Membrane and tissue distribution of folate binding protein in pig

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FOLATE HOMEOSTASIS is regulated in part by the uptake of folates by intestinal, hepatic, and renal membranes. The digestion of dietary polyglutamyl folates at the jejunal brush-border (JBB) membrane is followed by membrane uptake and transport of derivative monoglutamyl folates (7). Folate uptake at the liver plasma membrane (LPM) is followed by intracellular storage and metabolism (34) and then secretion of ~10% to an enterohepatic folate circulation and the remainder to the systemic circulation (35). Urinary folate excretion is regulated by >95% tubular reabsorption at the kidney brush-border (KBB) membrane (42). Candidate proteins that regulate the membrane uptake of monoglutamyl folates include the high-affinity folate binding protein (FBP) (1) and a low-affinity transporter, the reduced folate carrier protein (1, 8).

The overall goal of the present study was to study the potential role of FBP in folate homeostasis in the pig by comparing its activities, transcripts, and immunoreactivities in relevant membranes and tissues. We chose the pig as an experimental model because we have previously shown a similar process of hydrolysis of polyglutamyl folate and of JBB folate hydrolase in pig and human (7) and because we recently cloned and described the molecular sequence and properties of the cDNA of pig liver FBP (38).

MATERIALS AND METHODS

Chemicals. [3', 5'-7,9-H]Folic acid (28 Ci/mmol); [3', 5'-7,9-3H]5-methyltetrahydrofolic acid (27 Ci/mmol); and [3', 5'-7,9-3H]Methotrexate (26 Ci/mmol) were purchased from Moraveck Biochemicals (Brea, CA). The superscript preamplification system was purchased from Life Technologies (Gaithersburg, MD). Taq DNA polymerase was purchased from Fisher Scientific (Pittsburgh, PA). All other reagents were obtained from Sigma Chemical (St. Louis, MO) and various commercial sources. (α-32P)dCTP (3,000 mCi/mmol) and (α-35S)dATP (1,000 mCi/mmol) were purchased from Amersham Life Sciences (Arlington Heights, IL). The full-length cDNA for pig liver FBP was available from our recent study (38). cDNA probes for human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were obtained from ATCC (Rockville, MD). Hybridization-ready blots containing poly(A) RNA from multiple human and rat tissues were obtained from Clontech Laboratories (Palo Alto, CA). Protein A was obtained from Sigma. Purified bovine milk FBP was a gift from F. Kohouse, University of Colorado. Rabbit anti-human sera to KB human cancer cell FBP was a gift from S. Rothenberg, State University of New York Brooklyn.

Animals and tissues. Initial kinetic studies used tissues from 4-mo-old, 40-kg Yorkshire pigs of both sexes that were fed commercial diets ad libitum, whereas subsequent comparison studies used frozen tissues and membranes that were obtained from a prior study of five juvenile male Yucatan micropigs that had been fed a balanced control diet for 12 mo that contained all essential nutrients including added folic acid at 16.6 mg/kg body wt (39). For each experiment, tissues were obtained fresh at open surgery of anesthetized pigs. Following described protocols, liver samples were used for immediate preparation of LPM, which were then frozen at ~80°C before further use, whereas kidney and jejunal mucosal samples were initially frozen in liquid nitrogen and stored at ~80°C before subsequent preparations of KBB and JBB membranes (39). For each jejunal mucosal harvest, adjacent 10-cm sections of proximal jejunum were resected, opened longitudinally, and rapidly rinsed with ice-cold saline or saline containing 4 M guanidium thiocyanate to inhibit intestinal RNases before jejunal mucosa scraping and freezing.

Tissue folates. Serum and tissue folates were measured by conventional microbiological assay with Lactobacillus casei. Serum folates were assayed without heat denaturation of protein. Tissue folates were assayed following homogenization, heat denaturation, folate extraction, and treatment with purified exogenous hog kidney folate hydrolase to convert tissue polyglutamyl folates to their monoglutamyl derivatives (36).

Membrane preparations. LPM, KBB membranes, and JBB membranes were purified from fresh liver and from fresh and thawed kidney and jejunal mucosal samples as previously described (18, 39). According to the marker enzymes Na÷-K÷-ATPase, leucine aminopeptidase, and alkaline phos-
FOLATE BINDING PROTEIN IN PIG

Initial experiments assessed the effects of pH, temperature, incubation time, protein concentration, and hyperosmolarity on [3H]folic acid binding by ~90%, indicating that the FBP receptor in each membrane was for the most part occupied by folate molecules. Membrane pellets were resuspended in 100 mM phosphate buffer, pH 7.4, containing 150 mM NaCl, and used for the equilibrium binding assay. Concentrations of [3H]folic acid or other labeled folate derivatives ranging from 0.5 to 50 nM were added to duplicate samples containing 0.1 mg membrane protein, 150 mM NaCl, and 100 mM potassium phosphate buffer, pH 7.4, in a final volume of 1.0 ml. Following 30-min incubations at 37°C, 0.1-ml aliquots were removed and counted in a Beckman LS 3901 scintillation counter to determine specific activities. The samples were then centrifuged at 16,000 g for 10 min at 4°C. The concentration of unbound (free) folate was measured after accounting for 0.5 ml of the supernatant. After removal of the remaining supernatant, bound folate was measured in the pellet, which was resuspended in 1 ml of phosphate buffer, pH 3.5, and transferred to a scintillation vial for counting. By plotting the concentration of bound folate versus the concentration of bound folate divided by the concentration of free folate, the affinity constant (Kd) and binding capacities (pmol/mg protein) were calculated by Scatchard plot analysis (17).

Preparation of recombinant FBP. The amplified 759-bp open reading frame of pig liver FBP (38) was ligated into pProEX-T vector (Life Technologies) and then transformed into Escherichia coli. DNA was isolated from transformants using the standard mini-preparation procedure to verify the correct insertion of the open reading frame. A recombinant FBP fusion protein was induced according to the manufacturer’s protocol (Life Technologies) and purified by using TALON metal affinity resin (Clontech Laboratories) to release recombinant FBP under denaturing conditions in 8 M urea. The purity of the recombinant FBP was determined by SDS-PAGE (15) before and after dialysis against PBS, and protein was identified by Coomassie blue stain (Bio-Rad).

Immunoprecipitation. The specificity of rabbit antisera IgG was determined by its immunoprecipitation from recombinant pig FBP. Following an established protocol (13), 40 µg of recombinant FBP were mixed with 10 µg of the purified IgG fraction of rabbit antisera or preimmune serum and the volume was adjusted to 500 µl with Tris-buffered saline, pH 7.5. After overnight incubation at 4°C, 50 µl of protein A were added to the incubation mixture for an additional 1 h. After 1 min of centrifugation (200 g), the pellet was washed two times with PBS, pH 7.5, and once with 0.05 M Tris at pH 6.8, and was then resuspended with 20 µl Laemmli sample buffer (2% SDS, 10% glycerol, 0.05 M Tris at pH 6.8), heated for 5 min at 100°C, and resolved by 10% SDS-PAGE. Protein bands in the original recombinant FBP and the immunoprecipitate were detected by Silver Stain Plus according to the manufacturer (Bio-Rad).

ImmunobLOTS. In addition to confirming the size of recombinant pig FBP, immunoblots were used to validate the authenticity of the rabbit antisera IgG by comparing its ability to detect purified bovine milk FBP with that of a separate rabbit antibody to human KB cell FBP. Two micrograms each of recombinant FBP and purified bovine milk FBP were resolved on 12.5% SDS-PAGE and then electrotransferred to a polyvinylidene difluoride membrane (Immobilon-P; Millipore, Bedford, MA), followed by sequential incubations with the purified IgG fraction of rabbit antisera to recombinant FBP in 1:3,000 dilution or with antisera to human KB cell FBP in 1:1,000 dilution. Each primary antibody treatment was followed by secondary incubation with 1:3,000 goat anti-rabbit antibody linked to alkaline phosphatase (Bio-Rad).

Immunohistochemistry. Membrane FBP was detected by immunohistochemical staining of relevant tissues with rabbit antisera IgG. Liver, kidney and jejunal tissue samples were collected fresh from a Yorkshire pig and were stored in 10% formalin at 4°C for 2 weeks. Tissue samples were sectioned at 5 µm. Sections were deparaffinized, washed three times in PBS for 5 min each, and then treated with blocking solution containing 0.1% bovine serum albumin and 1% H2O2 for 1 h. After blocking, sections were incubated with 1:5,000 (vol/vol) of the IgG fraction of rabbit preimmune and antisera to recombinant FBP for 2 h at room temperature. After primary antibody incubation, sections were washed five times in PBS. Tissue slices were then incubated in 1:1,000 goat anti-rabbit serum linked to horseradish peroxidase for 1 h at room temperature, washed five times in PBS, and reacted for 10 min with True-Blue (KPL, Gaithersburg, MD). Sections were washed five times in distilled water and mounted on gelatin-coated slides. Microscopic images were obtained using an Olympus BH-2 microscope linked to Optonics CCD-color digital camera. Images were digitized to a personal computer (Macintosh Power-PC) and printed from a laser printer with photograde gray scale at 600 dots per inch.

Northern blots. Poly(A)+ RNA was isolated from micropig tissues using the Fast Tract 2.0 mRNA isolation system (Invitrogen, San Diego, CA) (3). Five micrograms each of poly(A)+ RNA from each tissue were separated by electrophoresis on 1.0% (wt/vol) agarose/2.2 M formaldehyde gels, transferred to nylon membranes, and hybridized with an amplified 32p-labeled 759-bp fragment of the open reading frame of the cDNA of pig liver FBP (38). The primers for cDNA...
amplification by the polymerase chain reaction were constructed from bases 89 to 109 (sense) and from bases 834 to 851 (antisense) (38). A $^{32}$P-labeled 0.8-kb XbaI-PstI fragment of human GAPDH cDNA was used as a positive internal control for mRNA. Following sequential hybridization with each probe, blots were washed twice for 15 min each in a solution of $6 \times$ saline-sodium phosphate-EDTA (SSPE)/0.1% SDS at room temperature and then twice in $1 \times$ SSPE/0.1% SDS at $37^\circ$C, and subsequently analyzed for $^{32}$P distribution and intensity using a Bio-Rad phosphoimager (37). Briefly, blotted dry membranes were exposed overnight with the high-sensitivity screen of a Molecular Imager System (GS-250, Bio-Rad) followed by screen scanning. The resulting images were processed and quantified using Phosphor Analyst software (Bio-Rad, v. 1.1). Counts accumulated by the radiation-sensitive screen of the phosphoimager were statistically analyzed after subtracting background from each sample.

Statistics. Results are expressed as means $\pm$ SE. Values from experiments that used micropig tissues and membranes were compared using Student’s $t$-test. The acceptable level of significance ($P$) was $<0.05$ for each analysis.

RESULTS

Tissue folate levels. Total folate levels were measured in serum, liver, and kidney tissues from each of five Yucatan micropigs by microbiologic assay with L. casei. The folate level was greatest in the serum at $10.2 \pm 3.3$ nmol/l and was twice as concentrated in liver at $6.5 \pm 0.8$ nmol/g as in kidney at $2.6 \pm 0.4$ nmol/g ($P < 0.005$).

Folate binding by membranes. Preliminary studies of LPM isolated from a Yorkshire pig showed that the binding of $[^3H]$folic acid at $37^\circ$C for 30 min was optimal at pH 7.5, was proportionate to protein concentration, and was not affected by increasing the osmolarity of the media by the addition of mannitol to the incubation medium (Fig. 1). Similar observations were made using purified KBB membranes (not shown). Figure 2 compares the kinetics of binding of $[^3H]$folic acid by LPM, KBB membranes, and JBB membranes that were isolated from a Yorkshire pig. In this representative experiment, $K_d$ values for $[^3H]$folic acid by LPM and

Fig. 1. Effects of pH (A), protein concentration (B), and buffer osmolarity (C) on binding of $[^3H]$folic acid by liver plasma membrane (LPM) prepared from liver of Yorkshire pig. Osmolarity was adjusted by adding different concentrations of mannitol to the buffer solution.

Fig. 2. Comparisons of $[^3H]$folic acid binding by LPM (A), kidney brush-border (KBB) membranes (B), and jejunal brush-border (JBB) membranes (C) prepared from tissues from a Yorkshire pig. All measurements were made in duplicate. Scatchard plots show affinity binding constant (1/slope) at 1.4 nM in LPM and at 0.98 nM in KBB membrane. Binding capacities are calculated from the x-intercept at 11 pmol/mg in LPM and 1.5 pmol/mg in KBB membrane. No measurable specific binding was detected in JBB membrane.
Table 1. Binding of different ³H-labeled monoglutamyl folates by LPM

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<tr>
<th>Substrate</th>
<th>Kₐ, nM</th>
<th>Maximal Binding, pmol/mg protein</th>
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<tr>
<td>5-Methyltetrahydrofolic acid</td>
<td>0.27 ± 0.02*</td>
<td>36.0 ± 6.02</td>
</tr>
<tr>
<td>Folic acid</td>
<td>0.53 ± 0.04*</td>
<td>19.7 ± 2.10</td>
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<tr>
<td>Methotrexate</td>
<td>4.72 ± 0.56†</td>
<td>14.4 ± 1.85</td>
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Values represent means ± SE of data using liver plasma membranes (LPM) isolated from Yucatan micropigs (39); n = 5. Kₐ, affinity binding constant. *P < 0.02; †P < 0.005.

KBB membranes were similar, whereas the total binding capacity of [³H]folic acid (pmol/mg membrane protein) by LPM was greater than binding by KBB membranes, and there was no measurable binding of [³H]folic acid by isolated JBB membranes.

Table 1 provides quantitative comparisons among the binding kinetics of [³H]folic acid, [³H]5-methyltetrahydrofolic acid, and [³H]methotrexate by LPM samples that were available from five different Yucatan micropigs (39). As shown, the binding affinity of purified LPM for [³H]5-methyltetrahydrofolic acid was twofold greater than that for [³H]folic acid (P < 0.02), whereas the affinity of LPM for [³H]methotrexate was 10-fold less than the affinity for [³H]folic acid (P < 0.005), and the total binding capacity of LPM was similar for each substrate.

The binding of [³H]folic acid was compared in purified LPM, KBB membranes, and JBB membranes from the native Yucatan micropigs (39). As shown in Table 2, binding affinities were similar, but the total binding capacity of LPM for [³H]folic acid was sixfold greater than that of KBB membranes (P < 0.005). As in the preliminary study (Fig. 2), there was no detectable binding of [³H]folic acid by JBB membranes from these animals.

Immunological studies. As shown in Fig. 3, recombinant pig FBP was identified by 10% SDS-PAGE as a single protein band at 30 kDa that was identical to the immunoprecipitate resulting from incubation with the IgG fraction of rabbit antisera complexed to protein A. Immunoprecipitation with IgG fraction of rabbit antisera identified recombinant FBP identified as a black band at 30 kDa following immunoprecipitation with IgG fraction of rabbit antisera complexed to protein A.

Northern blots of FBP transcripts. Figure 6 compares mRNA transcripts of FBP and GAPDH in liver and kidney samples from each Yucatan micropig. The FBP transcript intensity was consistently greater in micropig liver samples than in kidney samples, whereas membrane FBP was localized by immunohistochemical staining of liver, kidney, and jejunal mucosa using the IgG fraction of antiserum to the recombinant protein. FBP was detected on the surface of hepatocytes and renal tubular epithelial cells, whereas no staining was found in JBB membranes. FBP was identified incidentally within liver slices on the surfaces of intrasinusoidal red blood cells.

Table 2. Binding of [³H]folic acid by different membranes

<table>
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<tr>
<th>Membrane</th>
<th>Kₐ, nM</th>
<th>Maximal Binding, pmol/mg protein</th>
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<tr>
<td>LPM</td>
<td>0.45 ± 0.04</td>
<td>39.2 ± 9.0*</td>
</tr>
<tr>
<td>KBB</td>
<td>0.55 ± 0.02</td>
<td>6.8 ± 0.6*</td>
</tr>
<tr>
<td>JBB</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

Values represent means ± SE of data using membranes from Yucatan pigs (39); n = 5. KBB, kidney brush border; JBB, jejunal brush border; ND, none detected. *P < 0.005.
GAPDH transcripts appeared in equal intensity in samples from each tissue. FBP mRNA was absent from jejunal mucosa (not shown). Quantitative analysis of phosphoimager intensities showed that the ratio of FBP to GAPDH in mRNA extracts of liver samples, $4.32 \pm 0.48$, was threefold greater than the ratio of $0.87 \pm 0.07$ in mRNA extracts of kidney samples ($P < 0.001$). Figure 7 compares the abundance of mRNA transcripts of FBP in commercially obtained human and rat tissues. Transcripts of FBP were found in human heart, placenta, lung, and kidney (Fig. 7A) and in rat kidney (Fig. 7B), but were absent from liver of both species.

Fig. 6. Autoradiographs of Northern blots of mRNA prepared from 5 micropig livers (lanes 1–5) and kidneys (lanes 6–10). Blots were hybridized with cDNA probes of pig FBP (1.35 kb) and human glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 1.1 kb) as an internal standard. In each animal, the intensity of liver mRNA FBP was greater than that of kidney mRNA FBP, whereas GAPDH intensities were similar. Ratio of the intensity of FBP to GAPDH in liver was 3-fold greater than ratio in kidney ($4.32 \pm 0.48$ vs. $0.87 - 0.07; P < 0.001$).

Fig. 7. Northern blots of human and rat tissues. Human (A) and rat (B) Northern blots were obtained from Clontech Laboratories and were hybridized with $^{32}$P-labeled pig FBP cDNA open reading frame 759 bp fragment as described in the text. A: lane 1, heart; lane 2, brain; lane 3, placenta; lane 4, lung; lane 5, liver; lane 6, skeletal muscle; lane 7, kidney; lane 8, pancreas. B: lane 1, heart; lane 2, brain; lane 3, spleen; lane 4, lung; lane 5, liver; lane 6, skeletal muscle; lane 7, kidney; lane 8, testis. FBP transcripts were identified in human heart, placenta, lung, and kidney (A) and in rat kidney (B), but were absent from liver of either species.
DISCUSSION

FBP, or the folate receptor, was identified as a 37-kDa glycosylated protein with a high affinity for folic acid and was characterized at the molecular level in several human cancer-cell lines and in human placenta (1, 6, 14, 22, 24, 27, 28). Recently, we characterized the cDNA of FBP from pig liver, finding >70% nucleotide and amino acid sequence homology with FBP from other species (38). The different isoforms of FBP are present on chromosome 11 (1) and appear to share a common open reading frame sequence with a carboxy terminal glycosylphosphatidylinositol anchor sequence (26). The role of FBP in folate homeostasis has been questioned because of its preference for oxidized folic acid over normally circulating 5-methyltetrahydrofolinic acid (1) and the absence of its transcripts and immunoreactivity in autopsy-derived human intestine and liver (40, 41). Recently, a different putative transporter, the 57-kDa reduced folate carrier protein, was cloned from mouse L1210 cells (5), and its sequence and transcripts were found in human placenta and intestine (21, 23). Thus FBP and/or the alternative reduced folate carrier could act independently or synergistically in the transport of monoglutamyl folates across tissue membranes.

Previous studies suggested that different transport mechanisms could account for folate uptake by membranes of intestinal mucosal epithelial cells, hepatocytes, and renal tubular epithelial cells. Studies that used isolated JBB vesicles from rat, rabbit, human, and pig defined a saturable transport carrier that operates with Michaelis constants of 0.5 to 1.5 μM, prefers 5-methyltetrahydrofolate over folic acid as substrate, and demonstrates either $H^+$ cotransport or anion exchange (20, 29, 31, 33), consistent with recent evidence for the presence of the reduced folate carrier in intestinal epithelial cells (21). Prior studies of hepatic folate uptake by liver cells and LPM provide evidence for either or both binding and carrier-mediated membrane transport. In an early study, paraenteral $^{3}H$folic acid was bound initially to rat LPM before the intrahepatic appearance of labeled intracellular folate (43). Another group demonstrated high-affinity FBP in solubilized rat LPM that exhibited $K_d$ of ~7 nM and substrate preference for oxidized Pte-Glu (4). Other studies described minimal binding and predominant carrier-mediated transport of 5-methyltetrahydrofolate by LPM vesicles isolated from human and rat liver (9, 10). FBP was isolated and purified from pig KBB membranes at 38.5 kDa with an apparent equal specificity for folic acid and 5-methyltetrahydrofolate (11). In another study, FBP that was purified from rat KBB membranes at an estimated 30 kDa exhibited substrate preference for folic acid and was localized to rat KBB membranes by immunofluorescence histochemistry (32). Other studies of monkey MA104 and human renal proximal tubule cells provided evidence for either binding and/or carrier-mediated transport of 5-methyltetrahydrofolate acid (12, 16).

Within the context of membrane regulation of folate homeostasis in the pig, our studies suggest that FBP participates in folate uptake by the liver and to a lesser extent in the kidney, but plays no role in intestinal folate absorption. Previously, we isolated the cDNA for FBP from pig liver and identified its mRNA transcripts in a single experiment in both liver and kidney and its absence in the jejunum (38). The present studies extend these observations by 1) the quantitative demonstration that the binding capacity of purified pig LPM for $^{3}H$folic acid is sixfold greater in pig liver than kidney (Table 2), 2) by the immunohistochemical identification of FBP in both LPM and KBB membranes and its absence from JBB membranes (Fig. 5), and 3) by the finding of mRNA transcript of FBP in threefold greater concentration in pig liver than kidney (Fig. 6).

In the present studies, the specificity and authenticity of the IgG fraction of antiserum to recombinant pig FBP was established by its capacity to immunoprecipitate and immunoblot the recombinant protein (Figs. 3 and 4) and to immunoblot native bovine milk FBP with resultant bands identical to those detected by an independent antibody (Fig. 4). The molecular size of recombinant pig FBP at 30 kDa (Figs. 3 and 4) is predicted from the 253 amino acid sequence of pig FBP (38), whereas the larger protein band detected by immunoblot at 38 kDa in native bovine FBP (Fig. 4) is consistent with other estimates for the molecular size of native FBP from pig renal tubular epithelium (11). The immunohistochemical staining of pig LPM and KBB membranes and absence of staining of JBB membranes (Fig. 5) are in keeping with FBP binding by isolated pig LPM and KBB membranes (Table 2) and the pig liver and kidney distribution of FBP transcripts (Fig. 6). The immunohistochemical localization of FBP to renal tubular cells by antibody to recombinant pig FBP (Fig. 5D) is consistent with its renal tubular localization in the rat by antibody to purified native rat FBP (32). Similarly, the immunohistochemical staining of FBP on the surfaces of pig hepatocytes (Fig. 5B) is in keeping with prior observations on the presence and binding properties of FBP in rat LPM (4, 43). The incidental staining of red cell membranes within the sinusoids of pig liver slices is consistent with the prior identification of FBP in human red cell membranes (2).

The finding of FBP activity and immunoreactivity in pig KBB membranes and renal tubules (Table 2, Fig. 5D) and its transcripts in kidney samples from pig, human, and rat tissues (Figs. 6 and 7) suggests a universal mammalian requirement for FBP in the renal tubular reabsorption of folate. On the other hand, the absence of FBP activity and immunoreactivity in pig JBB membranes (Figs. 2 and 5F) and its transcript in pig jejunal mucosa (38) suggests that this protein is not involved in the intestinal absorption of folates. This finding contradicts a prior observation on the identification of FBP in pig intestine by an affinity labeling technique (25) and is consistent with recent evidence for the presence and alternate role of the reduced folate carrier in transport of folates across the intestinal mucosal epithelium (21).
The present findings on the transcript distribution of FBP in tissues of pig, human, and rat (Figs. 6 and 7) suggest that liver FBP is unique to the pig and are consistent with a potential role for FBP in the transport of monoglutamyl folate from portal venous blood into the hepatocyte in this species. Also, the present finding of somewhat greater binding affinity for reduced 5-methyltetrahydrofolic acid than folic acid by isolated pig LPM (Table 1) contrasts with prior observations on the preference of FBP for folic acid in various cell systems, placenta, and rat KBB membranes (1, 32) but is in keeping with studies of partially purified FBP from pig KBB membranes and of cell transfectants of human FBP, which showed similar binding specificity for folic acid and 5-methyltetrahydrofolic (11, 14). The nanomolar range of binding affinity for each substrate (Tables 1 and 2) and the neutral pH optimum for binding (Fig. 1) are consistent with the kinetics of folate binding demonstrated by others who used different membranes (1, 11) and with the observed concentrations of serum (nmol/l) and tissue folates (nmol/g) in the present study of micropigs.

Others (19) showed that two different folate species, tetrahydrofolic acid and 5-methyltetrahydrofolic acid, exist uniquely in pig plasma in an approximate 3:1 ratio, in contrast to the single presence of 5-methyltetrahydrofolic acid in the plasma of other species. The same group (30) demonstrated binding of tetrahydrofolic acid to a high-affinity pig plasma protein, which was proposed to stabilize this folate within the circulation and prevent its degradation. Although our studies demonstrated the preferential binding of 5-methyltetrahydrofolic acid by LPM (Table 1), the existence of a separate or integrated mechanism for transfer of high-affinity, protein-bound tetrahydrofolic acid from pig circulation into pig liver has not been established and remains speculative.

**Perspectives**

Knowing how folates are transported across cell membranes is essential to understanding the regulation of intestinal absorption, hepatic uptake, and renal tubular reabsorption and the maintenance of folate homeostasis. Although FBP has been studied previously in isolated cell systems and membranes, ours is the first study to compare the activity and potential significance of FBP in three different regulatory organs, the intestinal mucosa, liver, and kidney, in the same animal model. Although our findings underscore the importance of FBP for folate uptake by the liver and renal tubule and its insignificance to intestinal absorption in the pig, they must be tempered by the realization that the pig is an imperfect animal model for study of human folate homeostasis. Thus the species comparison showed FBP transcript uniquely in pig liver but in common in rat, human, and pig kidney (Figs. 6 and 7). The finding that 5-methyltetrahydrofolic acid appears as the preferred substrate for pig LPM (Table 1) is consistent with the presence of this folate in serum but runs contrary to established data on the preference of FBP for noncirculating folic acid (1). How FBP regulates the cellular uptake of 5-methyltetrahydrofolic acid cannot be inferred by the present studies; controversies over the separate or synergistic mechanisms of FBP and the reduced folate carrier are reviewed in detail elsewhere (1). Further insights into the regulation of human folate homeostasis will require application of the present approach in the pig model to the study of both proteins in human tissues and membranes.

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