Gene Transfection and Expression of the Ovarian Carcinoma Marker Folate Binding Protein on NIH/3T3 Cells Increases Cell Growth in Vitro and in Vivo

Federica Bottero, Antonella Tomassetti, Silvana Canevari, Silvia Miotti, Sylvie Ménard, and Maria I. Colnaghi

ABSTRACT

Theglycoprotein gp38 is overexpressed in 90% of ovarian carcinomas and recognized by monoclonal antibodies MOv18 and MOv19. This molecule is a high affinity folate binding protein (FBP) and a potential marker for ovarian carcinoma. We have developed a model to investigate the biochemical and biological properties of this folate receptor by transfecting NIH/3T3 cells, which do not endogenously express FBP, with a vector containing the complementary DNA for the gp38 cloned from the ovarian carcinoma cell line IGROV1. The FBP expressed shows features identical to those of the protein produced by IGROV1 cell. The FBP is expressed on the cell membrane in a glycosylphosphatidylinositol-linked form, since it is released by treatment with phosphatidylinositol-specific phospholipase C, and is shed into the culture medium of the NIH/3T3 transfectants. Immunoblot analysis with MAbs MOv18 and MOv19 showed that both the glycosylphosphatidylinositol-linked and the soluble FBP migrate at the same apparent molecular weight as the respective IGROV1 proteins. The FBP-transfected NIH/3T3 cells bound folic acid and internalized about 30-fold more folic acid than mock-transfected cells. Growth analysis revealed that FBP-transfected NIH/3T3 cells like IGROV1 maintained their growth rate after 10 days of culture in medium containing physiological or low folate concentration, and tumors arising after transplanting FBP-transfected NIH/3T3 cells in nude mice were 3-fold heavier than those arising after transplantation of non-FBP-expressing NIH/3T3 cells. These results suggest a correlation between human ovarian carcinoma growth and FBP overexpression.

INTRODUCTION

Folic acid and its reduced compounds are essential vitamins for cell growth. Several studies have been shown that the uptake of these vitamins into cells involves membrane protein receptors of two different classes: a membrane carrier which binds reduced folate in the μM range, and a high-affinity FBP, which binds preferentially to oxidized folate and other analogues with an affinity below 1 nm (for a review, see Ref. 1). Folate receptors form clusters organized in closed caveolae (2), and their assembly is maintained by cholesterol (3). Once the receptor-vitamin interaction occurs, the whole complex is internalized, and the vitamin, dissociated from the receptor probably by acidification of the caveolae lumen, is delivered to the cell interior, while the FBP recycled to the cell surface (4).

Two distinct FBP forms have been isolated: a membrane and a soluble form. The membrane-bound FBP is anchored to the plasma membrane by a fatty acid linkage, which has been identified in some cells as a GPI tail with a molecular weight of about 40,000 (5). The soluble FBP has been found in human serum, in bovine, goat, and human milk, and in some biological fluids (6), as well as in the spent medium of the KB epidermoid carcinoma cell line (7) and the IGROV1 ovarian carcinoma cell line (8). Although the biological role of the membrane form is fairly well known, the role of the soluble form is not well defined. Efforts to determine a possible precursor-product relationship between the two forms have suggested the possible involvement of proteases in the generation of the hydrophilic soluble FBP from the membrane form (1), and the GPI-linked FBP has been identified in human placenta and in rat, human, and porcine kidney (9).

Two monoclonal antibodies, MOv18 and MOv19, derived in mice immunized with a crude membrane preparation from an ovarian carcinoma specimen (10), recognize a glycoprotein, gp38, which is overexpressed in 90% of ovarian carcinomas. Purification of the protein and cloning of the cDNA from IGROV1 have shown that this protein is a FBP (11, 12). Use of these two monoclonal antibodies has identified the FBP in some normal tissues (13), in particular, the fallopian tube and choroid plexus which express the highest levels of FBP compared to other normal tissues tested (13-15).

In addition to ovarian carcinomas, brain tumors also express FBP. However, ovarian carcinomas homogeneously overexpress FBP on the membrane, and a soluble form has been detected in ascitic fluids and in the sera of ovarian carcinoma patients (15).

In order to study the precursor-product relationship between the membrane and the soluble FBP, and the possible biological role of FBP overexpression in ovarian carcinoma, we analyzed the biochemical and biological characteristics of the murine NIH/3T3 fibroblasts transfected with IGROV1 cDNA with a focus on the possible influence of FBP expression on cell growth.

MATERIALS AND METHODS

Cell Lines and Reagents. IGROV1 cells (I. Bénard, Institute G. Roussy, Villejuif, France) were cultured in standard RPMI 1640 containing 2.3 μM folic acid (Irvine Scientific, Santa Ana, CA) or in folate-depleted RPMI 1640 with 20 nM or less than 1 nM folate (GIBCO, Paisley, United Kingdom) plus 5% FBS (Irvine Scientific). NIH/3T3 cells were cultured in standard Dulbecco’s modified Eagle’s medium containing 9.2 μM folate (Boehringer-Manheim, Germany) supplemented, before transfection, with 10% calf serum (Colorado Serum Company, Denver, CO), or, upon transfection, with 5% FBS and 800 μg/ml of genetin G418 sulfate (GIBCO). Folate-depleted medium consisted of Dulbecco’s modified Eagles medium with or without 20 nM folic acid (GIBCO) plus 5% FBS (<1 nM folate). One percent of penicillin-streptomycin was added to each medium.

Antibodies. Murine monoclonal antibodies MOv18 and MOv19 were purified as described (10). Anti-gp38 rabbit antiserum was used as total purified IgG (11).

Vector Construction. Human FBP cDNA (11), named FBP-31, was cloned into the EcoRI sites of pBluescript II KS +/- (Stratagene, La Jolla, CA), to generate the HindIII/XbaI sites necessary for cloning in the expression vector. pBluescript II KS +/- was amplified in Escherichia coli XL-1 Blue. The HindIII/BglII FBP-31 insert was subsequently cloned into the pcDNA/neo vector and amplified in Escherichia coli MC1061/P3 (Invitrogen, San Diego, CA). Transcription of the relevant cDNA was under the control of the cytomegalovirus promoter.

Transfection. Cells were transfected using the DNA-calcium phosphate coprecipitation technique essentially as described (16). Briefly, NIH/3T3 cells were harvested by trypsinization and replated at a density of 5 × 10⁶ in 90-mm Petri dishes in Dulbecco’s modified Eagle’s medium plus 10% FBS. After 24 h, the medium was replaced and 3 h later, 10 μg of either the vector containing...
human FBP cDNA (FBP-31 NIH/3T3) or the vector alone (mock-niH/3T3), plus 10 μg of carrier NIH/3T3 DNA diluted in 1X HBS [0.05 M CaCl2 in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid-buffered saline] was added to the cells. After 3 days, transient expression was tested by indirect immunofluorescence with 10 μg/ml each of MOv18 and MOv19. Cells were stained using affinity-purified antibody fluorescein-labeled goat anti-mouse IgG (Kirkegaard and Perry Laboratories, Gaithersburg, MD) and analyzed by FACScan (FACS IV, Becton Dickinson, San Jose, CA). Cells were then seeded at a density of 100 cells/well in a 96-well plate, and geneticin G418 sulfate (Kirkegaard and Perry Laboratories, Gaithersburg, MD) was added to the growth medium. After incubating the cells with PI-PLC and Protein

Binding of Folic Acid. To eliminate endogenous folate, the PI-PLC-solubilized protein samples were dialyzed for 48 h at 4°C against 0.9% NaCl, adjusted to pH 3 with acetic acid, and further dialyzed against PBS to reach physiological pH. Binding to [3H]-folic acid (32 Ci/mmol, Amersham) was performed as described (8). The protein concentration of the PI-PLC-solubilized membrane preparation was determined according to Bradford (18). A standard curve was established using bovine serum albumin (Sigma, St. Louis, MO) at a concentration of 1 mg/ml.

Immunoprecipitation. IGROV1 cells (5 × 10⁵) were seeded and grown for 5 days in a T25 flask. FBP-niH/3T3 and mock-niH/3T3 cells (1 × 10⁶) were seeded in 90-mm Petri dishes and grown for 8 days. One ml of conditioned medium from each cell type was immunoprecipitated using MOv18 immobilized on CNBr-activated Sepharose CL4B as described (10). Samples were incubated overnight at 4°C, with gentle shaking. After removing the unbound fractions by centrifugation, the matrices were washed twice with buffer containing 1 μg NaCl, 0.02 μl Tris-HCl (pH 7.4), 0.001 μl EDTA, 0.5% Nonidet P-40, 1% Antisera, 0.001 μl phenylmethylsulfonyl fluoride, and twice with the same buffer without NaCl. The precipitated molecules were extracted from Sepharose using SDS-polyacrylamide gel sample buffer (8) containing 0.025% SDS. Samples were electrophoresed on a 12% SDS-polyacrylamide gel, transferred onto nitrocellulose, and assayed as described above. To avoid the high background due to the use of the anti-mouse IgG, blots were first incubated with the anti-gp38 rabbit IgG and subsequently with a goat anti-rabbit biotinylated antibody (Amersham).

DDIRMA. DDIRMA was performed using immobilized MOv18 and ¹²⁵I-MOv19 as described (19). IGROV1, FBP-, and mock-niH/3T3 PI-PLC-solubilized proteins were evaluated for FBP-immunoreactive units defined as the amount of immunoreactivity of 1 ml of the standard solution of IGROV1 supernatant (1). The same assay was also performed on 100 μl of the three conditioned media in serial dilutions (see above).

Cell Growth Analysis. For in vitro analysis IGROV1, FBP-, and mock-niH/3T3 cells, precultured in standard or folate-depleted medium, were

![Fig. 1. Expression vector construct. The pcDNAIneo/FBP-31 is the FBP-31 cDNA (1003 base pairs) (11) inserted in HindIII/XbaI sites of pcDNAIneo vector.](image1)

![Fig. 2. Characterization of the GPI-linked FBP. FBP- and mock-niH/3T3 cells were treated with PI-PLC and analyzed by indirect immunofluorescence assay using MOv18 and MOv19 (A) and by immunoblotting of PI-PLC-digested FBP (B). A, peak 1, mock-niH/3T3; peaks 2 and 3, FBP-niH/3T3 PI-PLC treated and untreated, respectively. In B, samples were electrophoresed (12% SDS-polyacrylamide gel), blotted onto nitrocellulose, and reacted with MOv18 and MOv19 using the enhanced chemiluminescence Western blot detection system.](image2)

![A](chart1)  ![B](chart2)
seeded at a density of $10^6$/dish in standard and folate-depleted medium with or without 20 nm folic acid. Each set of cells was harvested and counted daily for 5 days and again on day 10. For in vivo analysis FBP- or mock-tNIH/3T3 cell suspensions containing $4 \times 10^6$ cells in 0.2 ml 0.9% NaCl were inoculated s.c. into each of 10 4-5-week-old CD1 nude mice (Charles River, Calco, Como, Italy). Tumors were measured with a caliper on days 8, 13, and 16 after injection. Tumor volumes were calculated as

$$V = \frac{a \times b^2 \times 0.4}{2}$$

where $a$ and $b$ are the tumor length and width (cm), respectively. On day 16, mice were sacrificed and tumors were weighed. Differences in weight between the membrane and the soluble forms of FBP, the culture medium of FBP-tNIH/3T3 cells was assayed for the presence of soluble FBP. DDIRMA, performed on 5- and 8-day conditioned medium from IGROV1 and FBP-tNIH/3T3, respectively (Fig. 3A), indicated the presence of soluble FBP in the medium. No immunoreactivity was detected in the spent medium of mock-tNIH/3T3 cells.

**RESULTS**

**Biochemical Characterization of tFBP.** The FBP-31 cDNA corresponding to the human FBP was transfected into NIH/3T3 cells which do not constitutively express the FBP. The pcDNAIneo/FBP-31 expression vector construction is reported in Fig. 1. Three days after transfection, 1% of the transfected cells showed transient expression of the tFBP as determined by indirect immunofluorescence using both MOv18 and MOv19 (data not shown). After 1 month of growth in selective medium, 50% of the transfected cells were positive for FBP expression by immunofluorescence. To obtain a homogeneous cell population, cells were further selected using monoclonal antibody MOv18 and the resulting cells (virtually 100% positive by FACScan) were used to analyze the biological features of the newly expressed tFBP.

The tNIH/3T3 cells were treated with PI-PLC and analyzed by FACScan using MOv18 and MOv19 (Fig. 2A); reactivity on the PI-PLC-treated cells was reduced 95% confirming that tFBP is anchored to the FBP-tNIH/3T3 membrane by a GPI tail. Immunoblot analysis of PI-PLC-digested tFBP (Fig. 2B) revealed a single band of about $M_t 38,000$ migrating at the same position as the corresponding IGROV1 protein. The same samples were tested for their ability to bind folic acid after removing endogenous folate (Table 1). The amount of folic acid bound/ mg of PI-PLC-solubilized proteins from FBP-tNIH/3T3 is about one-half of that bound by FBP-tNIH/3T3 proteins, but the amount of tFBP is about one-third of the IGROV1 FBP. The discrepancy between the two ratios might be due to physiological folic acid still bound to IGROV1 protein.

To test the hypothesis that a precursor-product relationship exists between the membrane and the soluble forms of FBP, the culture medium of FBP-tNIH/3T3 cells was assayed for the presence of soluble FBP. DDIRMA, performed on 5- and 8-day conditioned medium from IGROV1 and FBP-tNIH/3T3, respectively (Fig. 3A), indicated the presence of soluble FBP in the medium. No immunoreactivity was detected in the spent medium of mock-tNIH/3T3 cells. Immunoprecipitation on immobilized MOv18, followed by immunoblotting with a rabbit antiserum raised against the IGROV1 FBP, revealed a $M_t 36,000$ protein in conditioned medium from both IGROV1 and FBP-tNIH/3T3 cells (Fig. 3B).

**Biological Activity of tFBP.** To investigate whether FBP overexpression could increase folate uptake and thereby affect cell proliferation, cell growth rate was evaluated. IGROV1, FBP-, and mock-tNIH/3T3 were cultured in standard, folate-depleted medium, or folate-depleted medium plus 20 nm folic acid and cell number was determined over 10 days. The growth curves of FBP-tNIH/3T3 and mock-tNIH/3T3 cells were the same when they were cultured in standard medium (Fig. 4A), whereas mock-tNIH/3T3 cells grown in 20 nm folic acid gradually decreased the growth rate (Fig. 4B). Mock-tNIH/3T3 cells in less than 1 nm folate showed a decrease in cell number already evident at 4 days and about 10-fold on day 5, when they began to die, as compared with FBP-tNIH/3T3 cells grown in folate-depleted medium (Fig. 4C). The proliferation rate of IGROV1 remained unaltered when the cells were cultured in standard (Fig. 4A), folate-depleted medium plus 20 nm folic acid (Fig. 4B), or folate-depleted medium (Fig. 4C). Thus, IGROV1 and FBP-tNIH/3T3 cells both maintained growth rates independent of folate in medium.

To test the effect of FBP expression on cell proliferation in vivo, FBP- and mock-tNIH/3T3 were s.c. transplanted into nude mice. Indeed, both cell lines, having been cultured for 8-9 months, were able

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**Table 1. Folic acid binding of PI-PLC-solubilized membrane protein**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>FBP-immunoreactive units$^a$</th>
<th>pmol $^3$H-folic acid/mg of protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>IGROV1</td>
<td>37</td>
<td>64</td>
</tr>
<tr>
<td>FBP-NIH/3T3</td>
<td>14</td>
<td>35</td>
</tr>
<tr>
<td>Mock-NIH/3T3</td>
<td>2</td>
<td>5</td>
</tr>
</tbody>
</table>

$^a$ See "Materials and Methods" (DDIRMA).

$^b$ Below detection limits of the assay.
to proliferate \textit{in vivo}. Tumor volume was monitored over a 16-day period, the mice were sacrificed, and the tumors were weighed. Only the tumors originating from FBP-tNIH/3T3 cells were already evident by touch after 8 days and grew regularly thereafter; tumors originating from mock-tNIH/3T3 grew more slowly and, by day 13, began to decrease in proliferation rate (Fig. 5A). The difference in proliferation capacity between the cell types was even more striking when tumor weights at day 16 were compared; tumors from the FBP-tNIH/3T3 had a mean volume 3 times greater than those from the mock-tNIH/3T3 \((P = 0.004)\) (Fig. 5B). The FBP expression was tested by immunohistochemical assay on cryostatic tumor sections by the anti-gp38 rabbit antiserum. On the tumors arisen after injection of FBP-tNIH/3T3 cells an intense immunostaining was detected in discrete areas whereas the remaining tumor tissue was mildly reactive in a diffuse pattern. No reactivity was found on tumors deriving from mock-tNIH/3T3 cells (data not shown).

**DISCUSSION**

The present report describes the transfection of the cDNA encoding the human FBP in the murine fibroblast cell line NIH/3T3. The transfected cells express a GPI-linked protein which is released in soluble form into the cell culture medium. Furthermore, the experiments demonstrate the involvement of FBP overexpression in cell growth \textit{in vitro} and, for the first time, \textit{in vivo}.

The FBP expressed on the surface of tNIH/3T3 is highly sensitive to the action of PI-PLC, demonstrating that the transfected cDNA contains the consensus sequences specific for the GPI protein. These include the 17-hydrophobic domain and the cleavage/attachment site at the COOH-terminus, constituted by serine 234/glycine 235, to which the GPI tail is attached after removing the COOH-tail in the endoplasmic reticulum (20). Other investigators (21) have hypothesized that a transmembrane FBP might be coexpressed with the GPI-anchored protein. Indeed, other cell surface proteins, such as N-CAM, can be expressed in the same cells in a transmembrane form and as a GPI-anchored protein. In the case of N-CAM, the two forms are expressed by two mRNAs derived from differential splicing (22). Whereas the presence of two forms of the membrane FBP can be excluded in FBP-tNIH/3T3 cells, based on the result of PI-PLC treatment, this possibility exists in ovarian carcinoma cells, which as cell lines as biopsy specimens show variable susceptibility to PI-PLC (23).

The soluble hydrophilic form of FBP has been reported to be present in the culture medium of tumor cell lines (7, 8) and in the ascitic fluids of ovarian carcinoma patients (15). The present data indicate that a soluble FBP is also detectable in the culture medium of tNIH/3T3 cells. Since a unique cDNA was transfected in this cell line, a precursor-product relationship between the membrane and the soluble forms of FBP can be hypothesized. Recently, we reported that the soluble form of FBP from IGROV1 appeared to migrate on SDS-polyacrylamide slab gel at a lower molecular weight than the membrane FBP (8). Present evidence that the transfected and IGROV1 FBP migrate at the same apparent molecular weight and that both share the same antigenic determinants suggests that posttranslational modifications of the two proteins are very similar. Experiments are in progress to verify the size of the native protein of both IGROV1 and FBP-tNIH/3T3 cells. These findings also suggest close similarity in the mechanism involved in FBP shedding. However, the enzyme(s) involved in this mechanism remains unknown. Some proteases have...
be reported to generate the soluble form in "in situ" models (26–28), but there are no data as yet to indicate whether they also act in physiological conditions.

Our experiments using medium that contains a high concentration of folic acid suggest that NIH/3T3 cells can access folate through a carrier and/or a mechanism of passive diffusion. Under these conditions, mock- and FBP-tNIH/3T3 cells show the same growth potential. However, mock-tNIH/3T3 cells grown in low-folate medium exhibited a dramatic decrease in growth potential since they cannot regulate constitutive expression of FBP and since the other mechanisms of folate uptake are probably not sufficient. By contrast, tFBP binds to folic acid and its reduced compounds and enables cell growth under conditions of folate deficiency. This observation is consistent with those of other studies (29–31) conducted with cell lines transfected with FBP cDNAs obtained from different sources.

IGROV1 cells also maintained their growth capability in the presence of very low amounts of folic acid in agreement with previous studies carried out on KB (32) and MA104 (33) cells. Together, the in vitro results obtained to date indicate that FBP overexpression allows cells to proliferate in folate-depleted medium.

Our in vivo experiments confirm the involvement of FBP in cell proliferation and reproduce the in vitro results obtained at physiological concentration of folate (about 20 nM). Tumors expressing FBP clearly grew faster than those not expressing the protein. Indeed mock-tNIH/3T3 decrease their growth rate at physiological condition as shown by in vitro experiment at 20 nM folate.

Based on our results, we can hypothesize that FBP expression contribute to the in vivo growth of ovarian carcinomas, although it remains unclear why FBP is overexpressed in those tumors. Since FBP is not detectable on normal ovarian epithelium, it is possible that its overexpression is a consequence of transformation. Due to metabolic changes, ovarian carcinoma cells might require higher amounts of folate for growth, or, alternatively, might become unable to utilize endogenously provided folate. In either case, the cells increase their folate uptake by activating an efficient system mediated by FBP.

In conclusion the FBP-tNIH/3T3 cell line appears to be a good model to investigate the biochemical and biological features of FBP. Moreover, considering the high percentage of ovarian tumors that overexpress FBP, the transfected cells could be exploited both in vitro and in vivo to study therapeutic approaches which utilize FBP as a target structure.

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REFERENCES

17. Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of Fig. 5. Effect of FBP overexpression on cell growth in vivo. Mock- and FBP-tNIH/3T3 cells were transplanted into 10 nude mice and tumor growth was monitored within 16 days by determining the volumes as described in “Materials and Methods.” Values represent mean tumor volume or weight ± SD. A, tumor growth curve of mock- and FBP-tNIH/3T3. B, weight of tumors derived from mock- and FBP-tNIH/3T3 cells. Bars, SD.
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