Ci/mmol) was obtained from ICN Biochemicals (Costa Mesa, CA). [^{3}H]folate (sp act 56 Ci/mmol) was purchased from Moravek Biochemicals (Brea, CA). [^{32}P] α dCTP (sp act 6,000 Ci/mmol) was purchased from New England Nuclear Research Products (Boston, MA). Restriction enzymes were obtained from New England Biolabs (Beverly, MA). Labeling of cDNA was done by the Random Primer Labeling kit from United States Biochemical Corp. (Cleveland, OH). All other chemicals used were of reagent grade.

Phospholipase C specific for phosphatidylinositol (PI-PLC), partially purified from *Bacillus thuringiensis* was generously provided by Dr. Sidney Udenfriend (Roche Institute of Molecular Biology, Nutley, NJ). The activity of the enzyme was determined as previously described (15).

Methods

Construction of the vector (pCAR 17) for transfection of the cDNA encoding the FBP in KB cells. Because the cDNA for the KB cell FBP cloned in our laboratory lacked the full-length nucleotide sequence encoding the signal peptide (14), the nucleotide sequence for this region of the protein, as determined by Elwood (16) for the same cDNA, was prepared by reverse transcription from KB cell poly(A)$^+$ RNA, followed by amplification of the 5′ coding fragment by the polymerase chain reaction (PCR) using specific oligonucleotides encompassing this domain. The integrity of the amplified fragment was confirmed by DNA sequencing.

The specific steps for the construction of this vector are as follows:

Oligonucleotide (RD7-22C) contains an Nco I restriction site (underlined) in the 5′ portion of the FBP signal sequence with the initiation codon shown in bold print. Its sequence is:

5′ - CAGCCATGGCTCAGCGGATGAC - 3′

Oligonucleotide (RD8-18C) contains a Sal I restriction site (underlined) and is complementary to a coding sequence within the FBP cDNA beginning 163 bp downstream from the ATG start site. Its sequence is:

5′ - CCAGGGTCGACACTGCTC - 3′

Whole-cell KB mRNA was incubated with oligo RD8-18C and the murine leukemia virus reverse transcriptase for 30 min at 42°C, thereby synthesizing RNA-DNA hybrids from FBP mRNA. This reaction was performed in PCR buffer (Cetus Corp., Emeryville, CA), 200 μM dNTPs, and 1 μM of the oligonucleotide RD8-18C. To this mixture, 5 U of Taq DNA polymerase, the primer RD7-22C (to attain a final concentration of 1 μM), and 50 μl paraffin oil were added. The reaction was incubated according to the following schedule: 5.0 min at 94°C (one cycle); 1.5 min at 94°C, 2.5 min at 50°C, 3.5 min at 72°C (35 cycles); and 10 min at 72°C (one cycle).

The reaction product was extracted twice into chloroform/isoamyl alcohol (24:1), precipitated in ethanol, resuspended in 30 μl 10 mM Tris/1 mM EDTA, pH 7.5, and 2 μl were subjected to electrophoresis in a 1.2% agarose gel to determine the yield.

The PCR fragment was cut with Nco I and Sal I and replaced the truncated 5′ end of the original cDNA which had been cloned in the Eco RI site of a pUC18 derivative. The FBP cDNA in this pUC 18 derivative had been previously modified to add the Kozak consensus sequence, which provided the Nco I restriction site into which the processed PCR-generated fragment was cloned. This complete cDNA coding region was then excised at flanking Sma I and Tha I sites and cloned between the Stu I and Sma I sites of pYZ28 (17, 18). The resulting plasmid, pCAR 17, contains the FBP cDNA sequence between an SV40 early promoter and polyadenylation signals (Fig. 1). As a control, a similar vector was constructed with the chloramphenicol acetyltransferase (CAT) gene, replacing the FBP cDNA.

Transfection of 3T3 cells with pCAR 17. Monolayer 3T3 cells were grown to 70% confluence in 60-mm plates and were co-transfected using the calcium phosphate precipitation method (19) with 10 μg of pCAR 17 and 1 μg of a control vector containing the hygromycin resistance gene under the control of an SV40 promoter. The cells were incubated for 16 h at 37°C in 3% CO_2 and then placed in fresh medium and incubated for 24 h in 5% CO_2. The cells were then harvested and seeded in fresh medium containing 100 μg/ml hygromycin B at either a 1:20 or a 1:40 dilution and incubated at 37°C in 5% CO_2 until the colonies developed (~ 2 wk). Ten colonies were transferred to individual plates and maintained under hygromycin selection. Cell lysates prepared in buffer containing 1% Triton X-100 were then tested for FBP expression by radioimmunoassay (20).

Southern blotting of transfected and wild-type 3T3 cells. Chromosomal DNA from the cultured cells (~ 15×10^6 cells) was prepared by a standard method of phenol/chloroform extraction and ethanol precipitation (19). The DNA (10 μg) was digested with Nco I, which is the linker restriction site for the insertion of the FBP cDNA into the transfection construct site in pCAR 17, and then subjected to electrophoresis in 1% agarose and Southern blotting (21) using the FBP cDNA as the probe.

Cell culture. Normal, folate-replete (FR) KB cells, and wild-type and transfected 3T3 cells were maintained as adherent cell monolayers in DME which contained 2.26 μM folate, as previously described (5). These cells were made folate deficient (FD) by culturing in DME containing 1 nM folate. Both FR and FD media were supplemented with 10% FCS, 2 mM L-glutamine, penicillin (100 U/ml), and streptomycin (100 μg/ml).

Measurement of the folate binding capacity (FBC) and total FBP. The cultured cells were released from the monolayer by incubation with 0.25% trypsin at 37°C for 2 min, washed three times in HBSS at 4°C and solubilized in 0.01 M K phosphate/1% Triton X-100, pH 7.4. The unsaturated FBC of the solubilized cells was determined by the binding of [^{3}H]folic acid using dextran-coated charcoal to remove unbound ligand as previously described (5). The total FBC was determined in a similar manner after removal of endogenous folate by dialysis of the solubilized cells against 0.01 M acetic acid/1% Triton X-100 for 4 h followed by dialysis against 0.01 M K Phosphate, pH 7.4 for 4 h. The total FBP (apo and holo forms) in these fractions was determined by noncompetitive radioimmunoassay (20).

PI-PLC treatment of intact cells. The media from the FD KB and the FD 3T3 monolayers were removed, the cell monolayers were then washed three times with HBSS and 1 ml of FD DME containing 2 mM glutamine, 10% dialyzed FCS, and PI-PLC (3 U/ml) was added. Control monolayer cells were treated similarly with the exception that no PI-PLC was added to the incubation medium. After a 6 h incubation at 37°C, the medium was removed and the monolayers washed three times with HBSS. The cells were then released from the monolayer by incubation with 0.25% trypsin at 37°C for 2 min, washed three times with HBSS at 4°C, and suspended in 1 ml of HBSS. Under these conditions, cell viability by trypan blue exclusion was > 90%. The FBC of the intact cells was determined by incubating increasing aliquots of the cells with 2.26 pmol of [^{3}H]folate for 15 min at 4°C. Unbound [^{3}H]-folate was removed by washing the cells with HBSS at 4°C on glass fiber filters and the bound radioactivity retained by the filters was determined by liquid scintillation counting.

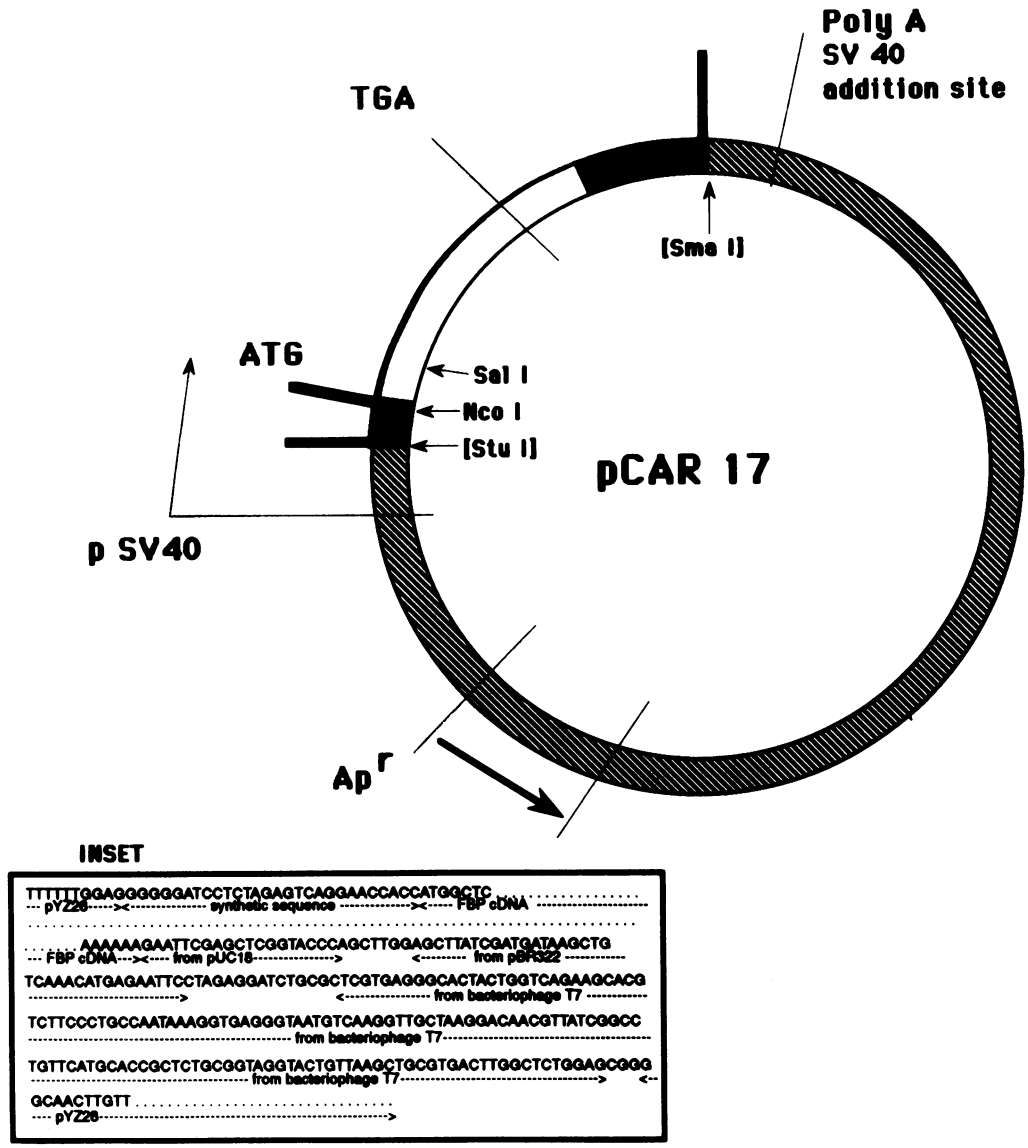

Figure 1. The vector derived from pYZ28 containing the cDNA for KB cell FBP used for the stable transfection of 3T3 cells. *Hatched box:* Sequence derived from pYZ28 between Stu I and Sma I sites. *Open box:* FBP cDNA. *Solid boxes:* Junction regions acquired in cloning. Abbreviations: Ap^r; ampicillin resistance gene; pSV40; SV 40 promoter. [ ]: these sites were destroyed in cloning the cDNA into this vector. The inset shows the origin of the nucleotide sequences that comprise the final vector: pYZ28 is the parent plasmid and contains the SV40 promoter and the bacteriophage T7 sequences (17, 18); the synthetic sequence contains the Nco I restriction site and the Kozak consensus sequence; pUC 18 is the derived plasmid into which the truncated FBP cDNA was cloned.

Growth of cells in low folate medium. Monolayer cultures of wild-type 3T3, 3T3 cells transfected with the vector containing the CAT gene (control transfectants), 3T3 cells transfected with the cDNA for the GPI-anchored membrane FBP of KB cells, and KB cells were seeded in 25-cm^2 flasks in FR medium at a concentration of 100×10^3 cells per flask. After the cells had adhered to the monolayer, the medium was replaced with either FR or FD medium and this was called day 0. The medium was changed every 3 d and the cell monolayers were reseeded at 100×10^3 cells per flask once weekly, when the monolayers approached confluence. Cell counts were determined every 2–3 d using the following protocol: cell monolayers were rinsed with HBSS three times, 1 ml of 0.25% trypsin was added to the flask which was incubated at 37°C for 3–5 min. When the monolayers were released from the flask, the action of the trypsin was stopped by dilution with 10 ml of ice-cold PBS, pH 7.4. The cells were then pelleted by centrifugation at 500 *g* for 5 min and resuspended in 2 ml of PBS at 4°C. The cell count was determined using a hemacytometer, and the cells were then pelleted at 2,000 *g* for 10 min. The PBS was removed and the cell pellet was prepared for the determination of intracellular folate concentration.

Measurement of intracellular folate concentration. Cell pellets were suspended in Ringer solution containing 0.05 M borate and 0.2% ascorbic acid, pH 8.0, and saved at −35°C. On the day of the folate assay, an aliquot of the cell suspension was removed for the determination of protein concentration and the remainder of the preparation was boiled for 10 min, the insoluble debris was pelleted by centrifugation at 12,000 *g* for 5 min and the supernatant was assayed for folate using the sequential noncompetitive radioassay described by Rothenberg et al. (22). The standard for the dose-response curve was (6S)-N^5-methyl THF. Oxidized and reduced folate monoglutamates and polyglutamates react similarly in this noncompetitive assay system except for N^5-formyl THF, the intracellular concentration of which is very low (23).

The total cellular protein concentration was measured by bicinchoninic acid protein assay reagent as described by the manufacturer.

Purification of the membrane FBP from transfected 3T3 cells. The membrane FBP was purified from cells cultured in FD medium using an epoxy-activated Sepharose 6B matrix coupled to folate (24), as previously described (5, 25).

[^{35}S]methionine labeling of KB cells and transfected 3T3 cells. KB cells and 3T3 cells transfected with the FBP cDNA, which had been cultured in FR medium, were metabolically labeled with [^{35}S]-methionine, as previously described for KB cells (26). Briefly, before labeling, the medium was removed, cell monolayers were washed three times with sterile HBSS and starved for 30 min in methionine-free DME supplemented with 2 mM glutamine and 10% dialyzed FCS. The

cells were then pulse-labeled for 60 min in the same medium containing 0.1 mCi of [^{35}S]methionine. At the end of the pulse period, the medium was removed, the cell monolayers were washed three times with sterile HBSS, and chased for varying time periods from 0 to 5 h in DME supplemented with 10% FCS, 2 mM glutamine, and 5 mM methionine. At the end of the chase period, the medium was removed, the cell monolayers were washed three times with HBSS at 4°C, solubilized immediately in 0.05 M Tris-HCl, pH 8.5/1% Triton X-100/5 mM EDTA/0.15 M NaCl containing aprotinin (200 U/ml) and centrifuged at 30,000 *g* for 30 min at 4°C.

Immunoprecipitation of the [^{35}S]methionine-labeled FBPs. The radio-labeled FBPs were isolated by binding to an immune serum-Protein A-Trisacryl complex, as previously described (26). Briefly, the sample was first incubated with 50 μl of Protein A-Trisacryl for 2 h at 4°C. The supernate of this mixture was then incubated sequentially with preimmune rabbit serum-Protein A-Trisacryl at 4°C for 16 h and immune serum-Protein A Trisacryl at 4°C for 16 h. The preimmune and immune serum-Protein A complexes were prepared by incubating 10 μl of the preimmune or immune serum, respectively, with 50 μl of Protein A-Trisacryl (1:2 dilution) in 90 μl of 0.01 M Tris-HCl/0.15 M NaCl, pH 7.5, for 2 h at 4°C. The matrix was then washed three times with 0.01 M Tris-HCl/0.15 M NaCl, pH 7.5.

After centrifugation at 1,000 *g*, the supernate was removed and the Protein A pellet was washed four times with 0.01 M KPO_4/0.15 M NaCl/1% Triton X-100/0.5% sodium deoxycholate/0.005% SDS, pH 7.5 and once with 0.01 M Tris-HCl/0.15 M NaCl, pH 7.5. The immune complex was then dissociated from the Protein A-Trisacryl by boiling for 5 min in 100 μl of 0.08 M Tris-HCl, pH 7.0/0.017 M EDTA/0.017 M dithiothreitol/3.3% SDS/15% glycerol. The matrix was pelleted and the supernate was subjected to SDS-electrophoresis by the method of Laemmli (27) on a 10% or 12% polyacrylamide slab gel, 1.5 mm in thickness. The gel was fixed, treated with Enhance or Autofluor, dried on a slab gel dryer (model SE 1160, Hoefer Scientific Instruments, San Francisco, CA) and developed at −80°C using Cronex 7 film (Dupont Co., Wilmington, DE).

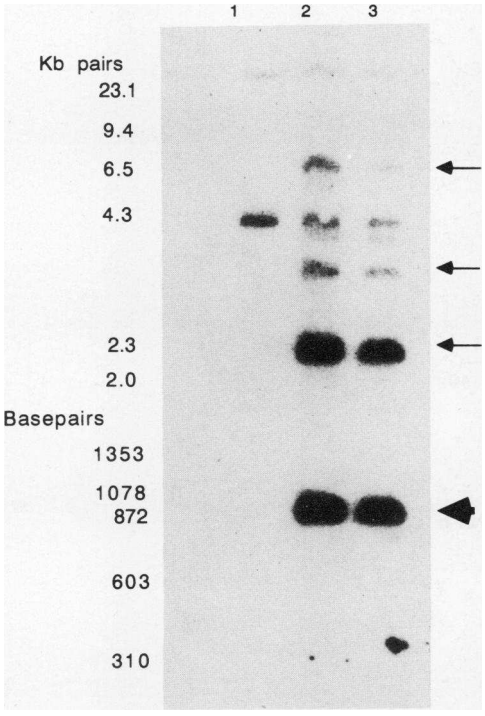

Figure 2. The autoradiograph of the Southern blot of chromosomal DNA prepared from wild-type 3T3 cells and digested with Nco I (lane *1*), and 3T3 cells transfected with the FBP cDNA and cultured in FR (lane *2*) and FD medium (lane *3*). *Large arrowhead:* The predicted 850 bp FBP cDNA insert. *Small arrows:* Restriction fragments of the vector which hybridized with some pUC 18 plasmid fragments.

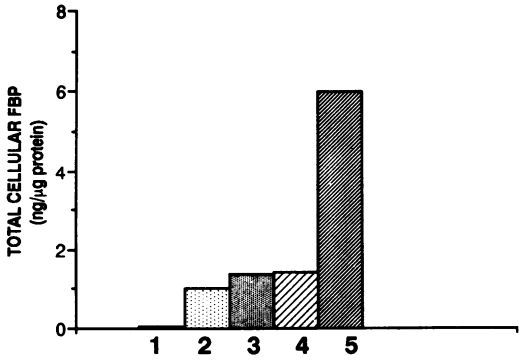

Figure 3. The FBP measured by radioimmunoassay of wild-type 3T3 cells (*1*), 3T3 cells transfected with the FBP cDNA and cultured in FR (*2*) and FD (*3*) media and KB cells cultured in FR (*4*) and FD (*5*) media.

Results

Southern blotting of transfected and wild-type 3T3 cells chromosomal DNA. Fig. 2 shows the autoradiograph of the Southern blot of the chromosomal DNA prepared from wild-type 3T3 cells (lane *1*) and 3T3 cells transfected with the FBP cDNA grown in FR (lane *2*) or FD medium (lane *3*), and digested with the restriction enzyme, Nco I. The predicted 850-bp FBP cDNA insert (*large arrowhead*) in the transfection construct can be identified in the chromosomal DNA from cells transfected with this vector (lanes *2* and *3*) and not in the wild-type 3T3 cells (lane *1*). The additional bands observed in the transfected cells (*small arrows,* lanes *2* and *3*) and absent in the wild-type 3T3 DNA are restriction fragments of the vector which hybridized with some pUC 18 plasmid fragments which co-purified with the FBP cDNA which was used as the probe. Only one band (4.3 kb) is observed in the genomic DNA from wild-type 3T3 cells digested with Nco I, which is the murine gene for the FBP and which has been observed in genomic DNA prepared from mouse liver (unpublished observations).

Total FBP and FBC of transfected 3T3 cells, wild-type 3T3 cells and KB cells. The total immunoreactive FBP of the solubilized, transfected 3T3 cells was similar to the total FBP of normal, FR KB cells, 1 and 1.4 ng/μg Triton solubilized protein, respectively (Fig. 3, columns *2* and *4*). The total FBP of the transfected 3T3 cells did not increase when these cells were cultured in FD medium for 4 mo (column *3*) (1.36 ng/μg Triton solubilized protein). In contrast, the total FBP of KB cells did increase when these cells were cultured in FD medium (column *5*) (6 ng/μg Triton solubilized protein) and this has been previously reported by our laboratory and others (3–5). Wild-type 3T3 cells contained no immunoreactive FBP (column *1*).

The total FBC of solubilized, transfected 3T3 cells cultured in FR medium and then dialyzed at pH 3.5 to remove endogenous folates, and transfected 3T3 cells cultured in FD medium was 0.015 and 0.011 pmol [^{3}H]folate bound/μg Triton solubilized protein, respectively, indicating that the immunoreactive FBP was functional, i.e., it bound folate. In contrast, wild-type 3T3 cells similarly prepared did not bind [^{3}H]folate.

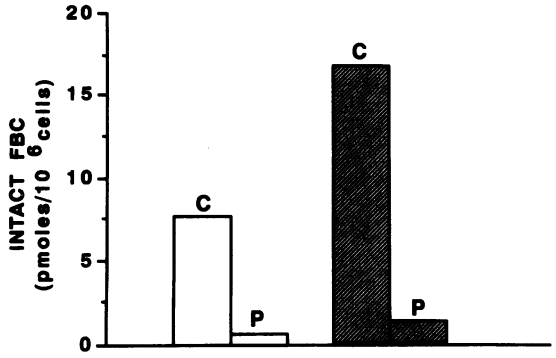

Figure 4. The FBC of intact 3T3 cells transfected with the FBP cDNA (*open columns*) and KB cells (*hatched columns*) before (*C*) and after treatment with PI-PLC (*P*).

FBC and PI-PLC treatment of intact cells. The FBC of intact 3T3 cells transfected with the FBP (Fig. 4) was 7.7 pmol[^{3}H]folate/10^6 cells (*C, open column*). In contrast, wild-type 3T3 cells did not bind [^{3}H]folate. The FBC of intact KB cells was 16.7 pmol[^{3}H]folate/10^6 cells (*C, hatched column*). After treatment of intact transfected 3T3 cells and wild-type KB cells with PI-PLC, the FBC of these cells was reduced 90%, to 0.68 and 1.3 pmol [^{3}H]folate/10^6 cells, respectively (*P, open and hatched columns*). Thus, the FBP in transfected 3T3 cells was not only functional but was localized to the plasma membrane and susceptible to cleavage by PI-PLC, a property of GPI-linked proteins (28).

Growth of transfected 3T3 cells in low folate medium. Fig. 5 shows the doubling times of wild-type 3T3 cells, 3T3 cells transfected with the control CAT gene, 3T3 cells transfected with the FBP cDNA, and wild-type KB cells. In each panel, the growth of cells was determined in both FR and FD media. At 13 d of culture in FD medium, both wild-type 3T3 cells and 3T3 cells transfected with the control CAT gene had a marked increase in doubling time. In contrast, both the 3T3 cells transfected with the FBP cDNA and the wild-type KB cells continued to grow in FD medium with no significant change in their doubling time even after 35 d of culture in FD medium. Although the doubling time was calculated for a period of 35 d, we have continued to observe a pattern of cell growth of the transfected 3T3 cells which is similar in FR and FD medium. Thus, the expression of the membrane FBP of KB cells in the transfected 3T3 cells is associated with the ability of these cells to survive when cultured in low folate medium.

The concentration of total folate in these cells is shown in Table I. In cells cultured in FD medium, the level of folate in wild-type and CAT transfected 3T3 cells was significantly lower (< 0.05 pmol/mg total cell protein for both) than the levels of folate in the FBP transfected 3T3 cells and KB cells (3.0 and 4.1 pmol/mg total cell protein, respectively).

Purification of the membrane FBP from transfected 3T3 cells. The purified membrane FBP of transfected 3T3 cells has an apparent M_r of 35,000 kD by SDS-PAGE (data not shown), which is smaller than the apparent M_r of 38,000 kD for the purified membrane FBP of KB cells (26). To investigate the reason for this discrepancy, transfected 3T3 cells and KB cells were metabolically labelled with [^{35}S]methionine and the radiolabeled FBPs were immunoprecipitated and analyzed by SDS-PAGE and autoradiography and the results are shown in Fig. 6.

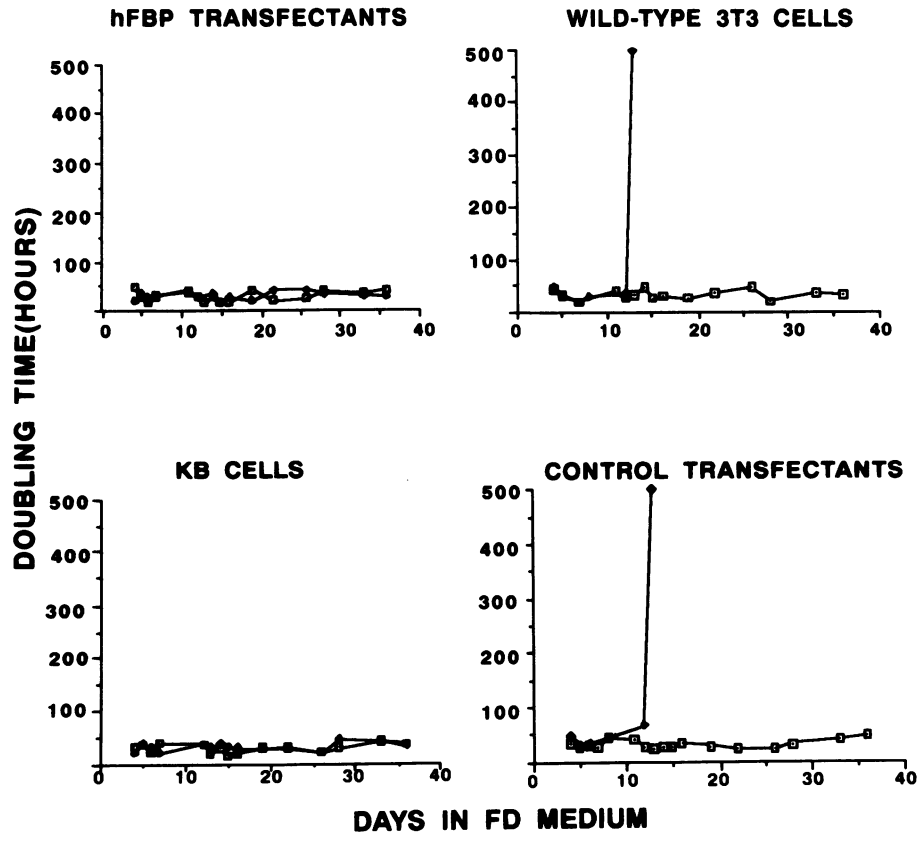

Figure 5. The doubling times of 3T3 cells transfected with the cDNA for the FBP (hFBP transfectants), wild-type 3T3 cells, KB cells and 3T3 cells transfected with the CAT gene (control transfectants) and cultured in FR and FD media. □ FR; ♦, FD.

*Table I. Total Cellular Folate of 3T3 and KB Cells**

	Total cell folate
	pmol/mg total cell protein
Wild-type 3T3	
FR	5.9
FD	<0.05
Cat transfected 3T3 cells	
FR	5.5
FD	<0.05
FBP transfected 3T3 cells	
FR	19.0
FD	3.0
Wild-type KB cells	
FR	37.1
FD	4.1

* Folate levels were measured in boiled extracts of cells after 15 d of culture in FR or FD medium.

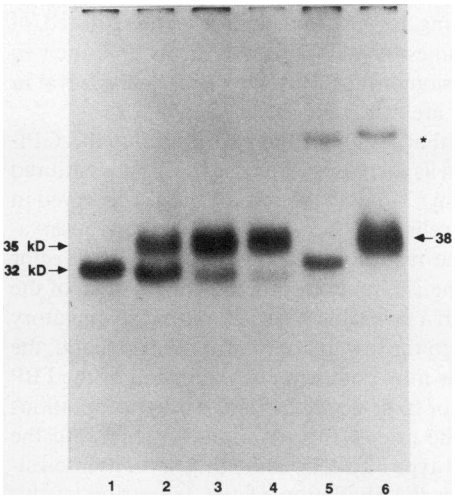

Figure 6. The autoradiograph of the SDS-PAGE of the immunoprecipitates obtained from 3T3 cells transfected with the FBP cDNA and KB cells after pulse-chase with [^{35}S]methionine and excess cold methionine. Lanes *1–4*—3T3 cells transfected with the FBP: 0 h of chase (lane *1*); 1 h (lane *2*); 3 h (lane *3*); 5 h (lane *4*). Lanes *5* and *6*—KB cells: 0 h of chase (lane *5*); 5 h (lane *6*). The bands at the asterisk (*) in lanes *5* and *6* are nonspecific protein bands which are also seen in samples precipitated with normal rabbit serum (data not shown). The indicated molecular mass of the proteins was determined by measuring the distance from the point of origin to the center of the autoradiographic band.

At 0 h of chase, a 32-kD radiolabeled band was seen in both the 3T3 cells transfected with the FBP cDNA (lane *1*) and the KB cells (lane *5*), indicating that the core-glycosylated FBP of the FBP-transfected 3T3 cells has the same apparent Mr as the corresponding species from KB cells. Over 5 h of chase, the 32-kD band disappeared in both cell lines and in the FBP-transfected 3T3 cells was replaced by a single radiolabeled 35-kD protein band (lanes *2* through *4*). In the KB cell line, the 32-kD species was replaced by a broader 38-kD band (lane *6*). Thus, the difference in the apparent molecular weights of the FBPs purified from the FBP transfected 3T3 cells and KB cells is not due to a difference in the molecular weights of the peptides but rather it is due to a difference in post-translational processing, probably involving complex glycosylation (29).

Discussion

We provide evidence in this report for the stable transfection of a murine 3T3 cell line with the cDNA for a human FBP and the expression of a functional and structurally similar protein to the one expressed by KB cells from which the cDNA was cloned (7, 14, 16). We also show that the FBP expressed in the transfected cells is: (*a*) localized to the plasma membrane; (*b*) susceptible to PI-PLC cleavage and therefore, GPI-anchored in the plasma membrane; (*c*) expressed at a level similar to the level of the FBP in KB cells cultured in FR medium; and (*d*) synthesized initially as a core-glycosylated species with an apparent M_r of 32 kD by SDS-PAGE, which is the same as the core-glycosylated FBP in KB cells. However, the FBP expressed by the transfected 3T3 cells migrates as a slightly smaller protein with an apparent M_r of 35 kD compared to the 38 kD FBP of KB cells. Metabolic labeling of the cells with [^{35}S]methionine demonstrates that this difference is due to a variation in the complex glycosylation of the two FBPs which may reflect species differences in post-translational processing capabilities between mouse and human cells but which does not affect the ligand binding function, GPI-linking, or translocation of the protein to the plasma membrane.

Reduced folate cofactors are essential for the biosynthesis of purines, methylation of deoxyuridine monophosphate to deoxythymidine monophosphate, the methylation of homocysteine to form methionine, glycine–serine interconversion, and the metabolism of histidine (30). These folate-dependent pathways are necessary for cell replication and viability and cultured cell lines die if the medium contains insufficient folate. The exceptions to this observation are KB (3–5) and MA104 cells (23) which express membrane FBPs. In addition, it has been possible to isolate sublines of certain cell lines, like L1210 (8–10) and CCRF-CEM leukemia cells (11) by slow adaptation to growth in low folate medium. Initially, these cell lines have decreased growth rates and gradually adapt with growth similar to the parental cell line. Concomitant with this adaptation, the cells express a membrane FBP and this has led to the conclusion that the two events have a cause-and-effect relationship. However, because the intracellular concentration of folate in KB cells cultured under low folate conditions (1 nM) is only 10% of the cellular folate of cells grown in FR medium, it is evident that expression of the FBP has a limited capacity to raise the intracellular folate when the extracellular folate concentration is low. This observation raises two possibilities. First, that the conditioning of cells to a low folate environment may involve more than one adaptation, i.e., expression of the membrane FBP with augmentation of salvage pathways to maintain the pool of nucleotides, or altered properties of the folate cofactor-dependent enzymes (i.e., lower K_m or higher V_{max}). Second, that the intracellular concentration of folate which is achieved by the expression of the FBP, although reduced, is sufficient to maintain intracellular folate metabolism for normal cell growth.

The results of these studies support the second hypothesis since the mouse 3T3 cells transfected with the KB cell cDNA for the GPI-linked FBP survive in low folate medium without

the slow conditioning to such an environment required of other cultured cell lines (8–11). This is evidence that the two phenomena, expression of the GPI-linked FBP and survival in low folate medium, are cause-and-effect related.

It is of interest that although the expression of the GPI-linked FBP in KB cells increases when these cells are cultured in low folate medium (3–5), no similar increase is observed in the transfected 3T3 cells. Two factors may explain this observation. First, since the incorporation of the transfected vector into the host genome is a random process, the location of the cDNA may not be in a favorable *cis* orientation to a regulatory element responsive to the low folate environment. Second, the transfected 3T3 cells may not augment expression of the FBP because of the lack of trans-active factors or other adaptations that can enhance the rate of transcription. KB cells and the transfected and wild-type 3T3 cells could thus serve as interesting models to study the regulation of the expression of this human FBP at the molecular level under normal conditions and during folate deprivation.

The concentration of cellular folate in the transfected 3T3 cells and KB cells cultured in low folate medium provides some indication of the minimum folate level that is required to maintain cell viability and replication. It is evident that the intracellular folate concentration in the wild-type 3T3 cells cultured in low folate medium rapidly falls below this essential minimum level and is responsible for the cell death that follows. Similar observations have been made by Watkins and Cooper (31) for K562 cells that were cultured in low folate medium. This cell line, derived originally from chronic myelogenous leukemia cells (32) and capable of erythroid differentiation, do not express a membrane-anchored FBP and the doubling time of these cells increases as intracellular folate decreases.

Although it is evident that the membrane FBP mediates folate transport and is of critical importance in the survival of cells in low folate medium, how it functions in the transport of the physiologic folate, 5-methyl THF, is unclear. In most cultured cell lines, reduced folates enter the cell via a transmembrane channel (33) which is inhibited by probenicid and other anions (33–35). Kamen and co-workers have postulated that the GPI-linked membrane FBP is coupled to the reduced folate transmembrane channel by demonstrating that after binding to the GPI-linked FBP of MA104 cells, the movement of 5-methyl THF into the cytosol is inhibited by probenicid (36). Since the GPI anchor of the FBP results in increased mobility of the protein in the plasma membrane (28), the FBP may function by concentrating folate in close proximity to a reduced folate channel through which 5-methyl THF enters the cytoplasm after it dissociates from the FBP. In support of this hypothesis, clustering of the FBP in invaginations of the plasma membrane called caveolae has been observed (37, 38). However, Westerhof and co-workers concluded from their studies (39) that these two transport systems, the membrane FBP and the reduced folate channel, transport reduced folates independently in L1210-B73 cells, a variant of murine leukemia L1210 cells grown in low folate medium, because folate did not inhibit the uptake of methotrexate which is internalized via the reduced folate channel. It is unlikely that the GPI-linked FBP transports folate through the cell membrane by providing a transmembrane channel, since it is anchored in the outer leaflet of the plasma membrane by the GPI tail which places the folate binding site at a distance from the cell surface.

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