Folate deficiency reduces the GPI-anchored folate-binding protein in rat renal tubules

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Folate deficiency reduces the GPI-anchored folate-binding protein in rat renal tubules. Am J Physiol Cell Physiol 278: C812–C821, 2000.—A folate-binding protein (FBP) anchored to cell membranes by a glycosyl phosphatidylinositol (GPI) adduct is constitutively expressed in some transformed and cultured cell lines. Its expression is upregulated when these cells are grown in medium containing low folate, but whether this occurs in vivo with nutritional folate deficiency is unknown. To address this question, the GPI-FBP in the liver, kidney, and brain of rats on control and folate-deficient (FD) diets was measured. The GPI-FBP in the kidney of FD rats decreased significantly in contrast to the upregulation of this protein in cultured cells. Northern blot analysis and nuclear run-on assays indicated that transcription of the GPI-FBP gene in the kidney was not reduced by folate deficiency. This decrease of the GPI-FBP appears to result from its proteolysis, similar to the enzymatic degradation of the apoprotein that occurs in vitro. Because the GPI-FBP is on the brush borders of the proximal renal tubules and provides for the reabsorption of folate, this function diminishes when the protein decreases in folate deficiency.

FOCAL POINTS
- Tubular reabsorption; endocytosis; transcription; proteolysis
- Folate deficiency reduces the GPI-anchored folate-binding protein in rat renal tubules.
- Northern blot analysis and nuclear run-on assays indicated that transcription of the GPI-FBP gene in the kidney was not reduced by folate deficiency.
- The GPI-FBP appears to result from its proteolysis, similar to the enzymatic degradation of the apoprotein that occurs in vitro.

MATERIALS AND METHODS

Four male and female 7- to 8-wk-old Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) were placed on a standard basal diet in which amino acids were provided from casein (Purina Mills) and contained 0.05 mg of folic acid/kg provided by the manufacturer. A similar group of rats was placed on a diet described by Clifford et al. (11) containing free amino acids (Dyets, Bethlehem, PA) that completely lacked folic acid as determined in our laboratory by competitive binding radioassay (12). The amino acids supplied by both diets were similar except that the Clifford diet had a lower content of the essential amino acids, isoleucine (25% less), leucine (44% less), tryptophan (30% less), and valine (37% less). Control rats were fed the experimental diet for 5 wk. Control and FD rats were killed by 10.220.33.4 on April 29, 2017
The GPI-FBP was measured by RIA as previously described (15) using an antiserum raised against the GPI-FBP purified from rat placenta (13). The GPI-FBP for the dose-response standard curve was prepared from solubilized rat placenta (13) and rat kidney (36) was also purified after solubilization and extraction with this detergent at 4°C. The 4°C temperature is essential to avoid proteolysis of the solubilized GPI-FBP, especially when it lacks the folate ligand. After solubilization, the insoluble components were pelleted by centrifugation at 100,000 g, and the GPI-FBP was assayed in the supernatant fraction. The protein concentration in the solubilized preparation was determined using the Bio-Rad Dc protein assay (Bio-Rad Laboratories, Hercules, CA).

The significance of the difference in mean values for serum and red blood cell folate, serum homocysteine, and GPI-FBP in tissues from the control FR and FD animals was computed using Student's t-test (StatView, Abacus Concepts).

Analyses of transcription of the GPI-FBP gene. The GPI-FBP RNA was identified and semiquantified by Northern blotting (34). Portions of the kidney, brain, and liver from the euthanized rats were immediately frozen in liquid nitrogen and stored at −80°C until processed. The total cellular RNA was prepared by homogenizing each tissue in 10 vol of 4 M guanidinium isothiocyanate-30 mM sodium acetate-0.1 M β-mercaptoethanol using a Polytron. The homogenate was centrifuged at 5,000 g for 5 min at 4°C to sediment the coarse debris. The supernatant fraction was then layered over 3.0 ml of 5.7 M CsCl with additional guanidinium isothiocyanate solution to fill the tube, and the samples were centrifuged at 30,000 g overnight at room temperature. Poly(A)⁺ RNA was prepared from the total liver RNA using the Biomag mRNA purification kit (PerSeptive Biosystems). The RNA preparations were electrophoresed in a 1% agarose gel in the presence of 0.66 M formaldehyde and then capillary blotted onto a Nytran membrane. The blots were hybridized for 2–18 h at 42°C with the random oligomer primed 32P-labeled cDNA.

Within 1 h and stored at −20°C after addition of ascorbic acid (5 mg/ml). All samples were assayed in duplicate at two dilutions. Homocysteine was measured using HPLC in-line with a spectrophotometer as described by Fortin and Genest (17) in serum or plasma separated from the blood sample within 1 h.

The GPI-FBP was measured by RIA as previously described (15) using an antiserum raised against the GPI-FBP purified from rat placenta (13). The GPI-FBP for the dose-response standard curve was prepared from solubilized rat placenta membranes in which the concentration of this protein was determined as the molar equivalent of the binding capacity for [3H]pteroylglutamic acid (PGA) after acidification to dissociate the bound endogenous folate as described for the purification of the protein (13). Figure 1 shows a typical standard curve obtained with this antiserum within 1 h.

Figure 1. A typical standard curve for RIA of glycosyl phosphatidylinositol/folate-binding protein (GPI-FBP). Antiserum was raised to GPI-FBP purified from placenta rat. Sensitivity of assay was 1 ng of GPI-FBP prepared from placental membranes. A plot of reciprocal of fraction of tracer ([3H]PGA-GPI-FBP) bound against concentration of competing GPI-FBP linearizes dose-response curve and provides a more accurate method to determine concentration of unknown (i.e., value for x) from value of y as indicated in equation.
(1.2 kb) encoding the rat homologue of the human KB cell GPI-FBP (30) in the presence of 10% dextran sulfate and 50% formamide. The filters were washed with 0.1× SSPE-0.1% SDS at 50–60°C and then exposed to reflection film (Eastman Kodak, Rochester, NY). Subsequently, the filters were stripped by boiling in 0.1× SSPE-0.1% SDS and hybridized with the random oligomer primed 32P-labeled glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA to assure comparable loading of the RNA into the gel. The cDNA encoding the GPI-FBP has been cloned from a rat placental cDNA library (unpublished observations).

The rate of transcription of the GPI-FBP mRNA in the kidney was determined as described by Greenberg and Ziff (19). The nuclei from the kidneys of control and folate-deficient rats were prepared as described by Derman et al. (16). A suspension of 2–4 × 106 nuclei in 200 μl of glycerol (40%) in 50 mM Tris-HCl buffer, pH 8.3, containing 5 mM MgCl2 and 0.1 mM EDTA was mixed with 2× reaction buffer containing 10 mM Tris-HCl (pH 8.0)-5 mM MgCl2-0.3 M KCl-5 mM dithiothreitol (DTT)-1 mM ATP, CTP, GTP and 100 μCi of [32P]uridine 5′-triphosphate. After incubation for 30 min at 30°C, RNase-free DNase was added, and, after incubation for 30 min at 42°C, the preparation was extracted twice with 1 ml of buffer saturated phenol-chloroform-isooamy alcohol (25:24:1). Two milliliters of water containing 50 μg tRNA and 3 ml of 10% TCA containing 60 mM sodium pyrophosphate were added, and the mixture incubated on ice for 30 min before filtration through a 25-mm Millipore filter (0.45 μm). The filter was washed three times with 10 ml of 5% TCA containing sodium pyrophosphate and then transferred to a 2.8-cm glass vial containing 1.5 ml 20 mM HEPES buffer (pH 7.5)-5 mM MgCl2-1 mM CaCl2 and 37.5 units of RNase-free DNase I and incubated for 30 min at 37°C. The addition of 45 μl of 0.5 M EDTA and 68 μl of 10% SDS terminated the reaction, and the vials were then incubated for 10 min at 65°C to release the nuclear RNA from the filter. The filter was treated for a second time with 10 mM Tris-HCl, pH 7.4, containing 5 mM EDTA/1% SDS, and the filter eluates were pooled and digested with 90 μg proteinase K for 30 min at 37°C and extracted with the phenol-chloroform-isooamy alcohol as indicated above. The aqueous phase was transferred to a glass centrifuge tube containing 0.75 ml of 1 N NaOH and kept on ice for 10 min. HEPES (1.5 ml of 1 M) was then added followed by the addition of 0.53 ml of 3 M sodium acetate and 14.5 ml of ethanol, and the mixture incubated overnight at −20°C. The precipitated nuclear RNA was pelleted and dissolved in 10 mM Tris-HCl, pH 7.4, 0.1 M EDTA, and 0.2% SDS. This 32P-labeled nuclear RNA was used to hybridize with 5 μg of the linearized plasmid containing the rat GPI-FBP cDNA, with the control plasmid containing human GAPDH cDNA and with the plasmid lacking an insert (negative control) that was on the Nytron filter. After 36 h hybridization at 65°C, the filters were washed and incubated with 80 μg RNase A in 8 ml of 2× sodium chloride-sodium citrate (SSC) for 1 h at 37°C to digest the free probes. After a final wash with 25 ml 2× SSC at 37°C, the filters were exposed to Dupont reflection film with an intensifying screen for 2–5 days at −80°C.

Preparation of proximal tubule brush-border vesicles. Two 8-wk-old rats were euthanized in a CO2 chamber, and the kidneys were rapidly removed and placed in a buffer containing 0.15 M NaCl-0.012 M Na2HPO4-0.0014 M KH2PO4 (pH 7.2) (PBS) at 4°C. The kidneys were processed as described by Bhandari et al. (3) to obtain brush-border membrane vesicles. The kidneys were cut into two coronal halves and the cortex separated from the medulla by careful dissection at the distinct boundary between these regions. The cortices were homogenized in 20 vol of 0.25 M sucrose in 10 mM Tris-HCl, pH 7.6, containing 0.1 mM PMSF and homogenized in a motor-driven Potter-Elvehjem homogenizer. The homogenate was centrifuged at 24,000 g for 20 min, and the layer above the pellet was carefully removed and suspended in the above sucrose buffer to which was added MgCl2 to a concentration of 15 mM. After 20 min at 4°C with occasional gentle vortexing, the preparation was centrifuged at 3,000 g for 10 min. The supernatant fraction was separated and centrifuged at 26,000 g for 20 min. The pelleted fraction containing the brush-border vesicles was resuspended in HEPES buffer, pH 7.3, containing 100 mM mannitol, gently homogenized again, and the vesicles pelleted at 26,000 g. The pellet was resuspended in this buffer and this step repeated. The final pelleted vesicles were resuspended in the HEPES/mannitol buffer and adjusted to a protein concentration of 3 mg/ml.

For preparation of the unsaturated GPI-FBP (apoform) on the brush-border vesicles, 80 μl of the suspension of vesicles was mixed with 600 μl of acetate buffer, pH 4.5, incubated at 4°C for 15 min, centrifuged at 13,000 g for 10 min, and washed twice with 10 mM potassium phosphate buffer, pH 7.4. The final pellet was suspended in 80 μl of 20 mM ethanalamine, pH 9.5, containing 0.15 M NaCl. Holo-GPI-FBP on the vesicles was prepared by incubating the acid-treated vesicle suspension with [14C]HPGPA for 10 min at 25°C. The unbound [14C]HPGPA was removed by washing the vesicles twice with the potassium phosphate buffer and resuspending them in the 20 mM ethanalamine/0.15 M NaCl mixture.

The effect of trypsin and a purified preparation of the renal protease, meprin (9), on the apo-GPI-FBP and the [14C]HPGPA was then determined. Because purified trypsin is readily available, a dose-response effect of the enzyme on the holo- and apo-GPI-FBP was determined at both 4°C and 37°C. Because we had only a small quantity of purified meprin, we assayed the effect of a single concentration of this enzyme on the apo- and holo-GPI-FBP. Ten microliters of the suspension of the brush-border vesicles containing the GPI-FBP in the apo- or holoform was incubated with 1.5 units of meprin in 89 μl of 20 mM ethanalamine (pH 9.5)-0.15 M NaCl-30 mM MgCl2 for 30 and 60 min at 37°C. In a parallel reaction, the buffer contained 40 mM EDTA and lacked MgCl2. The reaction was stopped by cooling to 4°C. 250 pmol [14C]HPGPA was added to the tubes containing the apo-GPI-FBP, and the incubation continued for 30 min at 4°C. Meprin was omitted from the incubation mixture in control assays. At the end of the incubation period, the vesicles were pelleted by centrifugation, washed three times with 0.01 M potassium phosphate buffer at 4°C, solubilized in 1 N NaOH, and transferred to scintillation liquid for measuring the radioactivity.

Reabsorption of folate in the kidney. The tubular reabsorption of endogenous folate (TRF) was determined in the rats on the FR and FD Clifford diets. The rats were fed fasted during the 24-h collection period (water provided ad libitum) and the total folate in the urine and serum folate were measured. The TRF was determined using the following computation:

\[
\text{TRF} (\%) = \frac{\text{total folate filtered} - \text{total urinary folate}}{\text{total folate filtered}} \times 100
\]

The total folate filtered in 24 h was computed using the glomerular filtrate rate of 1.01 ml·min⁻¹·kg body wt⁻¹ as reported (6). Based on our studies described below indicating that circulating plasma folate in the rat is not specifically bound to any protein, these calculations were made considering that 90% of endogenous plasma folate passes into the glomerular filtrate. This value is based on our studies indicating that 90% of folate in rat serum could be removed within 3
Table 2. Serum and red blood cell folate and serum homocysteine in rats on the FR and FD experimental diets

<table>
<thead>
<tr>
<th>Sample</th>
<th>Parameter</th>
<th>FR</th>
<th>FD</th>
<th>P</th>
</tr>
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<tbody>
<tr>
<td>Serum Folate, ng/ml</td>
<td>49 ± 3.5</td>
<td>3.6 ± 0.1</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>Red blood cell Folate, ng/ml</td>
<td>819 ± 124</td>
<td>356 ± 29</td>
<td>0.0027</td>
<td></td>
</tr>
<tr>
<td>Serum Homocysteine, µmol/ml</td>
<td>12.7 ± 0.7</td>
<td>19.4 ± 2.1</td>
<td>0.01</td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SE of the pooled values for 4 male and 4 female rats in each experimental group. Student’s t-test was used to determine level of significance for difference in values for the folate replete (FR) and folate-deficient (FD) groups.

RESULTS

Because the results obtained for the parameters measured did not differ significantly for the male and female rats in each experimental group, the data for both genders within each group were pooled and are presented in Table 2, for serum and red blood cell folate and serum homocysteine, and in Table 3 for tissue folate. Within 6 wk on the FD Clifford diet and 7 wk on the basal diet, serum and red blood cell folate decreased and serum homocysteine increased significantly. The increase in homocysteine in the rats on the FD Clifford diet was significantly greater than occurred in the rats on the FD basal diet (P = 0.0005), indicating a more severe perturbation of folate metabolism in the former group, as would be expected with the complete lack of any folate in the Clifford diet.

By RIA, no GPI-FBP was detected in the serum from the control group, whereas in only one of eight FD rats was the protein measurable, and it was the molar equivalent of 191 pg bound folate/ml serum. The binding of [3H]PGA by serum relative to the total folate concentration was not significant, ranging from 0 to 72 pg/ml in the control and from 0 to 104 pg/ml in the FD rats. These findings differ from the observations in human folate deficiency in which both immunoreactive FBP as well as an unsaturated FBP increase in serum (15).

The FD Clifford diet also impaired hematopoiesis in the female rats that developed anemia and a decrease in leukocytes and platelets. This was not observed in any of the rats on the FD basal diet. In neither experimental group was there any difference in the standard blood chemistries between the folate replete and folate-deficient diets. Surprisingly, the gain in weight of both groups of FD rats during this experimental period was similar to the animals on the folate-replete diets (data not shown).

The decrease in the folate content of the liver, kidney, and brain in the rats on the FD experimental diet is provided in Table 3. The percent decrease in the liver and kidney was similar, ~84% for each group. However, the decrease of brain folate for the group on the FD basal was 37% and for the FD Clifford diet was 68% (P < 0.0002), again indicating the greater severity of folate deficiency induced by the Clifford diet.

The GPI-FBP in the liver, kidney, and brain for the groups on the FR and FD experimental diets are shown in Fig. 2. The GPI-FBP in the kidney of the group on the experimental FR and FD basal diets decreased from 440 ± 24 ng/mg protein to 128 ± 12 ng/mg protein (71%, P < 0.0001), and the group on the corresponding Clifford diets decreased from 216 ± 20 ng/mg protein to 107 ± 10 ng/mg protein (50%, P = 0.0006). It should be noted that the GPI-FBP in the rats on the FR Clifford diet was substantially lower than the rats on FR basal diet (216 ± 20 compared with 440 ± 24, respectively; P < 0.0001). We do not have an unambiguous explanation for this finding, but two contributory factors may be that the rats on the Clifford diet were 1 wk younger than the rats on the basal diet, and the Clifford diet contains substantially less of the essential amino acids isoleucine, leucine, tryptophan, and valine (Table 1). In contrast to this effect of folate deficiency on the GPI-FBP in the kidney, there was no significant difference in the level of this protein in the liver or brain of the groups on the FR and FD experimental diets.

There was no binding of [3H]PGA by the solubilized membrane preparations from the liver, brain, and kidney of the control rats and barely detectable binding by the preparations from the liver and brain of FD rats. However, the kidney membrane preparations from the FD rats bound 128 pg to 308 pg of [3H]PGA per

Table 3. Tissue folate in rats on the FR and FD experimental diets

<table>
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<tr>
<th>Tissue</th>
<th>FR</th>
<th>FD</th>
<th>P</th>
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<tbody>
<tr>
<td>Liver</td>
<td>82 ± 11</td>
<td>12.8 ± 1.2</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Kidney</td>
<td>27 ± 1.8</td>
<td>4.2 ± 0.52</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Brain</td>
<td>5.0 ± 0.24</td>
<td>3.2 ± 0.29</td>
<td>0.0002</td>
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</tbody>
</table>

Values are means ± SE (folate in ng/mg protein) of the pooled values for 4 male and 4 female rats in each experimental group. Student’s t-test was used to determine level of significance for difference in values for the FR and FD groups.
milligram protein, which is less than the molar equivalent of the GPI-FBP measured by RIA, indicating that the protein still contained endogenous folate ligand. The major decrease of the GPI-FBP in the kidney could not be ascribed to a decrease in transcription as shown in the Northern blot (Fig. 3A). Of note is the expression of two GPI-FBP transcripts in the kidney (~1.25 and ~1.58 kb) compared with one transcript in the brain. A GPI-FBP transcript could not be identified in the liver even when probing 5 µg of poly(A)+ RNA. This is consistent with the very low expression of the protein in this tissue.

Though the steady-state level of the GPI-FBP transcript is apparently similar in the FR control and FD rats, the stability of the transcript may be reduced in folate deficiency, and this could be reflected in a compensatory increase in the transcription rate. Accordingly, nuclear run-on assays were carried out with isolated nuclei from folate-depleted and folate-replete rats to investigate this possibility. Rats fed the FD basal diet for 3 wk were used because preliminary experiments indicated that both folate and GPI-FBP in the kidney were substantially reduced within this time period. As shown in Fig. 3B, there was no apparent difference in the transcription rate of the GPI-FBP mRNA in the control and FD rats that could explain the decrease in the expression of this protein in folate deficiency.

Fig. 2. GPI-FBP in liver, kidney, and brain tissue from folate-replete (open bars) and folate-depleted (solid bars) rats maintained on respective basal and Clifford diets.

Fig. 3. Analysis of transcript encoding the GPI-FBP. A: Northern blot of total RNA from kidney and brain of rats on control FR (indicated as N) or folate-deficient (FD) basal or Clifford diet. B: nuclear run-on assays using nuclei prepared from kidney of folate-replete (normal) and folate-deficient rats. Intensity of autoradiograph signals for experimental groups was comparable to normalizing glyceraldehyde-3-phosphate dehydrogenase (GAPDH) signals.
indicating that neither an alteration in the processing of the hnRNA nor the stability of the transcript accounts for the reduction of the GPI-FBP.

In the absence of any evident reduction in transcription of the GPI-FBP gene(s) to explain the decrease of this protein in the kidney in folate deficiency, we considered proteolysis of the protein a likely possibility. In earlier studies in our laboratory of the FBP from human leukemia cells (33), we observed (unpublished observations) that the protein lacking the folate ligand (i.e., apoform) was rapidly digested by trypsin whereas it was resistant to such proteolysis if saturated with PGA (i.e., holoform). A conformational change induced by ligand binding (25) is likely to increase the stability and provide resistance of the protein to proteolysis. Because proteases, one of which is the metalloprotease meprin (9), have been identified on the brush borders of the renal tubules and are also secreted into the urine, holo- and apo-GPI-FBP on isolated proximal brush-border tubule vesicles were incubated with purified meprin or with trypsin, and the results are shown in Fig. 4. Incubation of apo-GPI-FBP on the brush-border vesicles with as little as 0.1 unit of trypsin rapidly degraded the protein, whereas the GPI-FBP saturated with [3H]PGA was substantially resistant to such proteolysis (Fig. 4A). Even the apo-GPI-FBP on membranes of intact KB cells grown in folate-deficient medium (30) is degraded by trypsin whereas the folate ligand provides resistance to such proteolysis (data not shown).

Proteolysis of the apo-GPI-FBP was also observed with meprin (Fig. 4B) whereas the [3H]PGA-GPI-FBP complex was resistant to this protease. Because meprin is a metalloprotease (9), it is puzzling that EDTA did not fully block proteolysis of the apo-GPI-FBP, but we did not have sufficient purified enzyme to investigate this further. It is also possible that the brush-border vesicle preparations contain other proteases that can degrade the apo-GPI-FBP.

To determine whether proteolysis in vivo generates immunoreactive and functional folate-binding protein in urine, one group of four rats (2 male and 2 female) was placed on the Clifford folate-deficient diet, and a second group on the Clifford folate-replete diet, for 4 wk. This duration of the folate-deficient diet is sufficient to induce severe deficiency in these animals. The rats were then housed in metabolic cages and the urine collected for 24 h into graduated glass cylinders containing aprotinin, leupeptin, and PMSF. As shown in Table 4, there is no significant difference in the excretion of immunoreactive FBP over 24 h in the folate-replete and folate-deficient groups. However, though not included in Table 4, the mean amount of immunoreactive FBP in 24 h for the four male rats (2,662 ng) was significantly greater (P = 0.01) than for the four female rats (858 ng); this is likely to be a consequence of the larger size of the male animals.

Although there is clear evidence for immunoreactive FBP in the urine, there was no specific binding of [3H]PGA either before or after the urine was treated at an acid pH to dissociate endogenous folate, which was then removed by charcoal adsorption (13).

A concern raised by these findings was that reduction of the GPI-FBP in the kidney might have been a consequence of degradation during the preparation of the tissue for these quantitative studies. To investigate...
Table 4. Immunoreactive FBP in urine

<table>
<thead>
<tr>
<th>RIA</th>
<th>Male</th>
<th>Female</th>
<th>Combined</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>FR 2</td>
<td>FD 2</td>
<td>FR 2</td>
</tr>
<tr>
<td>Immunoreactive FBP, ng/24 h</td>
<td>3,290 ± 984</td>
<td>2,035 ± 17</td>
<td>881 ± 246</td>
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</table>

Values are means ± SE; n, no. of rats. FR and FD indicate the folate-replete and folate-deficient groups, respectively. Combining values are the sum of the male and female rats for each group. P = not significant.

this possibility, the kidneys from four rats on the FD basal diet for 7 wk were removed and immediately frozen in liquid nitrogen. Each kidney was divided into two parts and the membrane fraction prepared as described, with one part homogenized in the standard buffer containing PMSF and leupeptin and the other part homogenized in a second buffer containing, in addition, 10 mM EDTA and 20 mM DTT, inhibitors of the meprins (9). The membrane fractions were solubilized in the respective buffers and the GPI-FBP assayed. The mean value for GPI-FBP in the membrane fraction prepared in the standard buffer was 247 ng/mg protein, and the GPI-FBP in the membrane fraction prepared in the same buffer containing EDTA and DTT was 223 ng/mg protein (P = 0.14). These results provide evidence that the decrease of the GPI-FBP in the kidney in folate deficiency occurred in vivo and not during the preparation of the membrane fraction.

A corollary to this observation that folate protects the GPI-FBP from degradation is that the rapid decrease in plasma folate that occurs with a folate-deficient diet, even for a short period, will reduce the folate in the glomerular filtrate and tubular fluid, and the apo-GPI-FBP that accumulates on the brush borders could be subject to proteolysis even before tissue folate is depleted. To examine this postulate, serum folate and the GPI-FBP in the kidney were determined in each of two groups of three rats (1 male and 2 female) fed the FR or FD basal diet for 1 wk. In this brief period, serum folate in the rats on the FD diet decreased 82% to 7.1 ng/ml, from a mean of 39 ng/ml in the rats on the folate-replete diet (P = 0.0001). The GPI-FBP decreased 41%, from 441 ng/mg protein in the control animals to 261 ng/mg protein (P = 0.14). These results provide evidence that the decrease of the GPI-FBP in the kidney of the FD rats begins within 1 wk of the FD diet and it is evident that reduction of the GPI-FBP in the kidney of the FD rats is quite similar (0.4 ± 0.04 and 0.54 ± 0.07, respectively, P = 0.35), supporting the notion that the reabsorption of folate from the tubules can be quantitatively related to the number of GPI-FBP molecules on the brush borders.

DISCUSSION

The initial objective and design of this study was to determine whether folate deficiency in vivo induces or
upregulates expression of a GPI-FBP as it does in a number of cultured cell lines that express this protein (7, 24). The rat was selected for this study because the GPI-FBP is highly homologous to the human protein (13) and it is expressed in similar tissues. There was no initial plan to measure the tubular reabsorption of folate by the kidney for two reasons; first, a decrease of the GPI-FBP on the brush borders of the proximal tubules was not expected and, second, the function of this protein in conserving folate that passes into the glomerular filtrate had already been reported (37).

Two folate-deficient diets, one containing a small amount of folic acid (basal), and the other lacking folic acid (Clifford), were selected for this experiment to avoid two potentially confounding results. One possibility considered was that the rats given the completely folate-deficient Clifford diet would become ill, as reported for weanling mice on this diet (4), and may, therefore, not be capable of upregulating expression of the GPI-FBP (or any proteins that require a healthy animal). On the other hand, the rats on the FD basal diet may be receiving just sufficient folate to maintain essential intracellular metabolic processes over this 7 wk experimental period but could still dampen the upregulation of expression of the GPI-FBP.

Two results of this study were unexpected. First, the normal liver, a major storage site for folate, expresses a very low level of this GPI-FBP (i.e., only ~1% and ~10% of that measured in the kidney and brain, respectively), and second, expression of the protein did not increase in folate deficiency. In a previous study from our laboratory, a FBP was identified in a membrane preparation from the liver of folate-deficient rats but the nature of the protein was not established (14). However, the total folate-binding capacity of this preparation from the deficient rats was similar to the total folate bound to this protein in the control rats, indicating that folate deficiency served to “unsaturate” this FBP rather than upregulate its expression.

The low expression of the GPI-FBP in rat liver may be unique for rodents. Vanhoozen and co-workers (40) have cloned the cDNA from a pig which encodes a GPI-FBP highly homologous to the human protein. Although the GPI-FBP was not quantified in the pig liver, Northern blot clearly indicated a level of expression that we could not find in the rat liver in this study.

The kidney, which contains substantially less folate than the liver, contains ~80-fold more GPI-FBP. This finding is compelling evidence that the GPI-FBP serves a specialized function in the kidney that complements the RFC in the conservation of folate. The GPI-FBP is topographically located on the brush borders of the tubules (27), where it functions to reabsorb folate from the renal tubular fluid (37). This protein has a high affinity for N’-methyltetrahydrofolate and can “sequester” this folate as the glomerular filtrate passes into the proximal tubules. After internalization by endocytosis, the folate dissociates from the GPI-FBP in the acidic endosome, exits the basolateral surfaces of the cell into the interstitial fluid, and passes into the plasma through the peritubular capillaries and may also be secreted back into the tubules (32). There is evidence that folate reabsorption may be passive diffusion through a high-capacity, low-affinity nonsaturable system (probably the RFC) when folate in the glomerular filtrate is high and via a saturable system (probably the GPI-FBP) when the folate concentration in the filtrate is low (as would occur in folate deficiency) (32).

The second unexpected finding in this study was that folate deprivation did not upregulate transcription of the GPI-FBP gene as it does in cell lines cultured in folate-deficient medium (7, 24). Indeed, the GPI-FBP in the kidney decreased by 64% in the rats on the FD basal and FD Clifford diets. This decrease in the tissue concentration appears to be the result of proteolysis of the GPI-FBP that becomes unsaturated as a consequence of the diminished concentration of folate in the glomerular filtrate as plasma folate decreases.

Another interesting finding is the release of immunoreactive but nonfunctional FBP in the urine, which, however, did not differ in amount in the folate-deficient and control groups, although males release more protein than female rats. Apparently, the folate status of the animals is not contributing to the urinary immunoreactive FBP, which is likely to be a consequence of the rapid turnover of the renal tubular epithelium. The GPI-FBP on the brush border could be released by either or both alkaline phosphatase, which will hydrolyze the phosphate bond that bridges the ethanolamine with the terminal mannose of the phosphatidylinositol moiety of the protein, or by phosphatidylinositol-specific phospholipase C, which will hydrolyze the phosphate bridge between the phosphatidylinositol and the lipid component of the GPI anchor. Because the urine contains no folate-binding component, the immunoreactive FBP may also be peptides released from the tubular epithelium after lysosomal degradation after endocytic uptake of the [folate]GPI-FBP complex (37). Clearly, because of these confounding possibilities, additional studies are necessary to characterize the nature of this nonfunctional immunoreactive FBP (or peptides) released from the renal tubular cells.

The reduction of the GPI-FBP in the brain of the folate-deficient rats was much less than in the kidney, and this relative preservation of the protein could explain why brain folate may not decrease (39), or decreases only moderately, in folate deficiency (8).

The results of this study may explain the pathophysiology of folate malabsorption that occurs with excessive intake of alcohol. Halsted et al. (20) showed that poor nutrition in association with chronic ingestion of alcohol in humans is responsible for folate malabsorption, because a normal hospital diet corrected the impaired absorption of [14C]PGA despite the continued daily consumption of alcohol. Because the GPI-FBP is expressed on the brush borders of the intestinal epithelium (38), when dietary folate is inadequate, the protein is likely to become unsaturated and could be subject to proteolysis by the meprin protease, which is also present on the intestinal brush borders (2), as well
as by trypsin secreted by the pancreas. This could contribute (in addition to the renal loss) to the development of folate deficiency by decreasing the absorption of folate from a diet deficient in this vitamin. This decrease in the GPI-FBP on the brush borders of the proximal tubular cells in folate deficiency is clearly antithetical to a teleologic adaptation to a deficiency of this important nutritional factor. This is evidently a physiological paradox because folate conservation by the kidney should increase, not diminish, as plasma folate decreases with lower folate intake. One can only propose that the susceptibility of the apoform of the GPI-FBP to proteolysis is a biological error that occurred in the coding region of the FBP gene during evolution, such that the tertiary conformation of the ligand-free protein exposes peptide sites that are readily hydrolyzed by proteinases.

We thank Lalitha Reddy for technical assistance and Bertha Wallace for help in preparing this manuscript. We are indebted to Marvin Nurnoef, President of Universal Diagnostic Laboratories, for the hemograms and standard chemistry analyses of the blood samples. Special thanks go to Dr. Judith Bond for the sample of purified meprin.

This work was supported by National Heart, Lung, and Blood Institute Grant RO1-HL-50874 and the Raymond and Frances Church Biomedical Research Fund.

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Received 17 June 1999; accepted in final form 18 October 1999.

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