Membrane folate-binding proteins are responsible for folate–protein conjugate endocytosis into cultured cells

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Folate–protein conjugates have been shown to bind to and enter HeLa and KB cells by receptor-mediated endocytosis [Leamon and Low (1991) Proc. Natl. Acad. Sci. U.S.A. **88**, 5572–5576]. Although these cells contain a membrane folate-binding protein (FBP) involved in the uptake of free folate, no studies have been conducted to evaluate whether the folate–protein conjugates enter cells via the same protein. To address this issue, HeLa cell monolayers were treated with folate-labelled ^{125}I-RNAase under various conditions characteristic of FBP-mediated folate uptake. Folate-labelled ^{125}I-RNAase was found to bind to cells with high affinity (K_d = 24 nM), and like the free vitamin, its binding could be competitively blocked by excess free folate. Furthermore, binding could be reversed by either washing the cells with acid/saline, pH 3.0, or by treating the cells with phosphatidylinositol-specific phospholipase C, an enzyme known to release FBP from cell surfaces. Because cells pretreated with anti-FBP serum were unable to bind folate conjugates, and since the same antiserum identified a single 65 kDa band reminiscent of FBPs found in many other tissues, we conclude that a classical FBP is responsible for the uptake of folate–protein conjugates by receptor-bearing cells.

INTRODUCTION

Because folates are required for the survival and growth of eukaryotic cells, their cellular uptake is assured by at least two independent transport mechanisms. Reduced folates are internalized via a carrier-mediated low-affinity (K_m 1–5 μM) anion-transport protein that is found in nearly all cells. Folic acid and 5-methyltetrahydrofolate, on the other hand, can enter cells in complex with a membrane-bound receptor called a folate-bind protein (FBP; for review see [1,2]). FBPs have been shown to bind folates with high affinity (K_d values in the nanomolar range) and to deliver them into the cytoplasm via receptor-mediated endocytosis [1,3–5].

Uptake studies of various dihydrofolate reductase and thymidylate synthase inhibitors have revealed that many cultured cells can express both FBP and the reduced folate carrier [6,7]. Consequently, it is believed that folate uptake occurs via an FBP when the exogenous folate concentration is at or below physiological levels (less than 50 nM), while the reduced folate carrier participates in folate uptake when cells are cultured in supraphysiological folate concentrations [1]. From studies involving MA104 cells, Kamen et al. [8] have theorized that FBPs are responsible for the binding and internalization of folates into membrane-bound caveolae, from which the vitamins are released and translocated into the cytosol by a co-localized anion channel. This 'receptor–transmembrane coupling' mechanism was reported to mediate 5-methyltetrahydrofolate uptake into MA104 cells [8] but presumably not folic acid, since these cells were unable to translocate this folate into the cytosol [9]. Importantly, folate uptake was not observed to occur via the aforementioned coupling process in an L1210 cell line known to express both FBP and the reduced carrier [7], suggesting that other mechanisms for folate translocation may exist.

Like the free vitamin, proteins conjugated to folic acid have been shown to enter cultured cells by a receptor-mediated process (10). Thus simple covalent attachment of folate to virtually any size macromolecule generates a conjugate capable of reaching the cytoplasm of receptor-bearing cells in an intact functional state [11]. Although we consider it unlikely that a folate-specific anion channel is responsible for translocating folate–protein conjugates into cultured cells, we have not been able to exclude the possibility that such conjugates might dock with these channels and enter the cells during the course of routine membrane cycling/turnover. Obviously the more likely route of folate conjugate entry would involve recognition of the protein-linked folate by FBP and the subsequent internalization of the complex by receptor-mediated endocytosis.

The purpose of the present study was to determine the role of FBP in the endocytosis of folate–protein conjugates into cultured cells. Using folate-labelled ^{125}I-RNAase A (^{125}I-RNAase–folate) as a model folate–protein conjugate and folate-depleted HeLa cells as a model FBP-containing cell line [12–14], we have examined the binding specificity and internalization characteristics of folate–protein uptake. We have also employed phosphatidylinositol-specific phospholipase (PI-PLC), an enzyme known to cleave FBP from cell membranes [15–19], and a specific anti-FBP serum to characterize folate–protein uptake further. The sum of our observations suggests that folate–protein conjugates bind to and are internalized by a membrane FBP similar to that found in a variety of other cell types [1,20–22].

EXPERIMENTAL

Materials

Na^{125}I and L-[4,5-^{3}H]leucine (120–190 Ci/mmol) were purchased from Amersham; RNAase, momordin, bovine milk FBP and rabbit serum were obtained from Sigma; IodoBeads and bicinchoninic acid (BCA) protein assay reagents were purchased from Pierce; Hunter's Titer Max was obtained from the CytRx Corporation; PI-PLC from *Bacillis cereus* was purchased from

Abbreviations used: FBP, folate-binding protein; ^{125}I-RNAase–folate, folate-labelled ^{125}I-RNAase A; PI-PLC, phosphatidylinositol-specific phospholipase C; FFMEM, folate-free Dulbecco's modified Eagle's medium; FDHeLa, folate-depleted HeLa cells; FDKB, folate-depleted KB cells; TBS, Tris-buffered saline, pH 7.5.

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Boehringer-Mannheim and contained less than 0.02% phosphatidylcholine-specific phospholipase C; all tissue culture products were obtained from Gibco BRL; and cultured cells were received as a gift from the Purdue Cancer Center (West Lafayette, IN, U.S.A.). All other chemicals were reagent grade from major suppliers.

Cell culture

HeLa and KB cells were grown continuously as a monolayer using folate-free Dulbecco's modified Eagle's medium (FFMEM) containing 10% heat-inactivated fetal calf serum, penicillin (50 units/ml), streptomycin (50 μg/ml) and 2 mM L-glutamine at 37 °C in a 5% CO_2/95% air humidified atmosphere, as previously described [10,11]. These cells will herein be referred to as folate-depleted HeLa cells (FDHeLa) or folate-depleted KB cells (FDKB).

Radioiodination

RNAase was dissolved in 0.1 M NaH_2PO_4, pH 7.0, and added to prewashed IodoBeads. $Na^{125}I$ was than added and the mixture incubated for 1 h at 23 °C, after which free unchanged ^{125}I was separated from labelled protein using a PD-10 desalting column (Bio-Rad) equilibrated in 0.1 M NaH_2PO_4, 0.1 M boric acid, pH 8.5.

Preparation of folate–protein conjugates

Folic acid was covalently attached to RNAase or momordin using a previously described protocol [10,11]. Briefly, folic acid was dissolved in anhydrous dimethyl sulphoxide and incubated with an excess of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide for 30 min at 23 °C in the dark. Protein samples were either dissolved in or dialysed against 0.1 M NaH_2PO_4, 0.1 M boric acid, pH 8.5. A 5- to 15-fold molar excess of activated vitamin was then added to the protein solution, and the labelling reaction was allowed to proceed at 23 °C for 1 h. Unchanged material was separated from the labelled protein using a PD-10 column equilibrated in PBS, pH 7.4. The extent of folate conjugation was determined as previously described [10]. This value varied from approximately 0.5 to 2 mol of folate/mol of protein among the many preparations required to complete the study.

Immunization

Bovine milk FBP (100 μg; Sigma) was dissolved in 300 μl of deionized water and added to an equal volume of Hunter's Titer Max. The mixture was sonicated for 30 s to form a homogeneous water/oil emulsion. Subcutaneous injections were made over seven sites on the back of a New Zealand White female rabbit. Booster injections were administered every 8 weeks, and blood was drawn from the marginal ear vein 10 and 25 days after each booster. Sera were stored at −80 °C.

Measurement of time-dependent uptake of ^{125}I-RNAase–folate

FDHeLa cells were suspended at 0.25×10^6–1×10^6 cells/ml in FFMEM containing the supplements mentioned above. Then 1 ml of suspension was deposited into individual 35 mm × 10 mm Falcon culture dishes, and the cells were incubated overnight (18–20 h) in a humidified incubator at 37 °C. The incubation medium was replaced with 1 ml of 5 mg/ml BSA in buffer A (113 mM NaCl, 10 mM dextrose, 5 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 1.7 mM $NaHCO_3$, 10 mM Na_2HPO_4, 0.6 mM $CaCl_2$, pH 7.4, with or without added excess folic acid), and the cells were incubated for 30 min at 37 °C. ^{125}I-RNAase–folate or ^{125}I-RNAase (control) was then added to designated dishes to yield 0.5 μg/ml RNAase, and cells were incubated for the indicated times at 37 °C. Monolayers were rinsed four times with 1 ml of ice-cold PBS, scraped into 0.5 ml of fresh PBS, centrifuged at 1000 ***g*** for 1 min, and then dissolved in 100 μl of cold 0.1 M KOH. Samples were counted for ^{125}I content in a Packard Cobra γ counter, and then total cellular protein was determined using a BCA protein assay kit. The values plotted represent the number of RNAase molecules associated per FDHeLa cell. They were calculated using an experimentally determined conversion factor of 2.23×10^{-10} g of protein per FDHeLa cell.

Measurement of concentration-dependent uptake of ^{125}I-RNAase–folate

FDHeLa cells were plated into Falcon culture dishes and incubated overnight as described above. Growth medium was replaced with 1 ml of buffer A containing 5 mg/ml BSA per dish, and monolayers were incubated for 30 min at either 4 or 37 °C. ^{125}I-RNAase–folate or ^{125}I-RNAase was added at the indicated concentrations, and the incubation was continued for 2 h at either 4 or 37 °C. Each dish was then rinsed with either cold PBS or cold 0.15 M NaCl adjusted to pH 3.0 with acetic acid and then harvested exactly as described above. The acid/saline rinses were necessary to demonstrate removal of externally oriented folate conjugates, as previously demonstrated [9–11,20,23].

Treatment of FDHeLa cells with PI-PLC

FDHeLa cells were plated into Falcon culture dishes and incubated overnight as described above. Growth medium was replaced with 1 ml of buffer A containing 2 μg of ^{125}I-RNAase–folate per dish, and cells were incubated for 10 min at 37 °C. Each dish was rinsed twice with 1 ml of FFMEM (no supplements), followed by the addition of 1 ml of fresh FFMEM. PI-PLC was added at the indicated concentrations, and cells were incubated for 30 min at 37 °C. Each dish was then rinsed with cold PBS and harvested exactly as described above.

Treatment of FDHeLa cells with anti-FBP serum

FDHeLa cells were plated into Falcon culture dishes and incubated overnight as described above. Monolayers were rinsed twice with 1 ml of buffer A before treatment with 1 ml (total volume) of fresh buffer A and increasing amounts of antiserum, as indicated. Cells were then incubated for 1 h at 23 °C without shaking. ^{125}I-RNAase–folate was then added directly to each dish (1 μg/ml final concentration), and cells were incubated for 15 min at 37 °C. Each dish was then rinsed with cold PBS and harvested exactly as described above.

Immunoblot detection of FBP

FDHeLa or FDKB cells were plated into one 75 cm^2 culture flask (Corning) and incubated in serum-supplemented FFMEM at 37 °C until the monolayer was 90% confluent. After three rinses with 15 ml of warm PBS, 5 ml of warm FFMEM (no serum) containing 0.1 unit/ml PI-PLC was added to the flask and the cells were incubated for 1 h at 37 °C. The incubation medium was collected, concentrated 10-fold using a Centricon-30 (molecular-mass cutoff, 30 kDa), and then sterile-filtered through a 0.2 μm syringe filter. The folate-binding capacity of the concentrated incubation medium was then determined as

described elsewhere [24]. About 100 ng of FBP equivalents was loaded per lane of an SDS/12% (w/v) polyacrylamide gel as described by Laemmli [25], and the system was run at 10 mA for 15 h. Protein was transferred to 0.2 μm nitrocellulose strips (8 mm × 135 mm) using 400 mA for 2 h at 4 °C. Strips were blocked in 1.5% gelatin in Tris-buffered saline, pH 7.5 (TBS), for 1 h at 23 °C, followed by incubation in a 1:250 dilution of primary rabbit antiserum or control rabbit serum in TBS for 3 h at 23 °C. After three rinses in TBS containing 0.5% Tween 20, strips were incubated in a 1:1000 dilution of goat anti-(rabbit horseradish peroxidase) conjugate in TBS for 1.5 h at 23 °C. After three TBS/Tween 20 and two TBS rinses, strips were developed in a solution consisting of 60 mg of 4-chloro-1-naphthol, 20 ml of methanol, 100 ml of TBS and 60 μl of 30% H_2O_2 for 3 min. The strips were then rinsed with an excess of deionized water, air-dried and stored for photography.

Momordin–folate protection experiments

FDHeLa cells (1.5×10^5) suspended in 1 ml of complete FFMEM were plated into individual 35 mm × 10 mm Falcon culture dishes and incubated for 18 h at 37 °C. Each dish was rinsed twice with 1 ml of FFMEM followed by the addition of 1 ml of fresh FFMEM. Then 0.1 unit of PI-PLC was added and cells were incubated for 1 h at 37 °C. After two 1 ml rinses with FFMEM, the indicated monolayers were pulsed for 15 min at 37 °C with 0.1 μM momordin–folate. Dishes were then rinsed twice with 1 ml of FFMEM and then chased for 48 h at 37 °C in FFMEM containing 10% heat-inactivated fetal calf serum.

Cell viability was assessed using a modification of the [^{3}H]leucine incorporation assay previously described [11]. Briefly, incubation medium from each dish was replaced with 1 ml of leucine-free FFMEM (no serum) containing 10 μCi of [^{3}H]leucine, and the cells were incubated for 2 h at 37 °C to incorporate the radiolabel into newly synthesized protein. The radioactive medium was then replaced with 1 ml of cold 0.1 M KOH per dish. After an overnight incubation at 4 °C, protein was precipitated by adding 250 μl of cold 100% trichloroacetic acid (20% final) for 30 min and then collected on to glass-fibre filter disks by vacuum filtration. Filters were rinsed three times with 1 ml of 5% cold trichloroacetic acid followed by two washes with 1 ml of ethanol. Filters were individually added to glass vials containing 5 ml of an aqueous scintillation cocktail and counted for 1 min in a Packard Tri-Carb liquid-scintillation analyser model 1600CA. The values shown represent the percentage of radioactivity per dish relative to the control (untreated) dish of cells.

RESULTS

Time-dependence of ^{125}I-RNAase–folate association with FDHeLa cells

Folate–protein conjugates were previously shown to associate with cultured FDKB cells with a half-time of approx. 3 min [10]. As shown in Figure 1, ^{125}I-RNAase–folate was also found to interact with FDHeLa monolayers with similar kinetics. Half-maximal binding occurred 10 min after mixing the conjugate with the cells, and steady-state uptake was reached within 1 h at 37 °C. The association was found to be specific for the attached folate moiety since (i) underivatized ^{125}I-RNAase did not associate with FDHeLa cells and (ii) a 100-fold molar excess of free vitamin competitively eliminated the association of ^{125}I-RNAase–folate with the cells (Figure 1). We conclude that some type of FBP must be involved in mediating the interaction of folate–protein conjugates with HeLa cells.

Concentration-dependence of ^{125}I-RNAase–folate association with FDHeLa cells

To support the hypothesis that folate-conjugated proteins associate with FDHeLa cells via a cell-surface folate receptor, the binding and internalization of folate-conjugated RNAase were evaluated as a function of the concentration of RNAase. As shown in Figure 2, cells exposed to ^{125}I-RNAase–folate for 2 h at 4 °C bound a maximum of 1.6×10^7 molecules per cell. Scatchard analysis of the data revealed the K_d for ^{125}I-RNAase–folate binding to be 24 nM, a value comparable with the K_d observed with KB cells [10]. Because endocytosis is blocked at 4 °C, we hypothesized that the conjugates in this study might be folate receptor-associated, but not internalized. Since surface-bound folates can be readily removed from receptor-bearing cells by a brief wash at pH 3 [9,10,20], this stripping procedure was conducted to evaluate the cellular disposition of the interacting conjugates. As shown in Figure 2(a), virtually all of the radioactivity was released by the acid wash, suggesting that the conjugates had indeed not yet entered the cells. In contrast, when FDHeLa monolayers were maintained at 37 °C for the 2 h

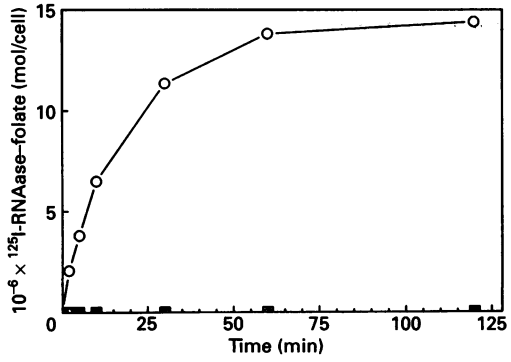

Figure 1 Time-dependent association of ^{125}I-RNAase–folate with cultured FDHeLa cell monolayers

Cells were incubated for the indicated times at 37 °C with 0.5 μg/ml ^{125}I-RNAase (□), ^{125}I-RNAase–folate (○) or ^{125}I-RNAase–folate plus a 100-fold molar excess of free folic acid (◇). After a wash, cell-associated RNAase was quantified as described in the Materials and methods section.

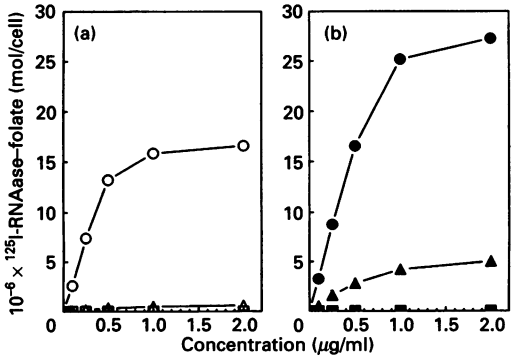

Figure 2 Concentration-dependent association of ^{125}I-RNAase–folate with FDHeLa cell monolayers

Cells were incubated for 2 h at 4 °C (**a**) or 37 °C (**b**) with the indicated concentrations of ^{125}I-RNAase (□,■), ^{125}I-RNAase–folate (○,●), or ^{125}I-RNAase–folate followed by acid/saline washes to remove externally bound conjugate (△,▲). Cell-associated RNAase was quantified as described in the Materials and methods section.

experiment, a maximum of 2.7×10^7 molecules of folate-labelled RNAase associated with each cell, and roughly 5×10^6 molecules of these remained after acid stripping (Figure 2b). This latter value closely approximates the reported level of folates inside other cultured cells when uptake was allowed to reach saturation [5]. Because we have directly observed that folate-labelled fluorescein–protein conjugates enter FDHeLa cells using confocal microscopy (results not shown), we suggest that this acid-inaccessible radioactivity corresponds to the endocytosed population of folate conjugates. Thus FDHeLa monolayers appear to have the same capacity to bind and internalize folate-labelled proteins as they do the free vitamin itself.

Removal of bound ^{125}I-RNAase–folate using PI-PLC

Folic acid has been reported to bind and enter many cells in complex with a membrane-associated FBP [1,2]. Interestingly, this receptor can be released into the culture medium as a soluble FBP if the cells are treated with PI-PLC, suggesting that glycosylphosphatidylinositol anchors the FBP to the membrane [15–19]. Because the data in Figures 1 and 2 imply the involvement of an FBP receptor in the binding and internalization of folate–protein conjugates, we have elected to examine the effect of PI-PLC on folate conjugate binding to FDHeLa monolayers. After a brief 10 min 37 °C pulse with ^{125}I-RNAase–folate, FDHeLa monolayers were treated with increasing concentration of PI-PLC for 30 min at 37 °C. As shown in Figure 3, very dilute enzyme concentrations (less than 1×10^{-4} units/ml) did not diminish the retention of ^{125}I-RNAase–folate on the membrane, since all of the initially bound 5×10^6 molecules per cell were recovered in the cell pellet. However, as the concentration of PI-PLC was raised, increasing amounts of ^{125}I-RNAase–folate were released into the supernatant (media) fraction. Maximum removal of bound conjugate (97.1 %) was achieved using 0.1 unit/ml PI-PLC, where only 1.5×10^5 molecules per cell remained cell-associated and probably intracellular. Importantly, PI-PLC treatment exerted a similar effect on FDKB monolayers (results not shown). Thus it appears that folate–protein conjugates associate with cell monolayers via a glycosylphosphatidylinositol-anchored membrane FBP receptor which may be similar to the FBPs reported to exist in other cultured cells [1,2,15–19].

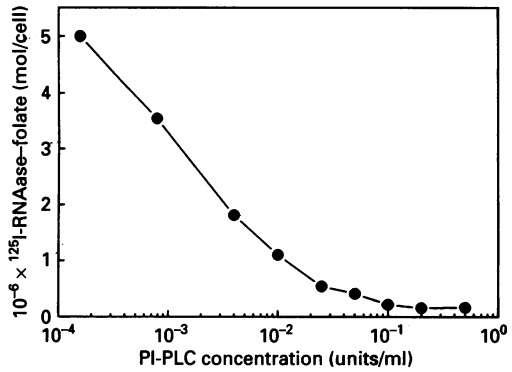

Figure 3 PI-PLC removal of bound ^{125}I-RNAase–folate from FDHeLa cells

FDHeLa cell monolayers were pretreated for 10 min at 37 °C with 2 μg/ml ^{125}I-RNAase–folate to permit the conjugate to bind to the cell surface. After a brief wash, PI-PLC was added at the indicated concentrations, and the cells were incubated for 30 min at 37 °C. The remaining cell-associated RNAase was quantified as described in the Materials and methods section. Note: FDHeLa cells not treated with PI-PLC bound a maximum of 5×10^6 molecules per cell when incubated under identical conditions.

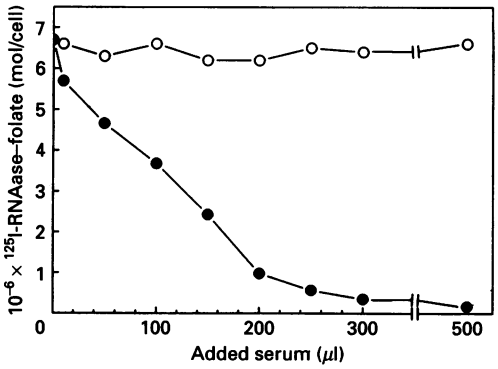

Figure 4 Inhibition of ^{125}I-RNAase–folate binding to FDHeLa cells with anti-FBP serum

FDHeLa cell monolayers were incubated for 60 min at 23 °C with the indicated amounts of rabbit anti-FBP serum (●) or control rabbit serum (○). Cells were pulsed for 15 min at 37 °C with 1 μg/ml ^{125}I-RNAase–folate and then cell-associated RNAase was quantified as described in the Materials and methods section.

Inhibition of ^{125}I-RNAase–folate association with FDHeLa monolayers by anti-FBP serum

To support our hypothesis that the folate receptor responsible for binding and internalizing folate–protein conjugates is a classic FBP, we elected to raise a polyclonal antiserum against a highly homologous commercially available FBP and used this antibody in competitive binding studies. Bovine milk FBP has been determined to have more than 90 % sequence identity with the membrane FBP receptors found in cultured cells [26]. For this reason, it was selected as the antigen for polyclonal antibody production in rabbits. As previously shown by Antony et al. [27], a polyclonal chicken antiserum raised against human placental FBP was effective at blocking 5-[^{3}H]methyltetrahydrofolate binding to KB cells in a concentration-dependent fashion [27]. Following a similar protocol, we have preincubated our FDHeLa cells with increasing volumes of antiserum (diluted in buffer such that the final volume was always 1 ml), and then pulsed the treated cells with 1 μg/ml ^{125}I-RNAase–folate for 15 min at 37 °C. As shown in Figure 4, progressively greater inhibition of ^{125}I-RNAase–folate binding to FDHeLa cells occurred as the antiserum content was raised. A maximum of 6.8×10^6 molecules per cell were bound in the absence of antiserum and complete inhibition was achieved at approx. 300 μl of antiserum (IC_{50} = 110 μl). This inhibition was found to be specific, since the same quantities of control rabbit serum were ineffective at reducing ^{125}I-RNAase–folate binding to FDHeLa cells (Figure 4). Therefore it appears that the PI-PLC-sensitive membrane folate receptor responsible for binding and internalizing folate–protein conjugates is a genuine FBP.

Immunoblot detection of FDHeLa FBP

FBPs have been purified in both soluble and membrane forms from a variety of sources [1,2]. The reported molecular masses vary considerably, a phenomenon attributed to differences in the extent of FBP glycosylation [2]. HeLa cells are known to contain a membrane FBP [12–14], yet the molecular mass of this receptor has never been published. Therefore we elected to analyse the PI-PLC-releasable proteins from intact FDHeLa cells in a Western-blot assay using our anti-FBP serum to determine the molecular

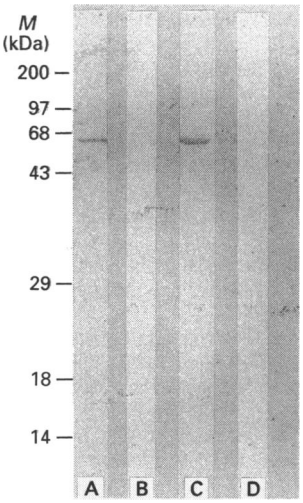

Figure 5 Immunoblot detection of FBP released from FDHeLa and FDKB cells by PI-PLC

Media from PI-PLC-treated FDHeLa (lanes A and B) or FDKB (lanes C and D) cell monolayers were collected and concentrated 10-fold before samples were loaded on to an SDS/12% polyacrylamide gel. Protein was transferred to 0.2 μm nitrocellulose strips and overlaid with a 1:250 dilution of rabbit anti-FBP (lanes A and C) or 1:250 dilution of control rabbit serum (lanes B and D) followed by goat anti-(rabbit horseradish peroxidase) conjugate. Immunoreactive bands were visualized using 4-chloro-1-naphthol as the chromogen. The migration position of standard molecular-mass (*M*) markers are shown to the left of lane A.

mass of the receptor for our folate–protein conjugates. For comparison, we also blotted the PI-PLC-releasable proteins from FDKB cells, a well-known FBP-containing cell line [4,13,15,20,26]. As shown in Figure 5, the immunoblot of the released proteins from FDHeLa cells revealed a single band at 65 kDa when probed with anti-FBP serum (lane A), but no bands when developed with a non-immune control (lane B). An identical 65 kDa band was also observed for FDKB cells (compare lanes C and D). These data suggest that FDHeLa cells contain a single membrane-bound PI-PLC-releasable FBP of molecular mass 65 kDa, similar to that found in FDKB cells.

Protection of FDHeLa cells from momordin–folate cytotoxicity

Recently, we demonstrated that simple attachment of folic acid to the impermeable ribosome-inactivating protein, momordin, rendered the resulting conjugate highly toxic to both FDKB and FDHeLa cells [11]. We interpreted this observation to indicate that folate–protein conjugates are not only internalized into cultured cells via an FBP, but are also capable of reaching the cytosol in a functionally active form. To confirm that the cytosol-directed population of internalized folate-conjugated proteins also enters cells via the classic FBP, we studied the effect of PI-PLC treatment on the toxicity of momordin–folate conjugates. As shown in Table 1, a single 15 min 37 °C pulse of 0.1 μM momordin–folate almost completely exterminated the FDHeLa cell population. Protein synthesis activity in the conjugate-treated cells was 4.7% of that measured in controls. In contrast, when the same cells were treated with sufficient PI-PLC to release nearly all of their membrane FBP receptors (0.1 unit/ml; see Figure 3), and then pulsed with momordin–folate, no significant cytotoxicity was observed. In fact, control cells treated with PI-PLC synthesized protein at the same rate (97% that of the untreated control) as the PI-PLC-treated cells pulsed with momordin–folate. Thus, it appears that folate–protein conjugates are internalized into cultured FDHeLa cells via a PI-PLC-releasable membrane FBP, the itinerary of which permits such conjugates to access the cytosol in a functionally active form.

Table 1 Protection of FDHeLa cell monolayers from momordin–folate cytotoxicity with PI-PLC pretreatment

FDHeLa cell monolayers were incubated in FFMEM supplemented with or without 0.1 unit/ml PI-PLC for 1 h at 37 °C. After a brief rinsing, the indicated monolayers were pulsed for 15 min at 37 °C with 0.1 μM momordin–folate, rinsed and then chased for 48 h at 37 °C in FFMEM containing 10% heat-inactivated fetal calf serum. Cellular protein synthesis was then measured as described in the Materials and methods section. Relative protein synthesis is expressed as a percentage of the total protein synthesis occurring in untreated control monolayers. Results are means ± S.D. of three experiments.

Treatment	Relative protein synthesis (%)
Control (untreated)	100
Momordin–folate	4.7 ± 0.26
PI-PLC	97 ± 4.2
PI-PLC + momordin–folate	97 ± 8.8

DISCUSSION

Previous reports suggested, but did not demonstrate, that folate-labelled proteins were endocytosed into cultured cells via a classical membrane FBP [10,11]. Together with these data, our current observations now provide strong evidence for this claim. First, folate conjugates of proteins such as RNAase, BSA, bovine γ-globulin and momordin bind and enter cultured FDHeLa or FDKB cells whereas the underivatized proteins do not [10,11]. Secondly, a 100-fold molar excess of free folic acid is capable of completely blocking folate–protein association with receptor-bearing cells [10,11], a phenomenon also observed for [^{3}H]folate uptake [5,27,28]. Thirdly, like the free vitamin [9,27], folate–protein conjugates can bind to, but not enter, cells at 4 °C, and this bound population is completely removable using brief acid/saline rinses [10]. This characteristic has also been demonstrated in a number of FBP-containing cell lines using ^{3}H-labelled folates [9,20,23]. Fourthly, bound conjugates can be quantitatively released from intact cells using PI-PLC, an enzyme that has been shown to release FBP from a variety of cell types [15–19,29]. And finally, anti-FBP serum was shown to block the binding of folate–protein conjugates to cultured FDHeLa cells. Taken together, the above observations strongly argue that a membrane FBP is responsible for the binding and internalization of folate-labelled proteins.

FBPs were recently identified as the surface antigen recognized in a number of cell types by tumour-specific monoclonal antibodies [14]. These antibodies identified a 38 kDa FBP which shared all of the characteristics of the FBP found in MA104 cells [14,30]. In contrast, higher-molecular-mass forms of FBP have been detected in a number of other cell types [1,18,21,22]. Although the differences in size have generally been attributed to differences in FBP glycosylation [2], the possible existence of multiple FBP isoforms has not been rigorously ruled out. In fact, placenta was recently found to contain two distinct types of FBP, a so-called 'fetal' form which is homologous to the 38 kDa FBP described above, and a higher-molecular-mass 'adult' form which is homologous to the FBP found in KB cells and human milk [31]. We assume that the PI-PLC-releasable FBP of FDHeLa and FDKB cells characterized in this study is more closely

related to the higher-molecular-mass class, since immunoblotting revealed its apparent molecular mass to be approx. 65 kDa.

Although both the 38 kDa and the 65 kDa FBP mediate the endocytosis of exogenous folates by receptor-bearing cells [1,3–5,10,11], some important mechanistic differences may exist between the two receptors. The 38 kDa FBP of MA104 cells can apparently bind and deliver 5-methyltetrahydrofolate into the cell's cytoplasm [9,17]. Folic acid, on the other hand, is found to bind and internalize, but is never released into the cytoplasm of the same cells [9]. Furthermore, the 38 kDa FBP receptor is reportedly not internalized into endosomes, but rather retained in association with the plasma membrane in a compartment termed the caveole [3,32]. Unlike the 38 kDa species, the 65 kDa receptor does enter endosomes and tubulovesicular elements dissociated from the plasma membrane [33]. Furthermore, folate and folate conjugates of proteins such as momordin, saporin, gelonin and ricin A are not only internalized but are also released by the 65 kDa protein into the cell's cytosol ([10,11] and data above). Thus important differences between folate-receptor classes may exist, and these differences may impact on the efficacy of targetting macromolecules for delivery into cells for medicinal purposes.

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