High-affinity folate binding in human choroid plexus

Characterization of radioligand binding, immunoreactivity, molecular heterogeneity and hydrophobic domain of the binding protein

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High-affinity [³H]folate binding in solubilized human choroid plexus homogenate displayed characteristics, e.g. apparent positive co-operativity, which are typical of specific folate binding. The highest folate-binding activity per g of protein was associated with the 27000 g membrane pellet where the membrane-marker enzyme γ -glutamyltransferase had its main localization. Ultrogel AcA 44 chromatography revealed two major folate-binding proteins (molecular masses > 110 kDa and approx. 100 kDa) and one minor one (molecular mass approx. 25 kDa) in the Triton X-100-solubilized membrane pellet. After exposure of the membrane pellet to phosphatidylinositol-specific phospholipase C there was only one large 25 kDa peak of folate binding. This could suggest that the folate-binding protein is anchored to the membrane by a glycosylphosphatidylinositol moiety, which can be inserted into Triton X-100 micelles and thus can give rise to forms of large molecular size on gel filtration. This notion was supported by the identical molecular masses of the > 110 kDa and 25 kDa folate-binding peaks determined by SDS/PAGE and immunoblotting. The folate-binding protein in choroid plexus cross-reacted with rabbit antibodies against the 25 kDa human milk folate-binding protein, and paraffin-embedded sections of choroid plexus showed immunostaining after exposure to rabbit anti-(human milk folate-binding protein) serum (1:8000 dilution).

INTRODUCTION

Choroid plexus, the anatomical locus of the blood–cerebrospinal fluid barrier, plays an important rôle in transport and homeostasis of vitamins and micronutrients in the central nervous system (Spector, 1977a, 1989). Folates are present in the cerebrospinal fluid and brain of mammals at higher concentrations than in plasma (Spector, 1989). Folates must therefore enter the cerebrospinal fluid/brain from the blood since they are not synthesized in mammalian tissues. It has been suggested that a mechanism for transfer of folates from blood into the cerebrospinal fluid might reside in the choroid plexus (Spector, 1977a, 1989).

A folate-binding macromolecule (340–400 kDa) has previously been identified in the solubilized particulate fraction of rabbit choroid plexus homogenate (Spector, 1977b). Folate binding was of a high-affinity type ($K = 10^{10}$ l/mol); it exhibited two peculiar characteristics, i.e. positive co-operativity and an affinity that increased with decreasing concentration of binder (Spector, 1979), both of which have been described for other high-affinity folate-binding systems, e.g. in bovine milk (Hansen *et al.*, 1978, 1983a; Salter *et al.*, 1981), human milk (Hansen *et al.*, 1977, 1980), serum (Holm *et al.*, 1980), granulocytes (Holm *et al.*, 1985) and urine (Hansen *et al.*, 1989a). A folate-binding protein (FBP) from hog choroid plexus solubilized with Triton X-100 has been purified to homogeneity (Suleimann & Spector, 1981). Interaction of Triton X-100 micelles with the protein was assumed to explain the large apparent molecular mass (200 kDa) of FBP on Sephadex G-200 gel filtration as compared with that observed on SDS/PAGE (51 kDa; Suleimann & Spector, 1981). A partially purified FBP from human choroid plexus (21 nmol of FBP/g of protein) seemed to be membrane-bound since it required the

presence of Triton X-100 above critical micellar concentration for optimum folate binding (Suleimann *et al.*, 1981). On SDS/PAGE its molecular mass ranged from 45 to 60 kDa, whereas an apparent molecular mass of 200 kDa was found on Sephadex G-200 gel filtration (Suleimann *et al.*, 1981).

We have established the presence of a high-affinity FBP in human cerebrospinal fluid (Hansen *et al.*, 1985). The protein was shown to cross-react with rabbit antibodies against low-molecular-mass FBP from human milk (Hansen *et al.*, 1989b). The present study deals with a high-affinity FBP in human choroid plexus, and describes its radioligand-binding characteristics, subcellular localization, immunological properties, molecular heterogeneity and hydrophobic domains. Because of the instability of 5-methyltetrahydropteroylglutamate, the major form of endogenous folate (O'Broin *et al.*, 1975), we preferred [³H]-pteroylglutamate as the radioligand.

MATERIALS AND METHODS

Tissue preparation

Human choroid plexus was homogenized with a Braun homogenizer (5 strokes at 1000 rev./min) in 5 mM-Tris/HCl buffer (pH 7.4, 4 °C) containing 5 mM-mannitol (5 ml of buffer/g of tissue). Homogenate solubilized (2 h, 4 °C) with Triton X-100 (20 g/l, Triton X-100/protein ratio 4.7) was centrifuged (1000 g, 30 min) and the supernatant analysed. Membrane pellets were prepared after CaCl₂ precipitation (Kessler *et al.*, 1978). Homogenate left at 4 °C for 20 min after addition of CaCl₂ (10 mM) was centrifuged (1000 g, 15 min), and the pellet (A) solubilized with Triton X-100 for 2 h at 4 °C (Triton X-100/protein ratio 11.4). The supernatant from A was centrifuged (27000 g, 30 min) and

Abbreviations used: FBP, folate-binding protein; PMSF, phenylmethanesulphonyl fluoride; PBS, phosphate-buffered saline (8 mM-sodium/potassium phosphate/0.5 M-NaCl, pH 7.2).

the pellet (B) solubilized (2 h; 4 °C) in Triton X-100 (Triton X-100/protein ratio 25.0).

The concentration of protein was determined by a commercial assay (Bio-Rad Protein Assay Kit, 500-0001). Measurement of γ -glutamyltransferase (EC 2.3.2.2) was by a method involving the use of an Ultrospec photometer (Pharmacia) (Committee on Enzymes, 1976).

Radioligand binding and dissociation

Tissue preparations were dialysed against 0.2 M-sodium acetate buffer (pH 3.5, 4 °C) to remove endogenous folate (see below). Equilibrium-dialysis experiments were performed as described (Hansen *et al.*, 1983a).

Portions (1 ml) of choroid plexus homogenate supernatant predialysed against [3 H]folate (1.0 nM) were dialysed in 1-litre volumes of folate-free buffer (0.17 M-Tris, pH 7.4, or 0.2 M-acetate, pH 3.5) according to a previously published procedure (Holm *et al.*, 1980).

E.l.i.s.a. for FBP in human choroid plexus

Rabbit antisera against low-molecular-mass FBP from human milk (Svendsen *et al.*, 1982; Hansen *et al.*, 1983b) were pooled, and the immunoglobulins were precipitated and employed in an e.l.i.s.a. (Høier-Madsen *et al.*, 1987).

Gel filtration

The molecular size of [3 H]folate-bound FBP was estimated by chromatography in the presence of Triton X-100 (1 g/l) on a column (5.3 cm² × 94 cm) of Ultrogel AcA 44 (IBF) (Holm *et al.*, 1980). Samples were treated with or without the proteinase inhibitor, phenylmethanesulphonyl fluoride (PMSF) (1 mM; BDH).

Immunoprecipitation

The experimental procedure was similar to that of Østerman *et al.* (1979). Briefly, 200 μ l of the 100 kDa FBP (see above) was mixed with 200 μ l of rabbit anti-(human FBP) serum diluted with Tris buffer (pH 7.4) containing 1 g of Triton X-100/l. The mixture was incubated for 1 h at 25 °C before addition of 50 μ l of rabbit serum (Dako, cat. no. 902) and 1 ml of an aqueous solution of 150 g of poly(ethylene glycol) 6000 l (Merck, cat. no. 807491). After centrifugation (2000 g, 20 min, 4 °C) 500 μ l of supernatant was taken for radioactivity measurements (control experiments with the peak of free [3 H]folate).

Treatment of choroid plexus B pellet with phospholipase C and phosphatidylinositol-specific phospholipase C

An aqueous solution of phospholipase C (*Bacillus cereus*, 245 units/ml) supplied by Sigma (P 6135) was mixed (6 μ l) with 62 μ l of 40 mM-Hepes buffer (pH 7.4) containing BSA (2 g/l) and 1 mM-EDTA. A volume of 50 μ l of solubilized B pellet treated with 1 mM-PMSF was added to the enzyme solution and the mixture of B pellet and enzyme (final enzyme concentration 12.5 units/ml) was left at 37 °C overnight (solution B). A parallel incubation of B pellet without enzyme (solution A) was performed. Further, to exclude the possibility that the enzyme possessed folate-binding activity, a solution of enzyme was exposed to [3 H]folate and subjected to gel filtration.

Solutions of B pellet preincubation with (B) or without (A) enzyme (see above) were incubated in 700 μ l of 0.17 M-Tris/HCl (pH 7.4) buffer containing Triton X-100 (10 g/l) and 10 nM-[3 H]folate for 1 h at 25 °C before Ultrogel AcA 44 chromatography.

A similar experiment was performed with phosphatidylinositol-specific phospholipase C (*B. cereus*, EC 3.1.4.10) from Sigma (P8804) (3 μ l; 125 units/ml) using a 3 h incubation.

SDS/PAGE (Laemmli, 1970) and immunoblotting (Kyhse-Andersen, 1984)

These were performed on fractions obtained after gel chromatography of solubilized choroid plexus homogenate concentrated 10-fold (Hansen *et al.*, 1989b). Rabbit anti-(human milk FBP) serum and normal rabbit serum (control) were diluted 1:50 in phosphate-buffered saline (PBS) containing 0.05 % Tween 20. The second antibody, anti-rabbit (total) Ig, (Dako), was diluted 1:1000 in PBS. *o*-Dianisidine was used as the chromogen.

Immunohistochemistry

Immunohistochemistry on paraffin-embedded 5–8 μ m sections of formalin-fixed choroid plexus was performed by a standard procedure (Hsu & Raine, 1981, 1984). A commercial kit (Vector Stain ABC kit, Vector Laboratories, Burlingame, CA, U.S.A.) was used. The primary antibody [rabbit anti-(human milk FBP) serum] was diluted 1 to 8000 with 12.5 % (w/v) BSA. Parallel controls using normal rabbit serum (diluted 1:8000) were performed.

RESULTS

Mechanism of radioligand binding and dissociation in choroid plexus

Saturation curves for radioligand binding in choroid plexus homogenate at two FBP concentrations are shown in Fig. 1 (Scatchard plots inserted). Binding data analysed in Scatchard and Hill plots (not shown) are summarized in Table 1. The

Fig. 1. High-affinity binding of [3 H]folate in solubilized choroid plexus homogenate (●) and a 5-fold dilution (○)

Equilibrium-dialysis experiments in 0.17 M-Tris buffer (pH 7.4, 37 °C). Scatchard plots of binding data are given as insets (left, diluted samples; right, undiluted samples).

Table 1. Parameters of high-affinity binding of [3 H]folate to choroid plexus homogenate in equilibrium-dialysis experiments (pH 7.4, 37 °C)

Binding data were analysed by Scatchard and Hill plots. *N*, maximum folate bound; *S*_{0.5}, the concentration of free folate at half-saturation of folate binding.

	<i>N</i> (nM)	<i>S</i> _{0.5} (nM)	<i>h</i> (S.D.)
Undiluted	1.8	0.028	1.24 (0.065)*
Fivefold-diluted	0.30	0.012	1.06 (0.049)

* The Hill coefficient (*h*) is significantly higher than 1.00: *P* < 0.005 (calculation of *h* based on 19 observations).

Fig. 2. Relationship between maximum [³H]folate binding (abscissa) and immunoreactive FBP determined by e.l.i.s.a. in human milk FBP (●), choroid plexus homogenate (○), choroid plexus 1000 g (A) membrane pellet (□) and choroid plexus 27000 g (B) membrane pellet (■)

The results are from duplicate determinations.

Table 2. Localization of FBP in human choroid plexus

Relative immunoreactivity is immunoreactive FBP/maximum [³H]folate binding. Concentration and units are per g of protein.

	γ -Glutamyl-transferase (units/g)	Maximum [³ H]folate binding (nmol/g)	Immuno- reactive FBP (nmol/g)	Relative immuno- reactivity
Choroid plexus homogenate	8	5.1	2.6	0.51
1000 g (A) pellet	36	8.0	3.0	0.38
27000 g (B) pellet	88	28.8	4.5	0.16

Fig. 3. Ultrogel AcA 44 chromatography of solubilized choroid plexus 27000 g (B) membrane pellet dialysate incubated with [³H]folate before column application

The column was calibrated as previously described (Holm *et al.*, 1980). The results are from duplicate determinations. Left ordinate, radioactivity (c.p.m.) (●) in the eluate. Right ordinate, immunoreactive FBP in eluate (○) determined by e.l.i.s.a. ($A_{492/630 \text{ nm}}$).

binding displayed apparent positive co-operativity as indicated by a Hill coefficient significantly higher than 1.00 and an upward convex Scatchard plot (Fig. 1) at FBP concentrations of 1.8 nM.

Fig. 4. Ultrogel AcA 44 chromatography of choroid plexus 27000 g membrane pellet dialysate

The samples were incubated in the absence (○) or presence (●) of phospholipase C before exposure to [³H]folate. The column was calibrated as previously described (Holm *et al.*, 1980). The results are from duplicate determinations.

Fig. 5. Separation by SDS/PAGE and immunoblotting [rabbit anti-(human milk FBP)] of the > 110 kDa (lane 1) and the 25 kDa peak (lane 2) obtained after gel filtration of choroid plexus 27000 g (B) membrane pellet (see Fig. 3)

Corresponding control experiments with preimmune rabbit serum are shown in lanes 3 and 4. Molecular-mass (M) markers are shown on the right.

The concentration of FBP seemed to affect binding affinity, i.e. concentration of free [³H]folate required for half-saturation of binding ($S_{0.5}$) decreased from 0.028 nM at 1.8 nM-FBP to 0.012 nM at 0.30 nM-FBP, corresponding to an increase in binding affinity ($1/S_{0.5}$) from 3.6×10^{10} to 8.3×10^{10} l/mol (Table 1). Further, at 0.30 nM-FBP the binding was non-co-operative, i.e. the Hill coefficient did not differ significantly from 1.00 (Table 1), and the Scatchard plot was rectilinear (Fig. 1).

No dissociation of [³H]folate from choroid plexus homogenate occurred at pH 7.4 even after dialysis for several days. At pH 3.5 a total dissociation of [³H]folate occurred within 24 h of dialysis (results not shown).

Immunoreactivity of FBP in choroid plexus

A two-site e.l.i.s.a. was used for quantification of FBP in choroid plexus preparations where FBP expressed as maximum [³H]folate binding was used as the reference. Titration curves for

Fig. 6. (a) Immunostaining of choroid plexus sections after exposure to rabbit anti-(human milk FBP) serum and (b) control choroid plexus sections exposed to preimmune rabbit serum

The dilution in each case was 1:8000.

purified human milk FBP, choroid plexus homogenate, A pellet and B pellet in Fig. 2 demonstrate a particularly low immunoreactivity of FBP in the B pellet. Exposure of tissue samples to folate before analysis had no effect on immunoreactivity (results not shown).

Localization of FBP in choroid plexus

Table 2 shows the localization of FBP and γ -glutamyl-transferase in the choroid plexus. A parallel increase in enzyme activity/g of protein and [3 H]folate binding/g of protein was seen from choroid plexus homogenate to B pellet. The modest increase in immunochemically determined FBP corresponded to a drastic fall in the relative immunoreactivity from 0.51 in choroid plexus homogenate to 0.16 in the B pellet.

Gel filtration of FBP in the B pellet

As shown in Fig. 3, [3 H]folate-bound FBP and immunoreactive FBP were eluted in three peaks: I, molecular mass > 110 kDa at 189 ml; II, molecular mass approx. 100 kDa at 225 ml; III, molecular mass approx. 25 kDa at 297 ml. Addition of 1 mM-PMSF to solutions of the B pellet had no influence on the gel-filtration pattern (results not shown). Further, rabbit anti-FBP serum (1:100 dilution) precipitated 75% of the 100 kDa FBP, but not the free [3 H]folate peak (results not shown).

Effect of phospholipase C and phosphatidylinositol-specific phospholipase C on the gel-filtration profile of FBP in the B pellet

After exposure to these two enzymes, peaks I and II disappeared from the gel-filtration diagram which now only contained one large peak III (25 kDa) of FBP (data obtained with phospholipase C are shown in Fig. 4). No FBP was found in the gel-filtration profile of enzyme solutions saturated with [3 H]folate (results not shown).

SDS/PAGE and immunoblotting

As shown in Fig. 5, SDS/PAGE and immunoblotting of > 110 kDa and 25 kDa FBP (see Fig. 3) gave rise to identical bands (molecular masses approx. 67000).

Immunohistochemistry

Fig. 6(a) shows immunostaining of choroid plexus cells exposed to rabbit anti-(human milk FBP) serum (1:8000 dilution). No staining reaction (Fig. 6b) occurred with normal rabbit serum (1:8000 dilution).

DISCUSSION

Folate binding in human choroid plexus exhibits characteristics typical of high-affinity folate-binding systems in milk, human biological fluids and granulocytes (Hansen *et al.*, 1977, 1978, 1980, 1983a, 1989a; Salter *et al.*, 1981; Holm *et al.*, 1980, 1985), for example (1) apparent positive co-operativity, which changed into a simple non-co-operative binding at relatively low FBP concentrations, (2) a binding affinity that seemed to increase with decreasing concentrations of FBP and (3) an extremely slow ligand dissociation at pH 7.4, which became rapid and complete at pH 3.5.

The highest concentration of FBP was associated with membrane preparations where the enzyme γ -glutamyltransferase has its main localization (Salgo & Szabo, 1982).

The gel-filtration profile of solubilized membrane pellet (B pellet) contained two major FBPs (> 110 kDa and 100 kDa) and one minor (25 kDa) FBP. Similar FBPs were found in human cerebrospinal fluid where the 25 kDa FBP, however, prevailed (Hansen *et al.*, 1985, 1989b). Phosphatidylinositol-specific phospholipase C decreased the apparent molecular size of the > 110 and 100 kDa FBPs to 25 kDa in the presence of the proteinase inhibitors PMSF and EDTA; apart from being a proteinase inhibitor, EDTA inhibits phosphatidylcholine-specific but not phosphatidylinositol-specific phospholipase C (Ikezawa & Taguchi, 1981). An enzyme-induced cleavage of a hydrophobic glycosylphosphatidylinositol moiety that inserts FBP into Triton X-100 micelles could account for this phenomenon as well as the identical molecular masses of > 110 kDa and 25 kDa FBPs on SDS/PAGE immunoblotting. By analogy, membrane and soluble forms of FBP from human KB cells were similar with respect to amino acid composition, N-terminal amino acid sequence and molecular mass on SDS/PAGE, but differed in that membrane FBP contained a glycosylphosphatidylinositol membrane anchor (Luhers *et al.*, 1986, 1987; Luhers & Slomiany, 1989), which explained its apparently larger molecular mass on gel filtration, and its release from membranes by phosphatidylinositol-specific phospholipase C (Luhers & Slomiany, 1989). Likewise, membrane FBP could be released from its glycosylphosphatidylinositol membrane anchor on human CaCo-2 cells (Lacey *et al.*, 1989).

By analogy with FBP in erythrocyte membranes (Antony *et al.*, 1987a,b), the variation in relative immunoreactivity (Fig. 2 and Table 2) could be due to the presence of immunoreactive precursor FBP which was unable to bind [3 H]folate. Alter-

natively, one might suggest the existence of immunoreactive holo-FBPs without vacant binding sites (no binding of exogenous folate), i.e. FBPs that do not release endogenous folate at low pH. The latter hypothesis seems attractive in view of the ability of the high-affinity receptor on the surface of monkey kidney cells to internalize folate by receptor-mediated endocytosis to an intracellular compartment where the bound material became resistant to acid release (Kamen & Capdevila, 1986). In contrast, surface-bound folate could be released by incubation of the cells at pH 3.5 (Kamen & Capdevila, 1986). The low relative immunoreactivity (Fig. 2 and Table 2) of FBP localized in choroid plexus membranes is thus in fair agreement with the latter theory.

We appreciate the valuable technical assistance of Mrs. Jytte Rasmussen, Mrs. Lotte Stummann and Mrs. Mona Pedersen. The study received financial support from the Bryde Nielsen Foundation.

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Received 25 February 1991/31 May 1991; accepted 6 June 1991