Folate binding in intestinal brush border membranes: evidence for the presence of two binding activities

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Binding of [^{3}H]folic acid by isolated rat jejunal brush border membranes (BBMs) was analyzed by chromatography on small Biogel P-30 columns. Folic acid binding to BBMs exhibited a prominent pH effect with a sharp maximum at pH 5.5 to 6.0. After acid treatment to strip the BBMs of bound folate, the membranes demonstrated a wider pH optimum (5.5 to 7.5) of folate binding and a higher binding capacity. Scatchard analysis of binding experiments performed at pH 6.0 revealed the existence of two components: one with a high affinity (kd = 12 to 25 nM) and low capacity (V_{max} for non-acidified BBMs = 0.259 to 0.264 pmol/mg protein, V_{max} for acidified BBMs = 0.41 to 0.71 pmol/mg protein) and the other with a low affinity (kd = 1.1 to 5.1 μM and high capacity (V_{max} for non-acidified BBMs = 0.93 to 1.93 pmol/mg protein, V_{max} for acidified BBMs = 4.05 to 7.69 pmol/mg protein). Phosphatidylinositol-specific phospholipase C preferentially detached the high affinity component from jejunal BBMs. Phosphatidylinositol-specific phospholipase C-released folate binding protein was precipitated by antibodies to the high-affinity folate-binding protein from rat kidney. These data suggest the existence of two different folate-binding proteins in isolated rat jejunal BBMs. The high-affinity folate-binding protein shares epitopes with the folate-binding protein in the kidney.

Keywords: Folate; binding; jejunum; brush border membranes; phosphatidylinositol-specific phospholipase C; pH.

Introduction

Folate transport in the intestine is a carrier-mediated, pH-dependent process with maximum transport occurring at a pH of approximately 6.0.[1-5] It is postulated that specific folate-binding protein(s) function as part of the carrier system which is responsible for the transport of folate across the membrane.[6]

In 1972, Leslie and Rowe[7] reported the presence of a low-affinity folate-binding protein in the brush border membrane (BBM) fraction of intestinal cells. Our laboratory subsequently reported the presence of a high-affinity folate-binding protein in rat jejunal BBMs.[8]

The present study was undertaken to reevaluate folate binding activity in isolated BBMs from rat jejunum and to determine if these activities are due to more than one protein species.

Materials and methods

Materials

[^{3}H]pteroylglutamic acid ([^{3}H]PteGlu, 26 to 45 Ci/mmol) was purchased from Amersham (Arlington Heights, IL, USA). Phosphatidylinositol-specific phospholipase C (PIPLC) was provided by Dr. Martin G. Low, Columbia University, New York, NY. Phosphatidylcholine-specific phospholipase C (PCPLC) and goat anti-rabbit IgG were obtained from Sigma Chemicals (St. Louis, MO, USA). Biogel P-30 was from Bio-Rad Laboratories (Richmond, CA, USA). Antibody to the high-affinity folate-binding protein in

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the kidney was raised in rabbits, as described by Selhub and Franklin.[9]

Preparation of brush border membranes from rat jejunum

The jejunum was removed from unfasted male Sprague-Dawley rats (150 to 300 g) and flushed with cold (4°C) saline. The jejunal mucosa was then removed by scraping the intestine. Brush border membranes were promptly prepared from these mucosal scrapings by the calcium precipitation method[10] as modified by Knickelbein et al.[11] Sucrase activity was enriched 14.2- ± 4.8-fold in the vesicle preparation compared with the crude homogenate when expressed in units per milligrams of protein.[12]

In those instances in which acidified membranes were used, the jejunal BBMs were treated with 0.05 M acetic acid (pH 3.0 to 3.5, at 4°C for 10 minutes) to remove endogenously bound folate[13] and collected by centrifugation at 37,000 × *g* for 15 minutes.

Binding of [^{3}H]pteroylglutamic acid by jejunal brush border membranes

Isolated BBMs, in both the acidified and nonacidified experiments, were first treated with 0.1% saponin in 0.3 M mannitol, 10 mM Hepes/Tris (pH 7.4) for 30 minutes at room temperature. Saponin, a mild detergent, has previously been shown to quantitatively eliminate intravesicular uptake of folate and, thus, to provide an accurate means of measuring the membrane-binding component of uptake.[14] Saponin-treated membranes were incubated at room temperature with 0.1 μM [^{3}H]PteGlu in 0.1 M sodium phosphate buffer (pH 6.0) or universal buffer (citrate-phosphate-borate/NaOH) of the desired pH (3.0 to 9.0) for 30 minutes. These incubation mixtures were extracted with 1% Triton X-100 in the same pH buffer at room temperature for 1 hour and centrifuged at 37,000 × *g* for 15 minutes. Supernatant fractions were analyzed for protein-bound [^{3}H]PteGlu by chromatography on Biogel P-30 columns (bed volume, 1.8 ml) which had been equilibrated with the same pH buffer. Separation of protein-bound versus nonprotein-bound [^{3}H]PteGlu by gel filtration Biogel columns has previously been described.[13]

Membrane detachment of folate-binding protein(s) by phosphatidylinositol-specific phospholipase C

After saponin treatment, acidified membranes were incubated with 0.08 μM [^{3}H]PteGlu at room temperature in mannitol buffer (pH 7.4) for 20 minutes. Excess free [^{3}H]PteGlu was removed with 20 volumes of cold mannitol buffer (pH 7.4) by centrifugation at 37,000 × *g* for 15 minutes. Thereafter, [^{3}H]PteGlu-labeled membranes were incubated at 37°C with either 100 mU/ml PIPLC, 100 mU/ml PCPLC, or no enzyme in mannitol buffer (pH 7.4). Aliquots were collected at desired time points and centrifuged at 37,000 × *g* for 60 minutes. Protein-bound [^{3}H] activity in the supernatant fractions was analyzed by Biogel P-30 columns at pH 7.4. Membrane-bound [^{3}H] activity in the pellet fractions was also measured.

In a parallel set of experiments, alkaline phosphatase activities both in the supernatant and pellet fractions were determined. Previous studies[15] have established that alkaline phosphatase is released from rat jejunal BBMs by treatment with PIPLC.

Immunoprecipitation of cleaved folate-binding protein by antibodies to kidney folate-binding protein

Rat jejunal BBMs were labeled with [^{3}H]PteGlu as described above. These membranes were treated at 37°C with 100 mU/ml PIPLC in mannitol buffer (pH 7.4) for 1 hour and centrifuged at 37,000 × *g* for 15 minutes. Control membranes were treated with 1% Triton X-100 in the same condition.

Thereafter, supernatant fractions were incubated at room temperature with increasing concentrations (0 to 7.2 pmol/ml) of polyclonal antiboides to the high-affinity folate-binding protein in 0.5 ml of 0.2 M potassium phosphate buffer (pH 7.4) containing 1.0% bovine serum albumin for 30 minutes. These incubation mixtures were then reacted at room temperature with 0.01 ml (which precipitates 0.02 mg of antibody) of goat anti-rabbit IgG for 10 minutes. These were precipitated twice by centrifugation at 2,000 × *g* in 3.7% polyethylene glycol (PEG 8000) for 10 minutes. Radioactivity in the pellet fractions was measured.

Binding of [^{3}H]pteroylglutamic acid by phosphatidylinositol-specific phospholipase C-treated brush border membranes

Brush border membranes were incubated at 37°C with or without 100 mU/ml PIPLC in mannitol buffer (pH 7.4) for 15 minutes in the absence of radioactive folic acid. After incubation, membranes were collected by centrifugation at 37,000 × *g* for 15 minutes and analyzed for residual folate-binding activity as described above. The results are expressed as picomoles of PteGlu bound per milligrams of protein.

Other assay methods

The alkaline phosphatase activity was determined with p-nitrophenyl phosphate as substrate.[16] Protein was determined by the method of Lowry et al.,[17] with bovine serum albumin as the standard.

Results

Effect of pH on binding of pteroylglutamic acid by jejunal brush border membranes

Pteroylglutamic acid binding to jejunal BBMs exhibited a prominent pH effect with a sharp maximum at pH 5.5 to 6.0. In a parallel set of experiments, membranes were first treated with 0.05 M acetic acid (pH 3.0 to 3.5, at 4°C, for 10 minutes) to remove endoge-

nous folate bound to the high-affinity folate-binding protein. Acid-treated membranes demonstrated a wider pH optimum at 5.5 to 7.5 and a higher binding capacity (*Figure 1*).

Concentration dependency on binding of pteroylglutamic acid

Pteroylglutamic acid binding in relation to substrate concentration was tested at pH 6.0. Scatchard analysis revealed the existence of two PteGlu-binding components in jejunal BBMs (*Figure 2*). One component has a high binding affinity and low capacity, while the other has a low binding affinity and high capacity. The dissociation constant for the high affinity component was 12 to 25 nM and that for the low affinity component was 1.1 to 5.2 μM. Values for V_{max} in nonacidified BBMs were 0.259 to 0.264 pmol/mg protein and 0.93 to 1.93 pmol/mg protein, respectively. In acidified BBMs, V_{max} values were 0.41 to 0.71 pmol/mg protein and 4.05 to 7.69 pmol/mg protein, respectively.

Detachment of folate binding protein from jejunal brush border membranes by phosphatidylinositol-specific phospholipase C

Figure 3 shows the time course of folate-binding protein detachment from jejunal BBMs. Phosphatidylinositol-specific phospholipase C cleaved approximately 2.5 times more folate-binding protein (50% of total) than controls at 37°C after 100 minutes of incubation. With PCPLC (or in the absence of enzymes), less than 20% of the total folate-binding protein was released.

Immunoprecipitation of cleaved folate-binding protein

In the studies shown in *Figure 4*, the antibody to the high-affinity folate-binding protein from the kidney

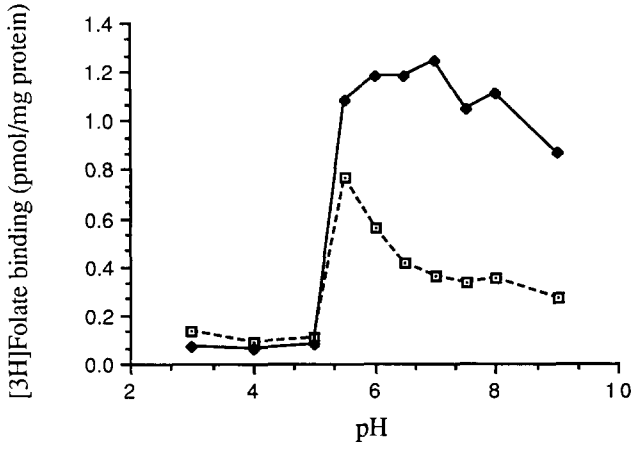

Figure 1 pH Dependency curves of PteGlu binding by BBMs. Brush border membranes were treated with (◆) or without (⊡) 0.05 M acetic acid, incubated with 0.1 μM [^{3}H]PteGlu in universal buffer (citrate-phosphate-borate/NaOH) of various pHs (3.0 to 9.0) for 30 minutes, and extracted with 1% Triton X-100 in the same pH buffer for 1 hour. Protein-bound [^{3}H]PteGlu was analyzed by chromatography on Biogel P-30 columns. Values are expressed in pmoles per milligram of protein.

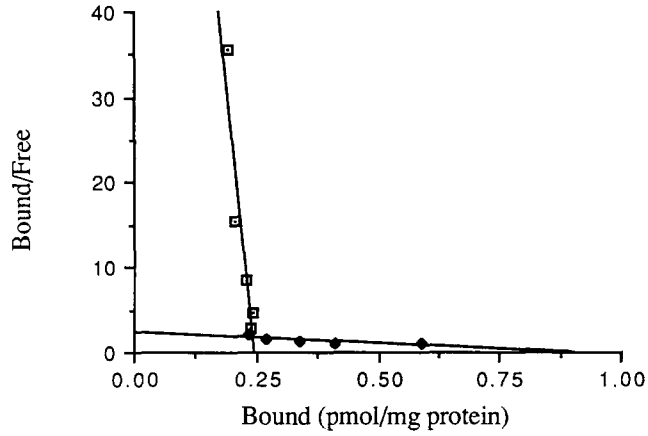

Figure 2 Scatchard analysis of PteGlu binding by jejunal BBMs. Nonacidified BBMs were incubated with various concentrations (0.01 to 2.5 μM) of PteGlu at pH 6.0 for 30 minutes and treated with 1% Triton X-100 in the same buffer for 1 hour. Protein-bound PteGlu was analyzed by chromatography on Biogel P-30 columns. ⊡, A high-affinity, low-capacity component; ◆, a low-affinity, high-capacity component.

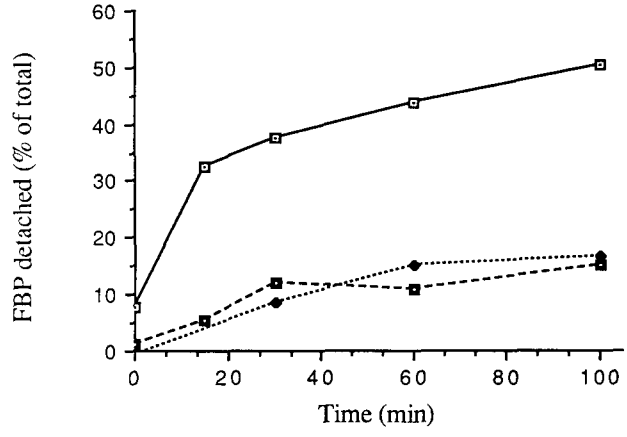

Figure 3 Membrane detachment of folate-binding protein by PIPLC. [^{3}H]pteroylglutamic acid-labeled rat jejunal BBMs were incubated with 100 mU/ml PIPLC (⊡) or PCPLC (◆) or without enzymes (■) in mannitol buffer (pH 7.4). Detached protein-bound [^{3}H]PteGlu from BBMs was measured by Biogel P-30 columns.

was tested for its capacity to interact with the binding protein in the jejunum. These studies demonstrate that the antibody interacts equally with the jejunal folate-binding protein solubilized by Triton X-100 and the activity which is cleaved from the membrane by the action of PIPLC.

Binding of [^{3}H] pteroylglutamic acid by phosphatidylinositol-specific phospholipase C-treated brush border membranes

Following incubation with or without PIPLC, residual folate-binding activity of the membrane was analyzed. The PIPLC treated membranes still demonstrated distinct high- and low-affinity binding activities. *Table 1* shows the V_{max} values obtained from Scatchard analysis of concentration dependency studies. Approximately 70% of the high-affinity folate-binding com-

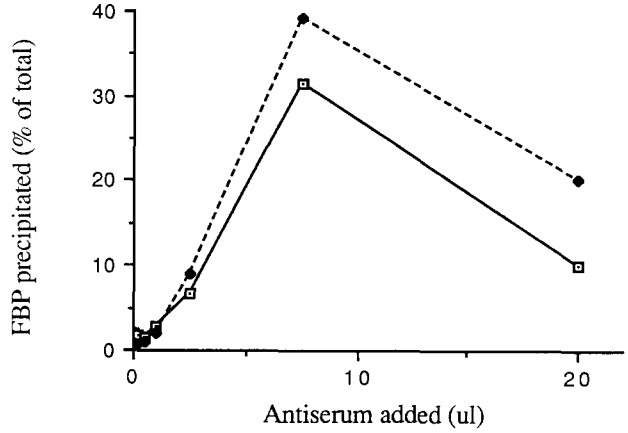

Figure 4 Precipitation of folate-binding protein from jejunal BBMs by the antibody against the high-affinity folate-binding protein in the kidney. Folate-binding proteins detached from jejunal BBMs by PIPLC (◆) or Triton X-100 (□) were incubated with various concentrations of antibody to the high-affinity folate-binding protein in the kidney in 0.2 M potassium phosphate buffer (pH 7.4). Protein-antibody complex was precipitated with goat anti-rabbit IgG in 3.7% polyethylene glycol (PEG 8000).

Table 1 V_{max} Values for folate binding in brush border membranes before and after treatment with phosphatidylinositol-specific phospholipase C

	V_{max} (pmol/mg protein)		
	Before	After	Percent of control
HFBP	0.41	0.13	32.0
LFBP	7.69	5.87	76.3

Abbreviations: HFBP, high-affinity folate-binding protein; LFBP, low-affinity folate-binding protein.
Acidified rat jejunal BBMs were treated at 37°C with or without 100 mU/ml of PIPLC in mannitol buffer (pH 7.4) for 15 minutes. Folic acid binding activities of these membranes were analyzed by Biogel P-30 columns. V_{max} values were obtained from Scatchard analysis of concentration dependency experiments at pH 6.0.

ponent was removed by PIPLC. On the other hand, the low-affinity binding component decreased by less than 25%. Values for the dissociation constant remained within the same order of magnitude (kd = 21.1 nM and 10.8 μM).

Discussion

Folate binding to specific membrane protein(s) is assumed to be an integral step in the intracellular uptake by cells of epithelial origin. In 1972, Leslie and Rowe[7] reported a low-affinity folate-binding protein in the cell membranes of isolated intestinal cells with a kd of 39.7 μM, similar to that described for folate transport (kd = 1.2 to 7.2 μM). More recently, high-affinity folate-binding protein was shown to be present in rat jejunal BBMs by the standard nitrocellulose filter assay.[8] The properties (affinity, pH dependency, and substrate specificity) of this high-affinity folate-binding protein resembled those properties observed in kidney BBMs.[9,13] The standard nitrocellulose filter assay is probably incapable of measuring [^{3}H]PteGlu, which binds weakly to protein(s). Moreover, during the long isolation procedure, the folate-binding activity of enterocytes can be lost to endogenous proteases.

For these reasons, we developed small Biogel P-30 columns, which are particularly suitable for analyzing weakly associated folate, to reevaluate folate binding in isolated jejunal BBMs.

The effect of pH on folate binding at rat jejunal BBMs (*Figure 1*) suggests two possibilities. One possibility is that there is only one binding protein in the membrane and that optimal folate binding to this protein occurs over a broad pH range. The apparent binding optimum at pH 5.5 with the untreated membranes would then be explained by increased exchange of endogenous and exogenous folate at lower pHs. A second possibility, and the one that seems more probable in light of our other observations, is that two binding proteins are present in the membrane, one possessing a pH optimum at 5.5 and the other possessing a binding optimum at a more alkaline pH. The latter hypothesis suggests that acid washing removes endogenous folate, enabling us to observe the combined binding characteristics of both proteins.

Scatchard analysis of concentration dependency of folate binding (*Figure 2*) showed two components. This indicates the presence of either two different folate-binding proteins or a single folate-binding protein with negative cooperativity.

To further investigate folate-binding characteristics in isolated jejunal BBMs, we used the phospholipase, PIPLC. A number of membrane proteins have been shown to be anchored to the membrane through a phospholipid.[15] In our laboratory, membrane detachment of folate-binding protein by PIPLC has recently been shown in rat kidney BBMs.* The present study demonstrated that folate-binding protein was released from rat jejunal BBMs by PIPLC (*Figure 3*). These data suggest that, unlike kidney BBMs,* jejunal BBMs contain a PIPLC-resistant folate-binding component. Moreover, compared with kidney BBMs, the intestinal preparation also demonstrates a substantial release of folate-binding protein even without enzymes. This may be due to the presence of endogenous phospholipase enzymes.[18] Scatchard analysis of PIPLC-treated membranes indicated that the high-affinity folate-binding component was selectively cleaved by this enzyme (*Table 1*). This observation is consistent with the existence of two different proteins.

The immunoprecipitation study indicated that the folate-binding protein cleaved from rat jejunal BBMs by PIPLC resembles the high-affinity folate-binding protein in the rat kidney. *Figure 4* demonstrates that

*Lee, H.C., Selhub, J., and Rosenberry, T. (1989). Folate binding protein in rat kidney has covalently bound phosphatidylinositol moiety which provides the sole anchoring domain to brush border membranes. *FASEB J.* 3, A1058.

the pattern of immunoprecipitation observed with Triton-extracted binding activity, which reflects the activities of both binding proteins, is quantitatively similar to the pattern observed with the high-affinity protein cleared by PIPLC. This suggests that the PIPLC-resistant binding protein does not possess immunoreactivity with the antibody to the kidney protein.

The present study demonstrates that rat jejunal BBMs contain two folate-binding proteins: a high-affinity, low-capacity protein and a low-affinity, high-capacity protein. Phosphatidylinositol-specific phospholipase C specifically removes the high-affinity folate-binding protein from the BBMs. The high-affinity protein in the intestine resembles the high-affinity folate-binding protein in the kidney: it has a similar affinity for folic acid and it shares antigenic determinants with the kidney protein. The function of these two binding proteins in the intestine and their relation to the transport of folate deserves further investigation.

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