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Isolation and biochemical characterization of the soluble and membrane forms of folate binding protein expressed in the ovarian carcinoma cell line IGROV1

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The human ovarian carcinoma cell line, IGROV1, produces two forms of folate binding protein (FBP), the membrane form that is anchored to the cell surface by a glycosylphosphatidylinositol tail and the soluble form that is shed into the tissue culture medium. Both forms are recognized by the monoclonal antibodies MOv18 and MOv19. Here we describe their purification and biochemical characterization. The purified soluble protein appeared as a single band with an apparent M_r of 36 kDa after SDS-PAGE, whereas the membrane form appeared as a single band with an apparent M_r of 38 kDa. The size difference between the two forms of FBP was confirmed by gel filtration of both the native and the *N*-glycanase-treated proteins. Both purified proteins had equal capacity to bind folic acid. The immunological cross-reactivity and the folic acid binding capability of the FBPs extracted from IGROV1 gave more evidence of the possible existence of a precursor–product relationship between them.

Folate binding protein; Ovarian carcinoma; Glycosylphosphatidylinositol linkage

1. INTRODUCTION

The monoclonal antibodies MOv18 and MOv19 recognize a glycoprotein molecule with a M_r of kDa that is over-expressed in ovarian carcinoma cells [1]. This membrane glycoprotein expressed by the IGROV1 ovarian carcinoma cell line can be released from the cell surface by treatment with PI-PLC, confirming the presence of a GPI linkage [2]. The cDNA corresponding to the GPI-linked protein was recently cloned by Coney et al. [3] from an IGROV1 cDNA library and by Campbell et al. [4] using cDNA libraries established from SKOv3 ovarian carcinoma and HT29 colon carcinoma cells confirming its identity as human FBP. A GPI-linked form was also reported to exist in the membrane of the KB epidermal carcinoma cell line [5] and human placenta [6]. A soluble form of FBP has also been found in the growth medium of KB [7]. Pulse-chase experiments have indicated a probable precursor–product relationship between the membrane-bound and soluble forms of FBP produced by KB cell line [8]. In addition, cDNA clones obtained from several different carcinoma cell lines, including the KB CaCo-2 and IGROV1 cell lines, share an identical coding region, supporting the existence of an interrelationship between the membrane and soluble forms [3,4,9,10]. We report here the purification of both proteins and a comparison of their biochemical properties.

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Abbreviations: sFBP, soluble folate binding protein; mFBP, membrane folate binding protein; GPI, glycosylphosphatidylinositol; PI-PLC, phospholipase C; PBS, phosphate-buffered saline; DDIRMA, double-determinant radioimmunoassay.

2. MATERIALS AND METHODS

2.1. *Cells*

The human ovarian carcinoma cell line IGROV1 was kindly supplied by Dr. Bénard (Institut G. Roussy, Villejuif, France). The cell line was grown in roller bottles and maintained in RPMI-1640 containing a standard concentration of folic acid (2.3 μM) supplemented with 5% fetal calf serum, 1 mM sodium pyruvate, 2 mM L-glutamine.

2.2. *Antibodies*

The murine MOv18 (1,k) and MOv19 (2a,k) monoclonal antibodies were used in a purified form [1].

2.3. *DDIRMA*

The concentration of relevant proteins was measured using a DDIRMA performed as described [1]. The standard solution was a purified membrane protein at a known concentration.

2.4. *Purification of the membrane and soluble proteins*

Membrane-bound FBP was released from IGROV1 cells by treatment with PI-PLC from *Bacillus thuringiensis* (American Radiolabelled Chemicals, St. Louis, MO) as described [3]. The supernate

derived from PI-PLC treated cells and the spent medium were independently purified on a MOv18 immunoaffinity column as described [3].

The purified proteins were loaded onto a non-reducing 12.5% SDS-PAGE essentially accordingly to Laemmli [11], containing 0.025% SDS. The gel was then stained using a Silver Stain kit (Bio-Rad) or transferred onto nitrocellulose as described [12]. The immunoreaction on the transferred protein with MOv18 and MOv19 was performed as already described [3].

2.5. *Deglycosylation*

Samples (50 μl) were incubated for 1 h at 37°C in the presence of 0.02% SDS, 10 mM EDTA, 1% β-mercaptoethanol, 10 mM potassium phosphate buffer, pH 7.4, in a final volume of 90 μl. β-Octylglucoside was added to a final concentration of 0.5%. The incubation with 0.5 units of endoglycosidase F/N-glycosidase F (Boehringer Mannheim, Germany) was performed overnight at 37°C. Control samples were incubated in the same conditions but in the absence of the enzyme. All samples were analyzed by HPLC as described below.

2.6. *Gel filtration*

The gel filtration by HPLC was carried out on a TSK G3000 SW column (7.5 × 600 mm, Pharmacia LKB, Uppsala, Sweden), equilibrated in 100 mM sodium phosphate buffer, pH 7.4. Samples of the native and deglycosylated proteins (0.1 ml) were filtered on a 0.45 μm HV filter (Millipore S.A. Malsheim, France) and separated at a flow rate of 0.5 ml/min. Fractions of 0.5 ml were collected and analyzed by DDIRMA for the presence of antigenic activity. The standards used for calibration of the column were bovine thyroglobulin (670 kDa), bovine gamma globulin (158 kDa), chicken ovalbumin (44 kDa), horse myoglobulin (17 kDa) and vitamin B-12 (1.35 kDa) supplied by Bio-Rad, CA.

2.7. *Binding to folic acid*

Each purified protein was incubated in PBS with a 2-fold molar excess of [^{3}H]folic acid (40.4 Ci/mmol, Amersham, UK) at 20°C in a dark room for 12–15 h. The protein–folic acid complex was separated from the free folic acid by gel filtration on Sephadex G-25 equilibrated in 50 mM borate buffer, pH 8, 150 mM NaCl and 0.25% gelatine to reduce non-specific binding. The radioactivity contained in each of the eluted fractions was diluted 1:10 (v/v) with Pico-Fluor 15 (Packard) and measured in a Beckman counter type LS 1801. Alternatively, each purified protein was pretreated with cold 0.3 M acetic acid (final concentration), pH 3, for 1 h at 4°C. The protein was separated from the endogenously bound folic acid by Sephadex G-25 as described above. The elution of the folate-free proteins was monitored by DDIRMA as described. The protein content of the acid-treated eluted samples was assumed to be equivalent to the content of the corresponding untreated purified proteins eluted from the same column. The eluted folate-free samples were assayed for folic acid binding as described above.

3. RESULTS

The FBPs were isolated from the membrane (mFBP) and the spent medium (sFBP) of IGROV1 cells by single-step immunoaffinity chromatography on the monoclonal antibody MOv18. Fig. 1A shows the silver staining of SDS-PAGE on the purified forms of the FBP. The protein purified from the IGROV1 conditioned medium (lane 2) appeared as a single band migrating to an apparent M_r of 36 kDa, whereas the purified protein, obtained after treatment of the IGROV1 membrane with PI-PLC (lane 1), migrated as a single band corresponding to 38 kDa. The immunoblotting of the purified proteins with MOv18 or MOv19 was consistent

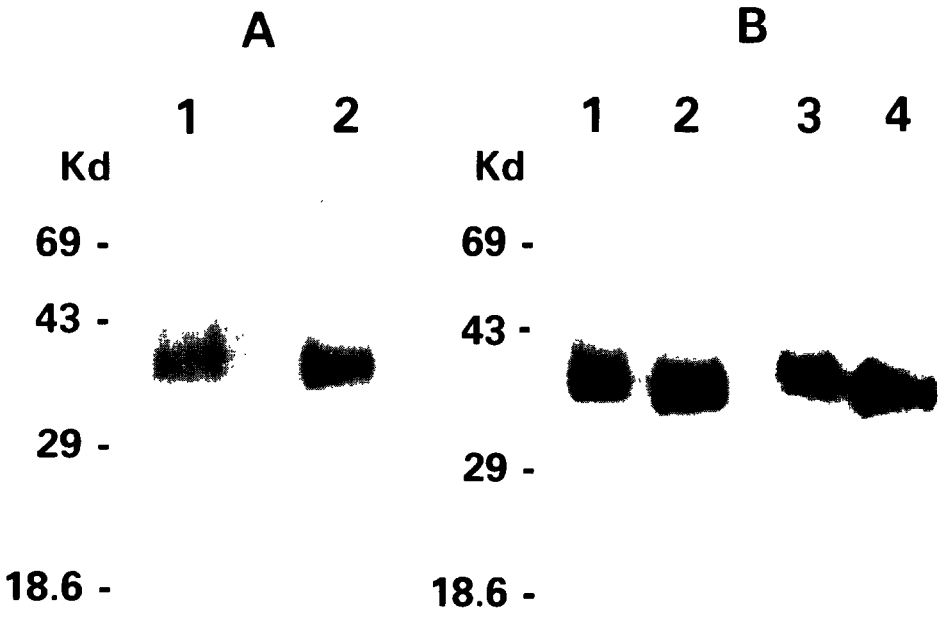

Fig. 1. Characterization of the mFBP and sFBP purified from IGROV1. (A) 12.5% SDS-PAGE of the purified FBPs. Samples were silver-stained following SDS-PAGE. Lane 1, mFBP; lane 2, sFBP. (B) Immunoblot of the purified FBPs: lanes 1 and 2, mFBP and sFBP incubated with MOv18, lanes 3 and 4, mFBP and sFBP incubated with MOv19.

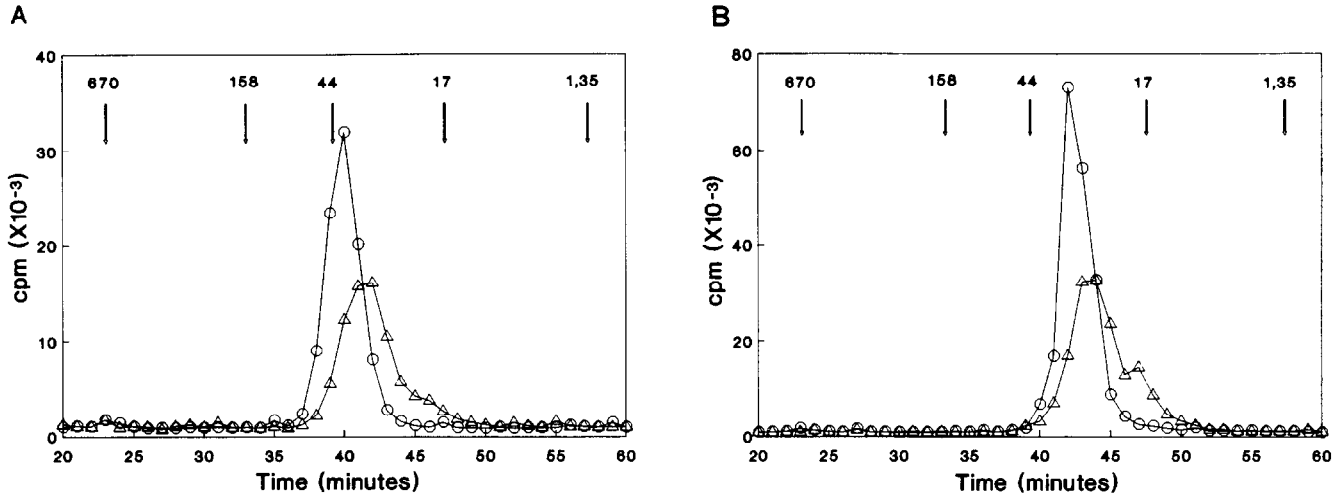

Fig. 2. Gel filtration by HPLC of the native and the deglycosylated FBPs purified from IGROV1. (A) Purified mFBP before (⊖–⊖) and after (△–△) the deglycosylation. (B) Purified sFBP before (⊖–⊖) and after (△–△) the deglycosylation. Arrows indicate where the molecular weight markers eluted.

with the results obtained by silver staining (Fig. 1B). To confirm the results obtained by SDS-PAGE, the purified mFBP and sFBP were submitted to gel filtration and the elution was monitored by DDIRMA. The mFBP was eluted as a single peak corresponding to 40 kDa and the sFBP also as a single peak corresponding to 32 kDa (Fig. 2. An aliquot of the obtained immunoreactive peaks, analyzed in immunoblotting, resulted to migrate at the same apparent M_r of the corresponding loaded sample (data not shown). The result obtained by gel filtration of the sFBP was confirmed in several experiments carried out on either the affinity-purified or the raw material. To investigate whether the difference in M_r was due to a different pattern in the *N*-linked carbohydrates, the membrane and the soluble forms were treated with *N*-glycosidase. After enzymatic treatment the digested proteins were analyzed by gel filtration chromatography. Again two single peaks were obtained corresponding respectively to the digested mFBP and sFBP. As shown in Fig. 2, the peak corresponding to mFBP was eluted in a range between 36 and 29 kDa and the peak of sFBP was eluted in a range between 22 and 18 kDa. By the way, the *N*-glycosidase-digested mFBP and sFBP analyzed by SDS-PAGE migrated as single bands, respectively of about 28 kDa [7] and 24 kDa (data not shown).

Table I

Source	Protein (μg)	pmol of folic acid bound to the cells*	
		Untreated	Treated
1×10^6 cells treated with PI-PLC (mFBP)	0.12	0.2	2.0
10 ml medium (sFBP)	0.11	0.099	3.2

*Acid treatment was utilized to released endogenously bound folic acid.

To test the capability of mFBP and sFBP to bind folic acid, the purified proteins were incubated with [^{3}H]folic acid as described in section 2 and the relevant results are reported in Table I. Both preparations bound very small amounts of folic acid. Assuming that the FBP is univalent for folate binding, only less than 10% of each protein was able to bind to folic acid. Since the MOv18 and MOv19 epitopes are different from the binding site of folic acid and analogues, as demonstrated by the binding of folic acid to the complexes of mFBP or sFBP with MOv18 or MOv19 (data not shown), the presence of endogenous folate still bound to the purified proteins could not be excluded. In order to investigate if the low folate binding capability of the FBPs was due to the occupancy of the binding site, an acid treatment was performed. The two preparations were treated with acetic acid and the uncomplexed proteins were separated from the free folic acid by gel filtration. After acid treatment one mol of the purified mFBP and of the sFBP (M_r of 40 kDa and 32 kDa, according to HPLC), respectively, bound 0.7 mol and 0.94 mol of folic acid (Table I). The lower folate binding capability of the mFBP could be attributed to a loss of the folate binding capability and/or of the protein amount due to manipulation.

4. DISCUSSION

High-affinity FBP is involved in the assimilation, distribution and retention of the vitamin folic acid. Two forms of FBP have been described, a membrane-bound form and a soluble form found in the culture supernates of some tissue culture cell lines. The membrane-bound form delivers 5-methyltetrahydrofolic acid to the cyto-

plasm of folate-depleted cells (reviewed in [13,14] and is over-expressed by 90% of ovarian adenocarcinoma tumors and tissue culture cell lines. The ovarian carcinoma cell line IGROV1 is a useful system to investigate the relationship between the different forms of FBPs. This cell line over-expresses a membrane-associated FBP which is solubilized by treatment with PI-PLC, and sheds a soluble form into the cell culture medium [2,15]. The two purified forms appear to have different sizes either as native, denatured or deglycosylated molecules. The M_r of the mFBP from IGROV1 was equivalent to that obtained previously by treatment with PI-PLC of the mFBP purified from KB cells [3]. On the other hand, the M_r of both preparations from IGROV1 calculated after gel filtration are different from those observed analyzing the FBPs from KB [4,5]. In the latter, however, the fractionation was performed under different condition [16].

Experiments carried out on metabolically labelled KB cells, indicates that sFBP originates from mFBP removed from the cell membrane [8]. This is in keeping with our finding that the mFBP and sFBP have properties indicating that they share at least three sites of protein sequence. In fact, they are both able to bind folic acid to the same degree. In addition, both proteins are recognized by two monoclonal antibodies, MOv18 and MOv19 [2,15], which bind to two different epitopes, thus indicating that they share two antigenic determinants. This fact, in addition to the lack of the cross-reactive determinant on the soluble protein [15] might suggest that the shedding of the protein could be due to cleavage by a protease. In vitro studies on erythrocytes FBP demonstrated that the hydrophobic form of FBP is converted to the hydrophilic form by trypsin [17]. Moreover, a Mg^{2+}-dependent protease from human placenta [18] and a metalloprotease from KB cell [19] have been identified which can generate the hydrophilic form of FBP. It is also possible that the sFBP is first released by the action of an endogenous phospholipase and then cleaved by a protease. Finally, the membrane and soluble forms of FBP could be encoded by different members of the same gene family or that they arise through alternative splicing of transcripts from the same gene as is a possible explanation for the origin of the polypeptide-anchored and phosphatidyl-inositol-anchored forms of the human immunoglobulin G receptor CD 16 [20].

A model has been suggested in which the folate receptor recycles while remaining tightly associated to the cell membrane [21]. However, it is still unclear why the receptor is shed from the cell membrane. The ovarian carcinoma seems to be a good model for studies concerning the folate metabolism and the biological importance of FBPs in tumor cells.

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