Homologous Membrane Folate Binding Proteins in Human Placenta: Cloning and Sequence of a cDNA†,‡

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ABSTRACT: A preparation of folate binding protein purified from human placental membranes in the presence of a variety of protease inhibitors followed by deglycosylation with *N*-glycanase gave a sharp band at M_r ~28 000 following SDS–polyacrylamide gel electrophoresis. The deglycosylated protein bound [^{3}H]folic acid as tightly as the native protein. Peptides obtained following digestion of the purified protein with staphylococcal V8 protease and HPLC purification were sequenced. Polyclonal antibodies against the protein preparation were affinity purified and used to screen a placental cDNA expression library. A full-length cDNA for a placental folate binding protein was thus obtained and the corresponding protein sequence deduced. This result, taken together with the peptide sequence data, indicates the expression of at least two homologous folate binding proteins in placenta, one of which appears to be identical with the folate binding protein from human milk and nasopharyngeal epidermoid carcinoma (KB) cells; the cDNA sequence obtained corresponds to the other protein. The deduced protein sequence is characterized by a putative 16-residue amino-terminal signal peptide that is cleaved, resulting in a 239-residue polypeptide. The mature protein exhibits two potential sites for N-linked glycosylation at Asn-99 and Asn-179, eight potential intramolecular disulfide bonds, and a stretch of hydrophobic residues at the carboxyl terminus that could form a transmembrane domain. The protein bears a 68% sequence homology with the KB cell folate binding protein and may represent a fetal folate transport protein. Evidence is discussed in support of the contention that the soluble folate binding protein in various tissues is not simply a product of proteolytic processing of the membrane protein and that the two proteins must differ by some type(s) of covalent modification.

In the past two decades an immense amount of data has accumulated on the pharmacokinetics of folate and antifolate transport in a large variety of normal and neoplastic tissues and cell lines [reviewed in Ratnam et al. (1989), Sirotnak et al. (1981), Dembo and Sirotnak (1984), Goldman and Matherly (1985), and Henderson (1986)]. Putative transport proteins for these compounds have been identified or purified from some sources such as milk (Antony et al., 1982), placenta (Antony et al., 1981), kidney (Kamen & Caston, 1986), human KB cells (Elwood et al., 1986), choroid plexus (Spector, 1977; Suleimann et al., 1981), and murine L1210 leukemia cells (Price & Freisheim, 1987) only in recent years. These proteins are all glycoproteins varying in apparent molecular weights (32K–51K). Membrane folate binding proteins (FBP)[1] from a variety of normal and neoplastic tissues display a range of absolute and relative affinities for folic acid, 5-substituted tetrahydrofolates (H_4folates),[1] and methotrexate (MTX).[1] Thus, a variety of cell lines that include mouse and human leukemias, mouse and rat sarcomas, mouse, hamster, and human carcinomas, and rat hepatoma display a characteristic saturable, high-affinity uptake system for 5-substituted reduced folates (K_t = 1–3 μM) and MTX (K_t = 3–26 μM) that has a low affinity for folic acid (K_t = 200–400 μM) (Ratnam et al., 1989). This transport system has a low capacity (influx V_{max} = 1–12 nmol min^{-1} g^{-1}, dry weight) and is sodium independent, ouabain insensitive, and highly temperature dependent. On the other hand, normal proliferative cells such as intestinal epithelial cells, rat hepatocytes, porcine and rabbit choroid plexus, and rabbit kidney cortical cells as well as mature rabbit and human reticulocytes and erythrocytes may possess low levels of such a transport system but take up folate compounds through routes that are quite diverse in their relative affinities for folic acid, 5-CH_3-H_4folate, 5-CHO-H_4folate, and MTX (Ratnam et al., 1989; Sirotnak et al., 1981; Dembo & Sirotnak, 1984). The well-studied human KB (nasopharyngeal epidermoid carcinoma) cells bind and transport folic acid more efficiently than the naturally occurring reduced folates and methotrexate (Antony et al., 1985).

The primary function of proteins that have a relatively high affinity for folic acid must also actually be to transport reduced folate coenzymes, which are the physiological substrates. This has been clearly demonstrated in KB cells (Antony et al., 1985; Kane et al., 1986). Several of these proteins, e.g., the membrane-associated folate binders in milk, KB cells, placenta, and erythrocytes, have been found to cross-react immunologically (Antony et al., 1981, 1982; Elwood et al., 1986). A common phenomenon in a number of systems examined is the occurrence of an integral membrane (or particulate) FBP and a soluble or cytosolic counterpart that in at least two cases (KB cells and milk) appear to have identical N-terminal amino acid sequences (Luhrs et al., 1987; Svendsen et al., 1982), although they have different apparent molecular weights.

While it would seem a priori that there is no obvious reason for the expression of such a large variety of tissue-specific membrane FBPs, it is quite possible that many tissues express

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[1] Abbreviations: SDS, sodium dodecyl sulfate; FBP, folate binding protein; MTX, methotrexate (4-amino-10-methyl-4-deoxyfolic acid); H_4folate, tetrahydrofolic acid; IPTG, isopropyl β-D-thiogalactopyranoside.

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the same protein or similar proteins that are glycosylated to different extents in different tissues or that may differ by some other types of covalent modification or, perhaps, due to partial proteolysis. Important aspects that require further study are the relationships between the soluble and membrane-associated proteins and among different types of tissue-specific FBPs, their structure, possible posttranslational modifications, function, and possible developmental regulation in the expression of these proteins.

The membrane-associated FBP isolated from human placenta was chosen for initial studies. This protein, purified essentially by affinity chromatography on folate–Sepharose, has an apparent molecular weight of 38 000 following SDS–polyacrylamide gel electrophoresis and reportedly contains 12% carbohydrate and binds 1 mol of folate/mol of protein (Antony et al., 1981). Herein are reported results of deglycosylation and peptide sequencing of a placental membrane FBP preparation as well as the cloning and sequence of a full-length cDNA for a membrane FBP.

Materials and Methods

Protein and Peptides. The membrane FBP from human placenta was purified essentially as described by Antony et al. (1981), i.e., with the major step being affinity chromatography on folate–Sepharose, but with several modifications including the use of protease inhibitors. Briefly, frozen (−70 °C) placentas were thawed and homogenized in 15 mM potassium phosphate buffer, pH 7.5, containing 10 mM EDTA, 10 mM EGTA, 1 mM phenylmethanesulfonyl fluoride, and 10 μM leupeptin. The membrane pellet obtained by centrifugation at 30000*g* for 30 min was solubilized in the above buffer containing 1.5% Triton X-100. Endogenous folate was removed by acid treatment as described (Antony et al., 1981). The solution was applied onto a column (3 cm × 5 cm) of folic acid–Sepharose 4B (35 mL), washed with 10 mM potassium phosphate, pH 7.5/1% Triton X-100/1 M NaCl, and eluted with 100 mL of 10 mM sodium acetate–acetic acid, pH 4.5/1% Triton X-100. The pH of the effluent was immediately neutralized with 1 M potassium phosphate, pH 7.5. After dialysis against 10 mM potassium phosphate, pH 7.5/1% Triton X-100, the preparation was rechromatographed as above but with 2 mL of folic acid–Sepharose. The final preparation was passed through 2 mL of Sepharose 4B to remove protein contaminants binding to Sepharose. The purified protein was freed of bound Triton X-100 by repeated extraction with 1-butanol.

To generate peptides from the protein, the detergent-free protein (200 μg) was dissolved in 0.3 M Tris-HCl, pH 8.5/6 M guanidine hydrochloride/0.2% EDTA. The protein was then treated with a 500-fold molar excess of dithiothreitol and alkylated by adding iodoacetic acid up to a 1.1-fold molar excess over the SH groups in the reducing agent. The carboxymethylated protein was chromatographed on a Synchropak C-8 reversed-phase HPLC column using a gradient of 0–60% acetonitrile. The major protein peak was collected and subjected to proteolysis with staphylococcal V8 protease (2 μg) in 10 mM sodium phosphate, pH 7.5/150 mM NaCl for 24 h at 37 °C. The resulting peptides were separated by HPLC under the conditions described above.

One of the peptide peaks (peak c in Figure 2B) was further purified by reversed-phase HPLC on a Model 130A separation system (Applied Biosystems, Inc.) at 35 °C using an RP-300 column (2.1 × 100 mm, Applied Biosystems, Inc.) as described (Marquardt et al., 1987).

Peptide Sequencing. Automated sequence analysis was performed on a Model 475A gas-phase sequencer (Applied Biosystems, Inc.) with an on-line Model 120A PTH analyzer (Marquardt et al., 1987). Data reduction and quantitation was performed by using a Nelson 760 interface, a Hewlett-Packard 9816 computer, and Model 900A/Model 475A chromatogram analysis software (User Bulletin No. 26, Applied Biosystems, Inc., 1987).

Deglycosylation. N-Glycanase (Tarentino et al., 1985) was a kind gift from Dr. Tony Tarentino, New York State Health Laboratories, Albany, NY. FBP (5 μg) was incubated in 50 μL of 0.25 M sodium phosphate buffer, pH 8.6/0.5% Triton X-100 with 5 milliunits of *N*-glycanase for 12 h at 37 °C.

Antibodies. The membrane FBP from human placenta was used as the immunogen. The protein (0.5 mg) was injected into a rabbit intradermally in Freund's complete adjuvant. Three weeks later a booster dose of 100 μg of protein was injected intradermally in Freund's incomplete adjuvant followed by bleeding a week later. The antibodies were affinity purified by chromatography on FBP–Sepharose prepared by coupling 0.5 mg of purified placental FBP to 0.5 mL of CNBr-activated Sepharose 4B (Pharmacia). The antibody was eluted with 0.1 M glycine hydrochloride pH 2.5.

Isolation of FBP cDNA Clones and DNA Sequencing. A human placental cDNA library constructed in the expression vector λgt11 was obtained from Clontech Inc. and plated on *Escherichia coli* RY1090 (Huyhn et al., 1985). Approximately 200 000 isopropyl β-D-thiogalactopyranoside (IPTG)[1] induced recombinant plaques were screened by using affinity-purified antibodies to placental FBP by standard procedures. The cDNA inserts in these recombinant clones were cut out with *Eco*RI or alternatively with *Kpn*I and *Sac*I. DNA sequencing was carried out by the M13 dideoxy sequencing method (Sanger et al., 1977). The M13 clones were generated by subcloning restriction fragments of the FBP cDNA into M13mp18 or mp19 (Yanisch-Perron et al., 1985).

Results and Discussion

Peptide Sequences of Placental Membrane FBP. An interesting observation made by Antony et al. (1981) is that antisera made against purified placental membrane FBP cross-react with both the soluble and particulate human milk FBP and with a cell surface protein on human erythrocytes. Further, these antibodies cross-reacted with a membrane FBP, inhibiting 5-CH_3-H_4folate uptake in human KB cells (Antony et al., 1985). To establish the extent of similarity between the placental FBP and FBPs from other tissues, the amino acid sequences of selected peptides derived from the placental membrane FBP were determined. When purified FBP that had been reduced and carboxymethylated was chromatographed on a reversed-phase C-8 HPLC column with a gradient of acetonitrile (Figure 1A), several protein peaks were obtained. The protein from the individual peaks migrated similarly on SDS–polyacrylamide electrophoretic gels, indicating that they may represent microheterogeneity in the extent of glycosylation of placental FBP. Protein from the major peak in Figure 1A was subjected to digestion with staphylococcal V8 protease, and the resulting fragments were chromatographed as before (Figure 1B). Peptides from peaks b–d (referred to as peptides b–d in Figure 1B) were sequenced. Peak c gave a major sequence (c_1) and two minor sequences (c_2 and c_3). The peptides in peak c were further purified (Materials and Methods) and sequenced. The amino acid sequences of the five peptides from placental FBP are indicated in Figure 2.

The complete amino acid sequence of the soluble FBP from bovine milk (Svendsen et al., 1984) and partial sequences of the homologous soluble and particulate FBPs from human milk

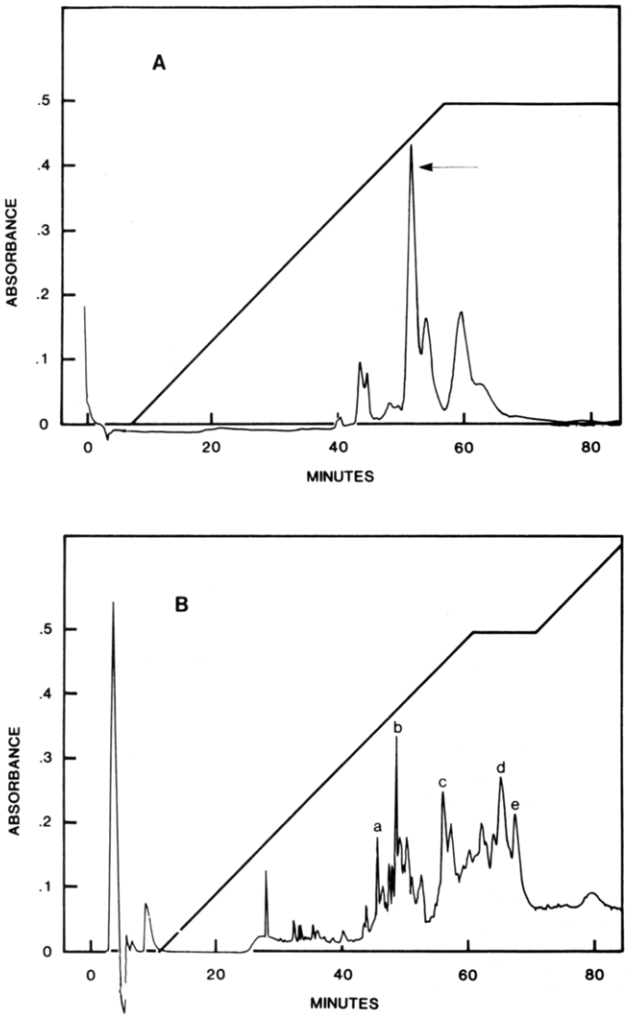

FIGURE 1: HPLC profiles of purified placental FBP and its proteolytic fragments. (A) Placental FBP was reduced with dithiothreitol, carboxymethylated with iodoacetic acid, and chromatographed on a C8 reversed-phase HPLC column (see Materials and Methods). The major peak (arrow) was used to obtain proteolytic fragments for sequencing. (B) The major peak in (A) was digested with *S. aureus* V8 protease and rechromatographed on a C8 HPLC column (see Materials and Methods). Peptides from peaks b, c, and d were used for sequencing. Absorbance wavelength is 220 nm.

(Svendsen, 1982) have been elucidated by sequencing overlapping peptides. In addition, the protein sequence of the human KB cell FBP has been deduced from the sequence of a partial cDNA (Sadasivan & Rothenberg, 1989). The sequences of the human milk and KB cell FBPs are identical. The peptides derived from human placental FBP are all homologous in sequence to corresponding regions of the human milk and KB cell FBPs (Figure 2). An interesting observation is that peptides c_1 and d are homologous but not identical with each other (6 of 18 nonidentical residues), have different lengths, and elute at quite different positions in the HPLC profile (Figure 1B). Further, unlike peptides b and d, peptides c_1, c_2, and c_3 are identical with a corresponding sequence in human milk and KB cell FBPs. Inspection of the milk and KB cell FBP sequence reveals no internal repeat of homologous peptides. These observations suggest that peptides c_1 and d represent two homologous forms of FBP in human placenta. From the nonidentity of peptides b and d with the milk or KB cell FBP it also appears that there is at least one form of FBP that is expressed specifically in placental tissue.

Deglycosylation of Placental FBP. The differences in apparent molecular weights between FBPs isolated from different tissues may be due to differences in glycosylation. It was

	PEPTIDE c_3	PEPTIDE c_1
KB CELL FBP	10 L L N V C M N A K 18	51 A H K D V S Y L Y R F N W N H C G E 68
PEPTIDE	L L N V C M N A K	A H K D V S Y L Y R F N W N H C G E
PLACENTAL FBP (cDNA)	L L N V C M D A K	L H K D T S R L Y N F N W D H C G K

	PEPTIDE b	PEPTIDE c_2
KB CELL FBP	51 A H K D V S Y L Y R F N W H H C G E M A 70	86 C S P N L G P W 93
PEPTIDE	L H K D T S R L Y N F N W D H C G K M E	C S P N L G P W
PLACENTAL FBP (cDNA)	L H K D T S R L Y N F N W D H C G K M E	C S P N L G P W

	PEPTIDE d
KB CELL FBP	157 F Y F P S P T I V L C N 167
PEPTIDE	S Y F P T P A A L C E
PLACENTAL FBP (cDNA)	S Y F P T P A A L C E

FIGURE 2: Comparison of peptide sequences of placental FBP with homologous sequences in KB cell FBP (Sadasivan & Rothenberg, 1989) and a placental FBP sequence deduced herein from its cDNA sequence. Peptides b and d were obtained from peaks b and d in Figure 1 and peptides c_1, c_2 and c_3 were obtained from peak c in Figure 1 (see Materials and Methods). Calculated yields of peptides b, c_1, c_2, c_3, and d were 300, 83, 50, 42, and 100 pmol, respectively. Differences in amino acid residues in the sequences compared are boxed. Amino acid residue numbers correspond to their positions in the KB cell FBP.

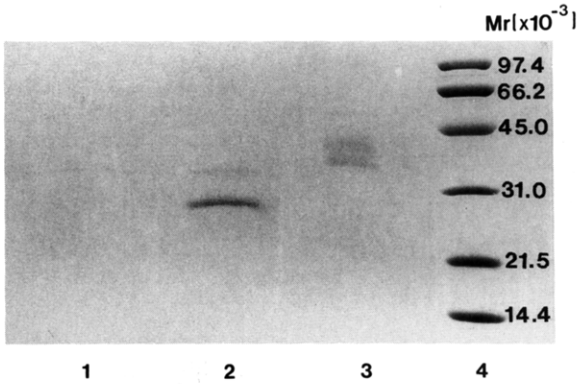

FIGURE 3: Deglycosylation of placental FBP with *N*-glycanase. Samples were treated and electrophoresed on a 12.5% sodium dodecyl sulfate–polyacrylamide gel (see Materials and Methods). (Lane 1) *N*-Glycanase (5 milliunits) alone; (lane 2) *N*-glycanase (5 milliunits) plus placental FBP (5 μg); (lane 3) placental FBP (5 μg) alone; (lane 4) molecular weight standards (Bio-Rad).

therefore of interest to determine the molecular weight of the placental FBP after deglycosylation of the protein. It was also reasoned that if there was a significant difference in the molecular weights of the two or more homologous FBPs in placenta that was masked due to glycosylation, one should be able to detect multiple bands by SDS–polyacrylamide gel electrophoresis after complete deglycosylation of the placental FBP preparation. Deglycosylation of placental FBP with *N*-glycanase converted the diffuse glycoprotein band of $M_r \sim 38\,000$ into a single sharp band at $M_r \sim 28\,000$ on SDS–polyacrylamide electrophoretic gels (Figure 3). This result indicates that the different FBPs in placental membranes are likely to be polypeptides of nearly the same length. The deglycosylated protein bound [^{3}H]folic acid as well as the native protein (results not shown), suggesting that different degrees of glycosylation could indeed account for a large part of the observed differences in molecular weights among FBPs from various tissues. This is also supported by the close correspondence of the molecular weights of KB cell and milk FBPs deduced from their amino acid sequences with that for the placental FBP.

cDNA Cloning and Sequence of a Placental FBP. The results of the peptide sequencing studies described above, which suggest the presence of at least two types of FBPs in placental

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GAATCAATTCCTCCAAACCGCAAGAACAGTAACATTTATTATTCAAAAAAACAAAAACCAGATTATAGGATATGACATTTGGTATAACAATAATGTTATTGAAAAATGGAAAAATGATC   119

CATTAATGGCTTGGGCTAAAAATTCGGGGGACAGCCTAGGGGCCTGGATCTATTGCCTACTTAGAGAGAGGCCAACTCAGACACAGCCGTGTATGCTCCCAGCAGCAACGGAGGTTCAC   238

GTCCGCCTGCAGGGACAGAAAGAC ATG GTC TGG AAA TGG ATG CCA CTT CTG CTG CTT CTG GTC TGT GTA GCC ACC ATG TGC AGT GCC CAG GAC AGG   334
                         Met Val Trp Lys Trp Met Pro Leu Leu Leu Leu Leu Val Cys Val Ala Thr Met Cys Ser Ala Gln Asp Arg
                                                -10                                  1

ACT GAT CTC CTC AAT GTC TGT ATG GAT GCC AAG CAC CAC AAG ACA AAG CCA GGT CCT GAG GAC AAG CTG CAT GAC CAA TGC AGT CCC TGG   424
Thr Asp Leu Leu Asn Val Cys Met Asp Ala Lys His His Lys Thr Lys Pro Gly Pro Glu Asp Lys Leu His Asp Gln Cys Ser Pro Trp
                                       20                                   30

AAG AAG AAT GCC TGC TGC ACA GCC AGC ACC AGC CAG GAG CTG CAC AAG GAC ACC TCC CGC CTG TAC AAC TTT AAC TGG GAC CAC TGC GGC   514
Lys Lys Asn Ala Cys Cys Thr Ala Ser Thr Ser Gln Glu Leu His Lys Asp Thr Ser Arg Leu Tyr Asn Phe Asn Trp Asp His Cys Gly
     40                                50                                   60

AAG ATG GAG CCC GCC TGC AAG CGC CAC TTC ATC CAG GAC ACC TGT CTC TAT GAG TGC TCA CCC AAC CTG GGG CCC TGG ATC CAG CAG GTG   604
Lys Met Glu Pro Ala Cys Lys Arg His Phe Ile Gln Asp Thr Cys Leu Tyr Glu Cys Ser Pro Asn Leu Gly Pro Trp Ile Gln Gln Val
      70                                80                                   90
  ○
AAT CAG ACG TGG CGA AAA GAA CGC TTC CTG GAT GTG CCC TTA TGC AAA GAG GAC TGT CAG CGC TGG TGG GAG GAT TGT CAC ACC TCC CAC   694
Asn Gln Thr Trp Arg Lys Glu Arg Phe Leu Asp Val Pro Leu Cys Lys Glu Asp Cys Gln Arg Trp Trp Glu Asp Cys His Thr Ser His
     100                               110                                  120

ACG TGC AAG AGC AAC TGG CAC AGA GGA TGG GAC TGG ACC TCA GGA GTT AAC AAG TGC CCA GCT GGG GCT CTC TGC CGC ACC TTT GAG TCC   784
Thr Cys Lys Ser Asn Trp His Arg Gly Trp Asp Trp Thr Ser Gly Val Asn Lys Cys Pro Ala Gly Ala Leu Cys Arg Thr Phe Glu Ser
     130                               140                                  150
                                                                  ○
TAC TTC CCC ACT CCA GCT GCC CTT TGT GAA GGC CTC TGG AGT CAC TCA TAC AAG GTC AGC AAC TAC AGC CGA GGG AGC GGC CGC TGC ATC   874
Tyr Phe Pro Thr Pro Ala Ala Leu Cys Glu Gly Leu Trp Ser His Ser Tyr Lys Val Ser Asn Tyr Ser Arg Gly Ser Gly Arg Cys Ile
     160                               170                                  180

CAG ATG TGG TTT GAT TCA GCC CAG GGC AAC CCC AAC GAG GAA GTG GCG AGG TTC TAT GCT GCA GCC ATG CAT GTG AAT GCT GGT GAG ATG   964
Gln Met Trp Phe Asp Ser Ala Gln Gly Asn Pro Asn Glu Glu Val Ala Arg Phe Tyr Ala Ala Ala Met His Val Asn Ala Gly Glu Met
     190                               200                                  210

CTT CAT GGG ACT GGG GGT CTC CTG CTC AGT CTG GCC CTG ATG CTG CAA CTC TGG CTC CTT GGC TGAGTTCAGTCCTCCCAGACTACCTGCCCTCAGCTT  1063
Leu His Gly Thr Gly Gly Leu Leu Leu Ser Leu Ala Leu Met Leu Gln Leu Trp Leu Leu Gly
     220                               230

GGATAACCAGGCTGGGCTCAGCTCAGCTCCCACAAATGACAGCCCCTTAAGCATGCTTCTATTAGTCACCTAACCCTCTGTCACCCAGTCTGTTGCTGCTCCATGGTGGGGCCAAGAGTC  1183

ACTTCTAATAAACAGACTGTTTTCTAATAAAAAAAAAAAAAAAAAA                                                                      1230
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FIGURE 4: Complete nucleotide sequence of the cDNA for a human placental FBP. The deduced amino acid sequence is indicated in the three-letter code; this sequence includes a putative signal peptide (−16 to −1) that is cleaved. The numbers on the right indicate the positions of nucleotides. The deduced amino acid residues are numbered above. (○) Potential sites for N-linked glycosylation.

membranes, led us to attempt to clone and sequence the cDNA for a previously unidentified FBP in placenta.

Affinity-purified polyclonal antibodies were used to screen a placental cDNA expression library constructed in the vector λgt11. Approximately 200 000 IPTG-induced recombinant plaques were screened. Four positive plaques were obtained, and restriction fragments from the largest cDNA insert (approximately 1.2 kb) were cloned into the vector M13mp18 or M13mp19 and sequenced by the dideoxy sequencing method. The complete nucleotide sequence of this cDNA is shown in Figure 4. The sequence has an ATG codon at nucleotide position 263, an open reading frame until nucleotide 1028, a 3′ noncoding region of 200 bp, and a poly(A) tail. A polyadenylation signal (AATAAA) is located 16 bp upstream from the poly(A) site.

The deduced amino acid sequence is indicated in Figure 4. A putative 16-residue signal peptide (−16 to −1) is proposed on the basis of general features of signal peptides, i.e., a basic residue close to the amino terminus, followed by a largely hydrophobic sequence (Figure 4). Further, the deduced molecular weight of the processed protein from the predicted sequence ($M_r \sim 27401$) corresponds closely to the molecular weight observed above for the deglycosylated protein ($M_r \sim 28000$). The proposed signal cleavage site is also very close to the positions of corresponding amino-terminal residues in the homologous amino-terminal peptides of bovine and human milk FBPs and of the soluble and membrane-associated KB cell FBPs. Attempts to directly determine the amino-terminal residue in the purified placental protein were unsuccessful, perhaps due to a chemical modification. The protein contains 17 cysteine residues which could potentially form 8 intramolecular disulfide bonds. Among a total of 12 asparagine

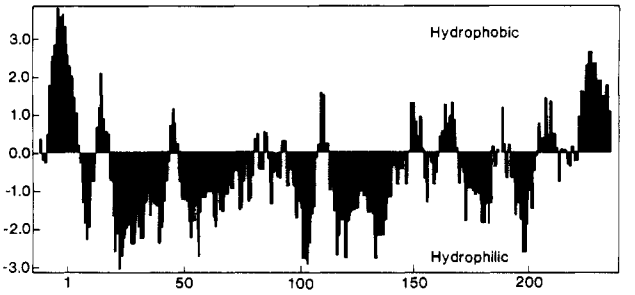

FIGURE 5: Hydropathy profile of a placental FBP. The amino acid sequence in Figure 4 was analyzed by the method of Kyte and Doolittle using GENEPRO, version 4.2 (Riverside Scientific Enterprises).

residues there are two potential N-linked glycosylation sites at Asn-99 and Asn-179. Hydropathy analysis of the protein sequence (Figure 5) indicates a strongly hydrophobic carboxyl-terminal sequence (19 residues) of sufficient length to form an α-helical membrane spanning domain. This sequence is tentatively assigned the role of anchoring the protein in the membrane.

Comparison with Other Proteins and with Peptide Sequences. A partial cDNA sequence of the human KB cell FBP was recently obtained (Sadasivan & Rothenberg, 1989) that does not contain either the 5′ untranslated sequence or a region coding for a signal sequence. The placental protein in Figure 4 shows an amino acid sequence homology of about 68% with this sequence (Figure 6). The 3′ untranslated regions of the two cDNAs bear no homology, and the codon usage for identical amino acids is frequently different. Thus, it is apparent that these two proteins are encoded for by different genes. As mentioned earlier, the KB cell protein sequence is

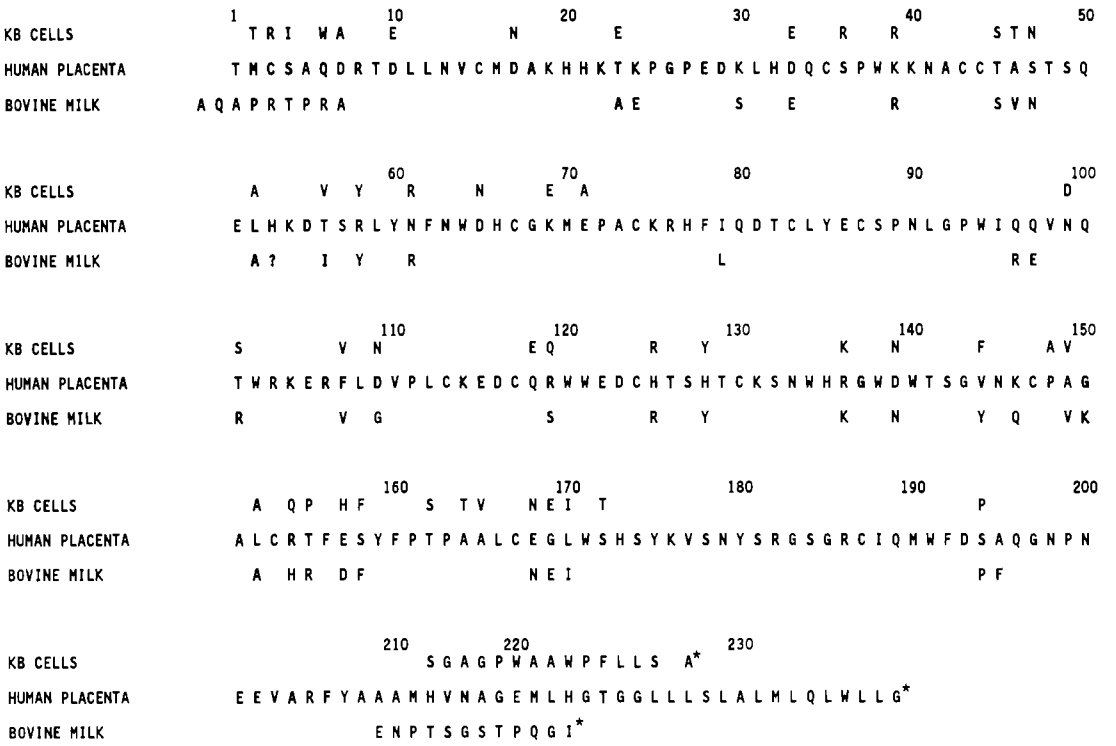

FIGURE 6: Aligned amino acid sequences of folate binding proteins. The human placental FBP (Figure 4) is aligned with the KB cell FBP (Sadasivan & Rothenberg, 1989) and bovine milk FBP (Svendsen et al., 1984). Only amino acid residues in the KB cell and bovine milk FBP sequences that are nonidentical with the placental FBP sequence are indicated. (*) Carboxyl-terminal residue for each protein.

identical with the known peptide sequences of the soluble and membrane-associated human milk FBPs and is homologous to the bovine milk FBP. The bovine milk FBP has a 69% amino acid sequence homology to the placental FBP (Figure 6). In all of these cases the amino acid sequence at the potential glycosylation site corresponding to Asn-179 in the placental FBP is conserved. The KB cell protein also displays a stretch of hydrophobic residues near the carboxyl terminus, similar to that found for the placental FBP. However, there is little sequence homology between these two proteins in the carboxyl-terminal region, and the placental FBP extends by a further 12 residues at the carboxyl terminus compared to the KB cell FBP. The possible significance of this difference between the two proteins with regard to their transport characteristics remains to be elucidated.

Comparison of the peptide sequences obtained from the placental FBP preparation (Figure 2) indicates that two of the peptides (b and d) are contained within the protein sequence of the cloned gene (Figure 4) but not in the KB cell FBP. On the other hand, peptides c_1 and c_3 are found in the latter protein but not in the former. A fifth peptide (c_2) is contained in both the KB cell protein and the cloned placental protein sequence. Thus, two homologous membrane folate binding proteins have been identified in human placenta. Since the placenta is formed by an intricate association of maternal and fetal tissues and since a single type of FBP appears to be present in KB cells and milk, it is attractive to hypothesize that the protein from which peptides c_1, c_2, and c_3 are derived represents an adult or maternal form of FBP and that the second FBP in placenta may represent a fetal form. Consistent with this view is a very recent paper that describes a cDNA obtained from a human placental library that is identical with the KB cell FBP cDNA (Elwood et al., 1988). These relationships are presently being investigated by using Northern blotting and in situ hybridization techniques.

In Table I the amino acid compositions of placental FBP reported by Antony et al. (1981) and the amino acid compositions calculated from the deduced amino acid sequences of the KB cell FBP (Sadasivan & Rothenberg, 1989) and of a placental FBP (Figure 4) are compared. The purified placental FBP differs significantly in amino acid composition from both the KB cell FBP and the placental FBP cloned and sequenced herein. This is a clear indication that the purified preparations of placental FBP reported by others (Antony et al., 1981) also must contain significant amounts of two or more folate binding proteins.

Table I: Comparison of FBP Amino Acid Compositions

amino acid	placental[a] (mol %)	KB cell[b] (cDNA) (mol %)	placental[c] (cDNA) (mol %)
Lys	6.6	7.1	5.9
His	5.6	4.9	5.0
Arg	5.9	6.6	4.6
Asx	11.1	12.0	10.5
Thr	5.9	5.5	5.9
Ser	10.8	8.2	7.1
Glx	4.2	13.1	9.6
Pro	4.5	6.2	4.6
Gly	6.9	6.0	6.3
Ala	6.9	10.4	6.7
Cys	4.2	7.1	7.1
Val	11.1	4.9	2.9
Met	1.4	2.2	2.9
Ileu	2.1	2.7	1.3
Leu	5.9	6.6	9.2
Tyr	3.5	4.4	2.5
Phe	3.5	4.9	2.9
Trp	ND[d]	5.3	5.0

[a] Calculated from data reported in Antony et al. (1981). [b] Values obtained from Sadasivan and Rothenberg (1989). [c] From Figure 4. [d] Not determined.

In a search using available databanks for amino acid or nucleotide sequence homologies with other proteins and cDNAs we found a significant amount (27% in a 177 amino acid overlap) of amino acid sequence homology of the placental FBP with the chicken riboflavin binding protein (219 residues) (Norioka et al., 1985). The functional significance of this

relatedness is unclear, at present, but warrants further study. The search revealed no significant homologies with folate-dependent enzymes.

Relationship between Soluble and Membrane-Associated FBPs. Membrane-associated FBPs differ from soluble FBPs in that they have a much higher apparent molecular weight due to their ability to bind significant amounts of Triton X-100. This has been attributed to an additional 5–10K_d hydrophobic segment in the membrane FBP which is proteolytically cleaved off to generate the soluble FBP (Antony et al., 1989; Antony & Verma, 1989). The data presented herein, taken together with recent data from other laboratories, indicate that soluble FBPs are not proteolytic products of membrane-associated FBPs, contrary to the earlier suggestion. The following observations support this view. (i) The membrane-associated FBPs from placenta were purified in this study in the presence of several protease inhibitors including EDTA, which was suggested to inhibit a specific protease that acts on the membrane FBP (Antony et al., 1989). Upon deglycosylation this protein gave a molecular weight corresponding to that of soluble or "low molecular weight" FBPs isolated from human KB cells and from human and bovine milk. (ii) The amino-terminal sequences of the membrane-associated and soluble FBPs from KB cells (Luhrs et al., 1987) and those from human milk (Svendsen et al., 1982) are identical, so that the only possible site of proteolytic processing would be near the carboxyl terminus of the membrane protein. (iii) The molecular weight deduced herein from the cDNA sequence for a placental FBP and that for the KB cell FBP (Sadasivan & Rothenberg, 1989), considering the amino-terminal sequences determined by peptide sequencing and the stop codons in the cDNA, are similar to that of soluble FBPs. This rules out cleavage of a large (5–10K_d) carboxyl-terminal peptide of the membrane FBP. The data do suggest, on the other hand, that the membrane-bound and soluble FBPs must differ by some type of covalent modification(s). It was recently reported (Luhrs et al., 1987) that the membrane-bound and soluble FBPs from KB cells have the same amino acid compositions and amino-terminal sequences but differ in that the membrane protein contains covalently attached fatty acids. Such a modification could account for the detergent binding properties of membrane FBPs. As discussed earlier, differences in glycosylation may also account for the different apparent molecular weights of soluble and membrane-bound FBPs.

Finally, in contrast to adult tissues, fetal or embryonic tissues may express an additional or alternate type of folate transport protein to cope with a higher demand for folate coenzymes in rapidly proliferating cells. The cDNA cloned and sequenced herein may code for such a fetal protein. Further studies will reveal the folate binding and transport properties of the two placental FBPs, their relative levels or tissue specificities in their expression, and their possible developmental regulation.

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