Folate binding protein: molecular characterization and transcript distribution in pig liver, kidney and jejunum

Chi Mai VAN HOOZEN, Erh-hsin LING and Charles H. HALSTED*

Division of Clinical Nutrition and Metabolism, TB 156, School of Medicine, University of California, Davis, Davis, CA 95616, U.S.A.

Folate-binding protein (FBP) was identified and characterized in a pig liver cDNA library by screening with a 0.6 kb fragment from the cDNA of FBP from a human KB cell cancer line. The cDNA of pig liver FBP included 1230 bp containing 759 bp in the open reading frame with 80% similarity to the human placenta FBP. The deduced 253 amino acid sequence showed 67–73% similarity to previous sequences and contained 16 conserved cysteine residues, 11 tryptophan potential folate-

binding sites, three sites for N-linked glycosylation and 14 hydrophobic C-terminal residues. Northern analysis and reverse transcriptase PCR identified transcripts in pig liver and kidney, but not in jejunal mucosa. Although defining the molecular structure of pig liver FBP, these studies suggest that this protein participates in the regulation of folate uptake by liver and kidney membranes but is not involved in folate absorption.

INTRODUCTION

Folate-binding proteins (FBPs) have a high affinity for monoglutamyl folates [1] and have been identified in human placenta [2,3], rat [4] and pig kidney [5], bovine milk [6], and human cancer cell lines including KB [2,7] and Caco-2 cells [8]. The nucleotide and amino acid similarities of FBPs from placenta and several different mammalian cells lines [1–3,7,8] suggest that they may play a role in folate homeostasis through regulation of transport across intestinal, liver and kidney membranes. In this context, previous investigators demonstrated FBPs by gel filtration of rat and human liver plasma membranes [9,10], affinity chromatography of rat [4] and pig [5] renal tubular membranes and by autoradiography of affinity-labelled pig jejunal mucosal proteins separated by gel electrophoresis [11]. So far, FBPs have not been described at the molecular level in these three tissues that regulate the intestinal absorption, hepatic storage and metabolism, and renal conservation of folates.

The goals of the present study were to identify and determine the molecular structure of FBP in pig liver and to elucidate and contrast its presence and function in three tissues relevant to folate homeostasis, including liver, kidney and jejunal mucosa. We report the molecular sequence of pig liver FBP, its deduced amino acid sequence, its predicted secondary structure and the tissue distribution of its transcripts. Although demonstrating high similarity of the pig liver FBP nucleotide and translated amino acid sequence to that described in other species and cells, our results suggest that FBP is essential for the membrane transport of monoglutamyl folates into liver and kidney but not into jejunal mucosa.

EXPERIMENTAL

Materials

Tissues were obtained by terminal surgery of adult Yucatan micropigs. For RNA isolation, portions of the liver were resected and freeze-clamped. A section of kidney was removed and

immediately frozen in liquid nitrogen. For isolation of jejunal mucosa, a proximal portion was isolated, clamped at both ends, resected, then opened longitudinally and rinsed thoroughly with ice-cold saline followed by mucosal scraping over a bed of ice and immediate freezing of mucosal portions in dry ice. All tissues were stored at -80°C before use. [α - ^{32}P]dCTP (3000 mCi/mmol) and [α - ^{35}S]dATP (1000 mCi/mmol) were purchased from Amersham Life Sciences. All endonuclease restriction enzymes were purchased from Life Technologies or New England Biolabs. Sequenase was purchased from United States Biochemical. *Taq* DNA polymerase was purchased from Promega. Human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA was obtained from ATCC.

Isotope labelling of the FBP fragment

A cDNA clone for FBP from the human KB cancer cell line [7] was a gift from Dr. S. Rothenberg (State University of New York, Brooklyn, NY, U.S.A.). The cDNA was double-digested with *Eco*RI and *Pst*I endonuclease restriction enzymes. The 0.6 kb fragment that contained most of the open reading frame was used as a probe. Isotope labelling of the probe was performed with the Random Primed Labelling Kit (Boehringer Mannheim Biochemicals) in accordance with the manufacturer's recommended protocol. Unincorporated nucleotides were removed with the Nuc Trap Probe Purification Column (Stratagene Cloning Systems).

Library screening and cDNA sequencing

An amplified λ gt10 cDNA library from Yucatan pig liver was a gift of Dr. R. MacKenzie (McGill University, Montreal, Canada). The library contained approx. 1.7×10^6 plaque-forming units/ml with an average insert size of 2–2.2 kb. The pig liver cDNA library was propagated in *Escherichia coli* strain C600 host cells (Stratagene Cloning Systems) at a density of approx. 2.5×10^4 plaque-forming units per 150 mm plate. Plaques were transferred to nylon membranes (Schleicher & Schuell) in ac-

Abbreviations used: FBP, folate-binding protein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RT-PCR, reverse transcriptase PCR.

* To whom correspondence should be addressed.

GAATTCGCGG CCGCTCCGGG AAGGGGGGAA GGGCACAAC CTCTCGGGAA GCTCGCGCT 60
 GCCTCTCTGA GCAGAAGGA GACAAAGCC ATG CCC TGG AAG CTG ACA GCC CTT CTG 116
 M^{*} P W K L T A L L
 CTC TTT CTG GCC GGG GTG GTC TCC GTG TGC CGC GCC CGG GCC AGG ACG GAC 167
 L F L A G V V V S C R A R A R A R T D
 CTG CTC AAC GTC TGC ATG GAT GCC AAG CAC CAC AAG GTA GAG CCA GGC CCT 218
 L L N V C M D A K H H K V E P G P
 GAG GAC GAG CTG CAC GAC CAG TGC GTC CCC TGG AAG AAG AAC GCC TGC TGC 269
 E D E L H D Q C V P W K K N A C C C
 TCC GCC AGA GTC AGC CAC GAG CTG CAC CGG GAC AAG TCC TCC CTG TAT AAC 320
 S A R V S H E L H R D K S S L Y N^{*}
 TTT TCC TGG GAG CAC TGC GGC AGG ATG GAG CCG GCC TGC AAG CGC CAC TTC 371
 F S W E H C G R M E P A C K R H F
 ATT CAG AAC AAC TGT CTG TAC GAG TGC TCG CCC AAC CTG GGG CCC TGG TTC 422
 I Q N N C L Y E C S P N L G P W F
 CAG GAG GTG AAC CAG AAG TGG CGC AAA GAG CGG TTC CTG AAC GTG CCC CTC 473
 Q E V N Q K W R K E R F L N V P L
 TGC AAA GAG GAC TGT CTG GAC TGG TGG GAA GAC TGC CGC ACC TCC TAC ACC 524
 C K E D C L D W W E D C R T S Y T
 TGC AAG AGC AGC TGG CAC AAG GGC TGG AAC TGG AGC TCA GGA TCT AAC CAG 575
 C K S S W H K G W N W S S G S N Q
 TGT CCC ACG GGG ACC ACC TGC GAC ACA TTT GAG TCC TTC TCC CCC ACA CCC 626
 C P T G T T C D T F E S F F P T P
 GCA GCG CTG TGT GAG GGC ATC TGG AAT CAC GAT TAT AAG TTC ACC AAC TAC 677
 A A L C E G Y K F T N Y^{*}
 AGC CGG GGC AGC GGC CGC TGC ATC CAG ATG TGG TTT GAC GCG GCC GAG GGC 728
 S R G S G R C I Q M W F D A A E G
 AAC CCC AAC GAG GAG GTA GCG AGG TTC TAC GCC TTG GCC TTG AGT GCG GGS 779
 N P N E E V A R F Y A L A L S A G
 ACC ATG TCC CTT GGG ACC GGG CCT CTC CTG CTC AGC GCA GCC CTG ATG CTG 830
 T M S L G T G P L L S A A L M L
 CCA CTT GGG CTC CTT GAC TGA GTCCGGCGTC TCCAGACGGT CCTCTGCCT GTCC 885
 P L G L L D²⁵³ -
 CCAGCTTTGA TGACGAGGCT GGTCTCAACT CAGCTCCAC CAATGAGGGA GCCCTAAGCC 945
 CGCCTCATCT GTTACCCATC CCTCTGTAT CAAGTTCCTG CCGTAGGGTG GGCCTTGGGG 1005
 TCTCTCTGAC AGCCAGTCTT AACAGGACGA TTAACAGCAC TGTGTCTGAT GGGCTGTGTTT 1065
 GGTGTGAGC TGGTGTGTGG CAGAGGACAG AGCCCATAGC TTTTGGATTC CTTTCAGCTTA 1125
 GAGAAATGAG ACCTGGGTTT GAATCCACAG TCTGCCACTC ACTATGTCAA GTGAAGCAGT 1185
 TCGCGCAGCG CTCTAAACCA TAGGCTCTCT CTCATTAABA TGAAG 1230

Figure 1 Nucleotide and deduced amino acid sequence of pig liver FBP

The complete nucleotide sequence (upper line) and deduced amino acid sequence (lower line) for the pig FBP cDNA clone are shown. A polyadenylation signal (AATAAA) is underlined. Amino acids are represented by their single-letter codes and the termination codon (TGA) is indicated by (-). The three potential sites for N-linked glycosylation (N-X-S/T) sites are marked (*).

cordance with the method of Benton and Davis [12]. Paired filters were then prehybridized in a solution consisting of $5 \times$ SSC, $5 \times$ Denhardt's solution, 0.1% SDS, 50% (v/v) formamide and 100 μ g/ml denatured salmon sperm DNA at 42 °C, and subsequently hybridized [13] overnight in identical conditions with the 32 P-labelled *EcoRI*-*PstI* 0.6 kb fragment of human FBP. After hybridization, membranes were washed with progressive stringency and autoradiographed overnight at -70 °C with an intensifying screen. Positive plaques were purified by secondary and tertiary screening. Tertiary clones were identified by Southern blot analysis [14]. After isolation and purification, cDNA inserts were subcloned into the *EcoRI* site of pBluescript II SK⁺ (Stratagene Cloning Systems). Both strands were completely sequenced by dideoxy chain termination [15] with either the T3 or T7 polymerase vector primer sequence or specific oligonucleotide primers, according to appropriate regions of the FBP cDNA. Each strand was sequenced independently by primer walking with oligonucleotide primers that were constructed from bases 288–306, 528–543, 700–716 and 969–985 (sense) and 1012–1026, 840–854, 744–759, 557–572 and 276–290 (anti-sense) according to the complete cDNA sequence (Figure 1).

Isolation of RNA and genomic DNA

Total RNA was isolated from jejunal mucosa, kidney and liver using TRIzol reagent (Life Technologies) and was quantified by spectrophotometry at 260 nm [16]. Each total RNA (10 μ g) was subjected to electrophoresis in 1.2% (w/v) agarose/2.2 M formaldehyde gel [17], stained with ethidium bromide and then photographed to show the same density of total RNA from each tissue. Poly(A)⁺ RNA was isolated with the FastTract 2.0 mRNA isolation system (Invitrogen). Genomic DNA was isolated from each tissue as described [18].

Northern blotting

Poly(A)⁺ RNA from jejunal mucosa, kidney and liver (5 μ g of each) were separated by gel electrophoresis in 1.2% (w/v) agarose/2.2 M formaldehyde gels, transferred to nylon membranes and hybridized with a 32 P-labelled pig FBP cDNA of a 759 bp fragment of the open reading frame that was obtained by PCR. The primers for PCR were selected from the sequence of pig FBP cDNA, including 5'-GGAATTCATGCCCTGGAAG-CTGACA-3' (sense) and 5'-GGGAATTCTCAGTCAAGGA-GCCCAAG-3' (anti-sense). 32 P-labelled human GAPDH cDNA 0.8 kb *XbaI*-*PstI* fragment was used as a positive quantitative control for mRNA in a parallel blot made from the same mRNA preparations from each tissue. The blots were washed as described above for the library screening and then autoradiographed for 3 h at -70 °C.

Reverse transcriptase PCR (RT-PCR)

First-strand cDNA was synthesized from 10 μ g of total RNA from each tissue by the SuperScript Preamplification System for First Strand cDNA synthesis (Life Technologies) [19]. An oligonucleotide from the cDNA for pig liver FBP was synthesized with 5'-GAGCTGCACCGGGACAAGT-3' (sense) and 5'-TAGAACTGGCTGTCAG-3' (anti-sense) as primers for the PCR of a 739 bp fragment. As described by the manufacturer, 2 μ l of each cDNA mixture was used for each reaction with 2.5 units of *Taq* DNA polymerase. Polymerization for 30 cycles at 94 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min was performed on a Hybaid thermal reactor (Labnet). Genomic DNA was treated with the same polymerase chain reaction to distinguish the separate identity of the 739 bp fragment in each amplified tissue cDNA after gel electrophoresis. Absorbance measurements were used to compare amounts between tissues.

RESULTS

Sequence of pig liver FBP

Screening the pig liver cDNA library with a probe prepared from the *EcoRI*-*PstI* digested fragment of human FBP cDNA sequence identified four positive clones out of 250000 plaques screened. Dideoxynucleotide sequencing of the 1.2 kb inserts from three clones resulted in a cDNA of 1230 bases that contained an identical 759-base open reading frame between bases 90 and 849 that encodes a peptide sequence of 253 amino acids (Figure 1). The similarity between the cDNA sequences in the open reading frame of pig liver FBP and the published sequence for human placenta FBP [3] was 80%. Back-translation of the open reading frame revealed 67–73% similarity of the deduced pig liver FBP sequence to previously published amino acid sequences [2,3,6–8,20] (Figure 2). The deduced amino acid sequence revealed cross-species conservation of 16 cysteine sites, 11 tryp-

Pig Liver	M P W K L T <u>A L L L F L A</u> G V V S V C R A R A R T D L L N V C M D A K H H
Human placenta	M V W K W M P L L L L L V C V A T M C S A Q D R T D L L N V C M D A K H H
Bovine milk	A Q A P R T P R A R T D L I N V C M D A K H H
Pig Liver	K V E P G P E D E L H D Q C V P W K K N A C C S A R V S H E L H R D K S S
Human placenta	K T K P G P E D K L H D Q C S P W K K N A C C T A S T S Q E L H K D T S R
Bovine milk	K A E P G P E D S L H E Q C S P W R K N A C C S V N T S I E A - K D I S Y
Pig Liver	L Y N F S W E H C G R M E P A C K R H F I Q N N C L Y E C S P N L G P W F
Human placenta	L Y N F N W D H C G K M E P A C K R H F I Q D T C L Y E C S P N L G P W I
Bovine milk	L Y R F N W D H C G K M E P A C K R H F I Q D T C L Y E C S P N L G P W I
Pig Liver	Q E V N Q K W R K E R F L N V P L C K E D C L D W W E D C R T S Y T C K S
Human placenta	Q Q V N Q S W R K E R F L D V P L C K E D C Q R W W E D C H T S H T C K S
Bovine milk	R E V N Q R W R K E R I L G V P L C K E D C Q S W W E D C R T S Y T C K S
Pig Liver	S W H K G W N W S S G S N Q C P T G T T C D T F E S F F P T P A A L C E G
Human placenta	N W H R G W D W T S G V N K C P A G A L C R T F E S Y F P T P A A L C E G
Bovine milk	N W H K G W N W T S G Y N Q C P V K A A C H R F D F Y F P T P A A L C N E
Pig Liver	I W N H D Y K F T N Y S R G S G R C I Q M W F D A A E G N P N E E V A R F
Human placenta	L W S H S Y K V S N Y S R R S G R C I Q M W F D S A Q G N P N E E V A R F
Bovine milk	I W S H S Y K V S N Y S R G S G R C I Q M W F D P F Q G N P N E E V A K F
Pig Liver	Y A L A L S A G T M S L G T G P L L L S A A L M L P L G L L D
Human placenta	Y A A A M H V N A G E M L H G T G G L L L S L A L M L Q L W L L D
Bovine milk	Y A E N P T S G S T P Q G I

Figure 2 Amino acid sequence alignment of three different FBP

Pig liver FBP is aligned with human FBP from human placenta FBP [3] and bovine milk FBP [6]. Amino acids that are identical with those in the pig FBP sequence are shown in bold. There are 16 conserved cysteine residues (C), and 11 conserved tryptophan sites (W). The N-terminal signal peptide sequence is shown in the box. Identical cross-species regions consistent with a glycosylphosphatidylinositol anchoring site at the C-terminus are underlined.

Figure 3 Hydrophobicity plot of pig FBP

A Kyte-Doolittle [22] analysis of the deduced amino acid sequence with a window size of 5 is shown. Prominent hydrophobic regions are found at both N- and C-termini.

tophan sites and three potential N-X-S/T sites for N-linked glycosylation at positions 77, 155 and 195. The N-terminal sequence ALLFLA is significant for a complete signal peptide sequence for endoplasmic reticulum translocation [21]. The terminal 14 hydrophobic C-terminal residues contain 64 % of the amino acid sequence as described for FBP in human KB and Caco-2 cells [2,7,8]. Hydrophobicity plot analysis described both hydrophobic domains at each terminus (Figure 3) [22]. Cleavage of the N-terminal signal peptide before membrane insertion would predict a predominant C-terminal membrane anchor.

Tissue distribution of transcripts

Northern blot analysis of poly(A)⁺ RNA with the 759 bp probe from the open reading frame of the pig FBP cDNA revealed a transcript at 1.35 kb in pig liver and kidney, whereas no transcript

Figure 4 Northern blot analysis of pig FBP transcripts in jejunum, kidney and liver

Poly(A)⁺ RNA from jejunum, kidney and liver (5 µg from each) was analysed with the ³²P-labelled pig FBP cDNA open reading frame 759 bp fragment. A parallel Northern blot of the same mRNA preparations was hybridized with a 0.8 kb fragment of human GAPDH cDNA for quantitative comparisons of mRNA from each tissue. The autoradiograph shows a 1.35 kb mRNA signal for FBP in kidney and liver but not in jejunum, and similar amounts of GAPDH signal in each tissue. Transcript size was determined by comparing migration distance with an RNA ladder (Gibco BRL). Lane 1, jejunum; lane 2, kidney; lane 3, liver.

was identified in pig jejunal mRNA (Figure 4). Absorbance measurements showed similar amounts of GAPDH mRNA in each tissue, whereas the FBP mRNA seemed to be more prevalent in liver than in kidney. RT-PCR with designated sense and anti-sense primers demonstrated amplified 739 bp fragments of liver cDNA that seemed somewhat more dense than similar fragments in kidney cDNA, whereas there was no detectable signal in jejunal cDNA (Figure 5). Finding a distinct genomic DNA size fragment at about 1.1 kb indicated the authenticity of the smaller fragments that were obtained by RT-PCR from liver and kidney cDNAs.

Figure 5 Identification of tissue transcripts by reverse-transcriptase polymerase chain reaction

Total RNA from jejunum, kidney and liver (10 μ g from each) was used for first-strand cDNA synthesis by reverse transcription, and 2 μ l of each cDNA mixture was used for PCR. A 739 bp fragment was amplified from kidney and liver cDNA by using sense and anti-sense primers as described, whereas no fragment was identified in jejunal mucosa. Genomic DNA was treated by the same PCR conditions as a size control to distinguish the amplified fragment from cDNA. Lane M, pGEM size marker; lane 1, jejunum; lane 2, kidney; lane 3, liver; lane 4, genomic DNA.

DISCUSSION

The current study has demonstrated the presence of a unique FBP in pig liver that shares 77% nucleotide similarity in the open reading frame with the human placenta cDNA [2,3] and 67–73% similarity of the deduced amino acid sequences with those previously described in human KB [2,7] and Caco-2 cancer cell lines [8] and placenta [2,3]. The finding of FBP cDNA in pig liver is consistent with prior findings of FBP by gel filtration of isolated rat and human liver plasma membranes [9,10] and by immunoblot and activity measurements of isolated liver plasma membranes from the present micropigs [23].

Previously, membranous FBPs were identified and fully characterized in isolated human KB [2,7] and Caco-2 cells [8], placenta [2,3] and spleen cells from patients with myelogenous leukaemia [20], each demonstrating polymorphism in the 5' untranslated and open reading frame with approx. 70–80% nucleotide similarity. Together with other studies on human placental [24] and rat [4] and pig kidney [5] FBP, these studies defined the membranous form of FBP as containing approx. 257 amino acids that, with glycosylation, give rise to a protein with a molecular mass of 38 kDa [1]. Other studies identified a slightly smaller soluble form of FBP in bovine and human milk [6,25] that might be a homologous cleavage form of the membranous FBP found in other tissues.

The isolated pig liver FBP reveals an identical deduced size and substantial similarity of the amino acid sequences when compared with those identified in human cancer cell lines, placenta and myelogenous cells [2,3,7,8,20]. As shown in Figure 2, the pig FBP contains all 16 conserved cysteine residues essential for the formation of disulphide bonds in the secondary structure of the protein. Two of the three N-linked glycosylation sites of pig FBP appear at the asparagine residues that are conserved in six of the seven reported sequences at Asn-155 and Asn-195. Eleven tryptophan residues appear in pig FBP at the same sites as those that occur in all of the other previously reported sequences. Other studies have implicated tryptophan residues in the protein binding of riboflavin [26], a vitamin with a pterin ring structure similar to that of the folate molecule [27]. It is possible that the tryptophan residues function in an analogous manner in the binding of folates to FBPs.

FBP might be inserted into the membrane by way of a C-terminal and cholesterol-regulated glycosylphosphatidylinositol anchor [28]. Conceptually, the folate–FBP complex might be internalized in caveolae by a process termed potocytosis, following which folate is dissociated by low pH and crosses into the cytoplasm by an anion carrier and is polyglutamylated while the FBP recycles back to the cell surface [29]. Although this concept was developed from studies with isolated monkey kidney MA104 cells [30], it is also applicable to the proposed mechanism for folate uptake by Caco-2 cells [8] and the rat kidney proximal tubule [31] in which uptake was prevented by the cleavage of membrane-bound [3 H]folic acid from transfected cells and isolated rat kidney brush border membranes by phosphatidylinositol phospholipase C [8,31]. The sequence analysis of pig liver FBP might be consistent with the glycosylphosphatidylinositol anchoring system, because 9 of the 14 C-terminal residues were identical with those described in isolated human Caco-2 and KB cells (Figure 2) [2,7,8]. However, hydrophobicity plots of KB cell and placental cDNA species showed several hydrophobic and presumably membrane-spanning regions, without convincing evidence of sufficient length of the C-terminus for membrane insertion [2,3,7]. An alternative proposal that accounts for regional hydrophobicity holds that membrane anchoring of FBP is dependent on ester linkages to membrane fatty acids [32].

Our results are partly at variance with some of the previously published studies that used Northern blots with cDNA probes from isolated cells to localize FBPs to human placenta, thymus, brain, choroid plexus, lung, thyroid and kidney, but not liver, pancreas or intestine [2,33]. The reported immunohistochemical locations of FBP to epithelial cells in placenta, choroid plexus and renal tubules support a homeostatic role of FBP in folate conservation by these tissues [34]. Supporting our findings of FBP transcripts in liver and kidney, others isolated FBP from rat, pig and human kidney tubules by affinity chromatography [4,5,35] and from rat and human liver plasma membranes by affinity labelling of proteins after gel filtration [9,10]. In contrast, the absence of FBP transcripts from pig jejunal mucosa (Figures 4 and 5) does not support a prior report of FBP in isolated pig jejunal brush border membranes [11].

The present findings on the tissue localization of FBP transcripts are consistent with our recent results on the sites of membrane FBP activity [23] and suggest that this protein is essential for the hepatic uptake of folates by liver plasma membranes and in renal conservation by kidney brush border membranes. Because we could not detect FBP in jejunal mucosal preparations, it is unlikely that FBP is involved in the jejunal absorption of folates. However, a reduced folate carrier protein was recently characterized in isolated L1210 mouse leukaemia cells [36] and mouse intestinal mucosa [37] that has a deduced size of 58 kDa, which is nearly identical with that shown by affinity-labelling electrophoresis of pig jejunal mucosa [11], and may provide for transport of monoglutamyl folate across the intestine.

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