Annu. Rev. Nutr. 1990. 10:319–35

FOLATE-BINDING PROTEINS

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KEY WORDS: 5-methyltetrahydrofolate, methotrexate, transport of folate compounds, human milk, endocytosis

CONTENTS

INTRODUCTION

Folate-binding proteins are crucial to the assimilation, distribution, and retention of the vitamin folic acid (16) and have been identified in various cells, extracellular fluids, and tissues from mammalian sources (12, 27, 64, 91, 92). These proteins often differ in amount, can have alternative functions, and can form distinct classes based on their biochemical properties. High-affinity folate-binding proteins (FBPs) constitute a major class: They are characterized by their preferential binding of folate relative to reduced folate com-

0199-9885/90/0715-0319$02.00

pounds and by dissociation constants for folate below 1 nM. The antifolate methotrexate is bound relatively poorly by this class of folate-binding proteins. The FBPs can be separated further into soluble forms (sFBPs) and membrane-bound forms (mFBPs) that are structurally related but differ in function. A second major class is composed of membrane-associated folate-binding proteins, which are structurally unrelated to the high-affinity binders and function in the transport of folate compounds across cell membranes. The affinity of the latter proteins for folate compounds is generally in the micromolar range. Cytoplasmic-binding proteins with a high affinity for specific reduced-folate compounds are a third major class of binders. These proteins consist of specific enzymes involved in one-carbon metabolism. The properties of the cytoplasmic-binding proteins have been reviewed recently (91, 92) and are not considered further in this review.

HIGH-AFFINITY FOLATE-BINDING PROTEINS

Distribution

Membrane-associated high-affinity folate-binding proteins (mFBPs) have been detected on the plasma membranes of various cells and tissues. A survey of cultured cells and patient-derived leukemic cells (55) showed that mFBP could be detected in varying amounts from each source tested. KB human nasopharyngeal epidermoid carcinoma cells grown at limiting concentrations of folate expressed the highest level of binder, and the amount (936 pmol/mg membrane protein) greatly exceeded the minimum folate concentrations (2–10 pmol/mg cellular protein) required by cells for growth (32, 46). HeLa and Molt 4F cells contained lesser amounts of binder, whereas a relatively low level was observed in isolated human leukemic cells (55). MA104 monkey kidney epithelial cells (42, 44) represent another abundant source of mFBP, and researchers have isolated sublines of cultured tumor cells that have acquired elevated levels of mFBP (33, 40, 41). Erythrocytes contain only a trace of binding protein (5), and CCRF-CEM T-cell derived leukemic cells have no detectable FBP (32). mFBPs have been found in tissues from placenta (4, 6, 17, 25, 60, 89, 90), proximal kidney tubules (13, 77–81), choroid plexus (86), small intestine (79), and liver (14). sFBPs are present in human (3, 7, 58, 76, 87, 94), bovine (7, 26, 87, 88), and goat (66, 72) milk, in the growth medium from human KB cells (18, 45) and cultured human lymphocytes (15), in normal human serum (57, 93), and in umbilical cord serum (43). Milk and umbilical cord serum are relatively abundant sources of sFBP, whereas relatively low levels of sFBP occur in normal serum (43, 92). sFBPs and mFBPs from various sources show immunological cross-reactivity and a precursor/product relationship (45) and hence have common structure fea-

tures. The description of FBPs as folate receptors should be discouraged, since none have been assigned a regulatory or signalling function.

Structural Properties

Soluble folate-binding proteins (sFBPs) have been isolated by affinity chromatography from bovine (73, 87), goat (66), and human (3, 94) milk. These proteins bind folate preferentially to reduced-folate compounds and have a much higher substrate affinity at pH 7.4 than at pH 5.5 (92). Binding measurements (26, 73, 92) have been complicated by the presence of multiple forms of sFBP in milk and by the tendency of sFBPs to aggregate and to exhibit varying substrate binding affinities. Analyses by gel electrophoresiss have been complicated by band broadening that is due to microheterogeneity in charge and carbohydrate content (92). Heterogeneity is also seen in N-terminal amino acid sequences (87). Estimates of molecular weight range from 25,000 to 40,000; carbohydrate accounts for 10 and 22% of the molecular weight of bovine and goat milk, respectively, and 35% of the human sFBP. An essentially complete sequence for the bovine milk binding protein (88) and a partial sequence (45%) for the human milk binder (87) have been obtained by standard biochemical procedures. The homology between the bovine and human milk binders is 83% when the partial sequences are compared (Table 1). Homology is also apparent between the sFBP from bovine milk and from a chicken riboflavin-binding protein (97). These proteins have six conserved tryptophan residues, they share eight of nine pairs of cysteines, which are involved in disulfide bonds, and they have an overall homology of greater than 30%. A soluble form of FBP has also been isolated

Table 1 Sequence homology for high-affinity folate-binding proteins[a]

	Percent homology				
Source	KB/ Placenta	Fetal placenta	Caco-2	Human milk	Bovine milk
KB/placenta	100	68	96	100	80
Placenta (fetal)	*[b]	100	75	79	69
Caco-2	*	*	100	97	73
Human milk	*	*	*	100	83
Bovine milk	*	*	*	*	100

[a] Published amino acid sequences from KB (17, 69), placenta (17, 60), fetal placenta (60), Caco-2 (49), human milk (87), and bovine milk (88) were aligned and compared for identity. Terminal amino acids with no counterpart were counted as different. Only known amino acids in the partially sequenced human milk-binding protein were considered in the comparison.

[b] Table shows diagonal symmetry. Values at lower left were omitted for clarity since the same values appear in upper right portion of the Table.

from human placenta and characterized (4), although subsequent studies (6) revealed that this soluble binding protein had been generated from membrane-associated binder by a cleavage reaction during the solubilization step.

mFBPs have been isolated by affinity chromatography from human KB cells (18, 52) and rat kidney (78). The purified proteins contain covalently bound carbohydrate and fatty acids (52) and exhibit molecular weights in the range of 40,000, which can be reduced to 28,000 following exposure to *N*-glycanase (60). Binding affinities and substrate specificities exhibited by mFBPs are comparable to those of sFBPs and are not altered by removing covalently bound carbohydrate (92). Anti-FBP antibodies and oligonucleotides derived from FBP peptide sequences have led to the screening of KB (17, 60, 69), placental (17, 60), and Caco-2 (49) cDNA libraries and to the isolation of full-length or near full-length cDNA clones for the binding protein. Analysis of the isolated cDNAs established the amino acid sequences of binding proteins from each source and showed the presence of a leader sequence that had lengths of 16 (60), 24 (49), or 25 (17) amino acids. Consensus sequences identified two (60) or three (17, 49) asparagine residues that could be glycosylated in the native protein. KB and Caco-2 cells expressed a single species of mFBP, whereas two distinct mFBPs were identified in placenta (60). One of the placental binding proteins had a sequence identical with mFBP from human KB cells, whereas the other was 68% homologous to the KB-binding protein (Table 1). The latter protein was proposed to have a fetal origin (60). mFBP from Caco-2 cells was 96 and 75% homologous with the corresponding proteins from KB cells and placenta (fetal), respectively (Table 1). The cDNA sequences predict molecular weights of 26,252, 27,401, and 28,256 for the protein portions of the binding proteins from KB, placenta (fetal form), and Caco-2 cells, respectively. Computer analysis of secondary structure revealed that each of the sequenced binding proteins (excluding the leader sequence) contain only a single stretch of hydrophobic amino acids that could span or integrate into the membrane. This sequence is located at the extreme C-terminal portion of the protein, but it lacks the hydrophilic tail that characterizes other proteins with known transmembrane sequences (49). The hydrophobic C-terminal sequences of the protein from Caco-2 and KB cells also contain proline, which disrupts transmembrane α-helices.

Researchers have proposed that the hydrophobic C-terminal domain of mFBPs functions as a recognition site for anchoring the binding protein via fatty acid (glycosylphosphatidylinositol) linkages (49). Treatment of the membrane-associated form of the binding protein from MA104 (49) and KB (52a) cells with phosphatidylinositol-phospholipase C releases a soluble form of the binding protein. Data from consensus sequences suggest that the site of fatty acid linkage resides in the hydrophobic C-terminal portion of the protein

(49). In other cells, studies show that proteolysis can also generate a hydrophilic form of the binding protein (5, 6). Because sFBP from bovine milk (88) lacks the short hydrophobic sequence observed at the C-terminus of mFBPs, a single proteolytic cleavage that removes a C-terminal hydrophobic peptide containing the attached fatty acids appears to be the most direct mechanism for converting the membrane-bound binding protein to its hydrophilic form. Elwood (17) suggested that a sequence as long as 31 amino acids might be removed during conversion to the soluble form, but cleavage of a large portion of polypeptide from the membrane-bound protein is unlikely because the polypeptide components of the two protein forms are very similar in size (52). Prior reports of large differences in apparent molecular weight of the two protein forms can be explained by different levels of glycosylation (60).

Milk Folate-Binding Proteins

Lactating cells of the mammary epithelium produce substantial quantities of mFBP, and the processing of mFBP to soluble forms allows bound folate compounds to accumulate in milk. Bovine and human milk contains sFBP, aggregates of sFBP, and a hydrophobic form of FBP that may represent incompletely processed binder (26, 92). The majority of the FBPs contain bound substrate, although some apo-binder is usually present. The principal folate compound attached to the human milk binder is 5-methyltetrahydrofolate, and bound folates consist of approximately equal mixtures of monoglutamate and polyglutamate forms (10, 58). Since polyglutamates of 5-methyltetrahydrofolate are not released by cells and are not formed extracellularly, the binding protein appears to acquire its bound folate substrates intracellularly prior to secretion (76). The presence of a leader sequence (17, 49, 60) suggests further that the protein is transferred out of these cells by established secretory processes.

Soluble forms of FBP in human milk are likely to serve multiple functions. These high-affinity binders afford a convenient mechanism for concentrating folate compounds in milk, and the bound reduced-folate substrates are protected from oxidation during ingestion by infants. The mechanism of sequestering folate in human milk is very efficient, since lactation alone causes a decrease in maternal folate status (58, 84) and can cause maternal folate deficiency (56). Studies in 6-day-old goat kids (72) showed that the FBP of goat milk is resistant to proteolysis by pancreatic enzymes and can survive along the length of the small intestine. Protease inhibitors inherent to milk may also assist the passage of intact binders into the small intestine (51). Bound folate substrates are probably released transiently in the acidic environment of the stomach and then are reabsorbed after passage into the upper small intestine.

The uptake mechanism for bound folate in the small intestine of neonates is different from that of free folate compounds. A study of suckling rats (53) found that the absorption of folate bound to sFBP from rat milk occurred more slowly and preferentially in the ileum, whereas free folate compounds were absorbed more readily and primarily in the jejunum. Inhibitors of free folate absorption were also ineffective in inhibiting the uptake of bound folates (53). These results led to the suggestion that in neonates the absorption of bound folate occurs via a process that resembles the endocytotic absorption of macromolecules. Suckling mammals are known to absorb intact proteins by a process that decreases markedly at the time of weaning and is more active in the ileum than in the jejunum (1, 95). A possible mechanism of uptake is that milk sFBP is absorbed directly, and intact, by the enterocytes of the ileum and that subsequent proteolytic digestion inside the enterocyte releases the bound folate for export into the portal circulation. After weaning, endocytosis of folate-binding proteins and other macromolecules ceases, and folate absorption becomes the responsibility of intestinal membrane-associated folate transport systems (9, 64).

The presence of unsaturated folate binders in the small intestine of milk-fed neonates has the additional effect of reducing the bioavailability of folate compounds to gut bacteria (21). The prevention of folate uptake by intestinal bacteria could increase the amount of folate available to the neonate and it could alter the nature of the gut microflora and possibly reduce the occurrence of gastroenteritis. Pasteurization reduces the amount of folate-binding protein in cow milk by greater than 90% and total folate by 50% (7), but neonatal folate deficiency should not arise with the use of processed cow milk because the concentration of unbound folate compounds in the latter remains high relative to total folate in unprocessed human milk (7, 58). Pasteurized or sterilized cow milk does not affort infants the potential benefits of protein-bound folates, but its use should not lead to folate deficiency.

Serum Folate-Binding Proteins

The function of sFBPs in serum is less clear. These proteins are derived from cells of unknown origin and are present in normal serum at levels that correspond to a small fraction of the total serum folate (92, 93). Increased levels of binding protein have been observed with folate deficiency (93), in patients with leukemia (68), and during pregnancy (11), but the physiological importance of these elevated binding activities has not been determined. Hepatic cells contain receptors that appear to mediate the clearance of sFBP from serum (65), although receptors that could function in the uptake of folate compounds by other cells have not been reported. It is possible that the serum folate binder has no significant physiological function and arises only by random or accidental cleavage from the surface of cells (92). Alternatively,

serum sFBP could act as a minor storage protein to conserve folates (20, 93), or it could serve as a means for selectively clearing the serum of folic acid or other oxidized folate compounds (65).

Transport via Membrane-Associated Folate-Binding Proteins

Membrane-associated high-affinity folate-binding proteins in cells other than the mammary epithelium are not readily processed to soluble forms and accumulate primarily on the cell surface. The binding protein of cells replete with folate is mostly saturated with folate compounds, whereas folate-deficient cells contain primarily apoprotein lacking bound substrate (42, 55). The amount of binder can be increased substantially by growing cells in folate-deficient medium (42).

mFBPs can mediate the transport of folate compounds into cells. The assignment of a transport function is supported by the sensitivity of folate compound uptake to anti-FBP antibodies, by similarities in the kinetics of substrate binding and transport (2, 42, 44, 46), and by the isolation of sublines that acquire in parallel the ability to transport folate compounds at low concentrations and elevated levels of high-affinity binding protein (33, 40, 41). Slow transport rates are also characteristic of mFBPs. The FBP-mediated transport rate for folate in L1210 cells is less than one molecule per binding site per hour (33), whereas a slightly higher rate of one turnover per binding site per hour has been observed for 5-methyltetrahydrofolate in MA104 cells (42). Methotrexate is also transported by the high-affinity binder of KB cells, and its uptake leads to growth inhibition (46). High-affinity binders, however, probably do not represent a pharmacologically important uptake mechanism for methotrexate for the following reasons: FBPs exhibit a relatively poor binding affinity for methotrexate (33, 42); physiological levels of reduced-folate compounds should compete strongly with methotrexate for binding and transport by FBPs (33, 42); and growth inhibition by methotrexate has been shown only in cells that have very high levels of FBP (46).

The mechanism of transport via mFBPs appears to involve endocytosis. Substrate internalization is sensitive to weak acids and other agents that disrupt endosomal function (42, 44); the low pH within the endosome, which reduces the substrate-binding affinity of FBPs (33, 55, 92), could afford a mechanism for releasing bound folate compounds. Binding protein appears capable of performing multiple transport cycles (44, 80); it may proceed to endosomes, release bound substrate, and then return to the cell surface. The lack of multiple transmembrane segments on the binding protein (17, 49, 60, 69) also argues against a classical transport mechanism in which binding sites on a carrier protein reorient between the outer and inner plasma membrane surfaces. Receptors for the FBP/substrate complex have not been identified, which suggests that substrate internalization may not be facilitated by the

receptor-mediated endocytosis pathway but, instead, may rely upon random endocytosis of the plasma membrane. Variation in transport rate between cells of diverse origin might then be explained by differing inherent rates of random endocytosis. A relatively slow mechanism like endocytosis could provide cells with sufficient folate for growth, since the folate requirement of some cells containing mFBP could be met by the intracellular delivery of only a single bound substrate per binding site per doubling time (2, 55).

mFBP on the brush border membrane of kidney proximal tubules serves a crucial physiological role in maintaining the folate status of mammals. Renal clearance studies in rats (77, 80) have shown that the reabsorption of folate compounds is a saturable process and that the efficiency of reabsorption of folate compounds is proportional to their affinity for mFBP. Regeneration of unsaturated binders after the uptake of folic acid is much more rapid in kidney tubules ($t½$ = 0.5 min) than in other cells, but the transfer of internalized folate into the renal capillaries remains a relatively slow process ($t½$ = 47 min) (80). Studies with membranes (13) or vesicles (81) derived from kidney tubules of rats confirmed the presence of a mFBP in the brush border and suggested that these binders are involved in transport. The rapid rate of association of folate compounds with the mFBP of kidney tubules, and its presence in high amounts, combine to provide an efficient mechanism for preventing the loss of folate compounds in the urine.

Placenta also contains high amounts of mFBP. A soluble form of the protein has been purified (4), and subsequent cDNA cloning established the amino acid sequences of two distinct placental mFBPs (17, 60). The function of the placental mFBPs in vivo, however, remains less clear. Studies with perfused guinea pig placenta (89, 90) and with human placenta obtained at delivery (25) have shown that folate and methotrexate can be taken up by the placenta and that the process exhibits the specificity of high-affinity folate-binding proteins, although translocation of free folate across the placental membrane via these binding proteins could not be demonstrated during short-term incubations. The properties of mFBPs from other cells suggest that the placental mFBP probably functions in the transfer of folate compounds across the maternal blood barrier to the fetal circulation. The possibility of a fetal form of mFBP (60) suggests further that the transfer of folate compounds across the placenta may involve a shuttling mechanism between two mFBPs.

OTHER MEMBRANE-ASSOCIATED FOLATE-BINDING PROTEINS

Distribution and Function in Transport

Mammalian cells require an external source of folate compounds for growth and hence have developed transport systems for accumulating available forms

of this vitamin (9, 27, 64). Binding proteins with accessibility to the external membrane surface are required components of these transport systems. The high-affinity folate-binding proteins afford one route for the uptake of folate compounds, but other transport systems are also present in cells. L1210 and other leukemic cells of hematopoietic origin contain a transport system whose primary component is a binding protein with a specificity substantially different from FBPs. Preferred substrates include reduced folate compounds and methotrexate, but not folate, and affinities for substrates are in the micromolar range. L1210 cells also contain a second, less-active transport system for folate compounds which can be distinguished from the major route and from FBP-mediated uptake by differences in substrate specificity and pH dependence. Additional transport systems for folate compounds with unique characteristics can be found in intestinal enterocytes and in hepatocytes from liver.

Transport in Leukemic Cells

L1210 (27, 35, 82) mouse and CCRF-CEM (31, 32) human leukemia cells represent cell lines of hematopoietic origin that have been examined extensively for the ability to transport folate compounds. These cell lines each contain a transport system with similar characteristics. Reduced-folate compounds and methotrexate are transported with K_t values (concentrations for half-maximal rates) that range from 1 to 5 μM, whereas the transport system is much less able to accommodate folate. Maximal rates of transport for various folate substrates are approximately the same in each cell line (27), although the V_{max} in L1210 cells is about fourfold higher than in CCRF-CEM cells (32). This transport system is broadly inhibited by a variety of large organic anions including thiamine pyrophosphate, bromosulfophthalein, and phthalate (27, 31, 34, 38). Various criteria (27, 34, 38) indicate that transport proceeds via an anion exchange mechanism and that extracellular folate compounds probably exchange with various intracellular anions including phosphate and AMP. The binding protein has been identified by direct binding measurements (28, 31) and by affinity labeling with an *N*-hydroxysuccinimide ester of methotrexate (36) and by an azidosalicyl derivative of methotrexate (59). The amount of binder in L1210 cells (1 pmol/mg protein) (28, 36) is about threefold higher than in CCRF-CEM cells (0.3 pmol/mg) (31). Workers have isolated cell lines that overproduce the reduced-folate transport protein of L1210 cells by as much as 40-fold (96), but neither the binding protein nor its cDNA has yet been isolated.

The reduced-folate transport system of L1210 cells can function as the sole uptake route for folate compounds during growth under physiologically relevant conditions. Even though serum folate concentrations normally range from 10 to 40 nM (92, 93) and transport via this system with the physiological form of folate (5-methyltetrahydrofolate) occurs half-maximally at a substrate

concentration (1000 nM) that is much higher than serum levels, the moderate amount of binding protein (1 pmol/mg protein) and a V_{max} of 16 cycles/min per binding site, combined with the modest folate requirement of cells (2–10 pmol/mg protein) (32, 46), indicate that a 10-fold excess of 5-methyltetrahydrofolate could be accumulated via this system alone during the course of one cell doubling (12 hr). Similar calculations indicate that the reduced-folate transport system of CCRF-CEM could provide these cells (which have a doubling time of 18 hr) with a fourfold excess of folate compounds for growth at a physiological concentration (20 nM) of 5-methyltetrahydrofolate (32). The reduced-folate transport system of L1210 is also the major route for the uptake of methotrexate and can account for half-maximal growth inhibition of L1210 cells at methotrexate concentrations in the 10-nM range. Transport measurements have shown that the uptake of methotrexate at both nanomolar (33) and micromolar (27, 35) substrate concentrations occurs via the reduced-folate carrier system, and cell lines that have become resistant to methotrexate via a reduction in methotrexate transport lose the activity of the reduced-folate transport system (54, 75, 83). L1210 cells also retain the ability to grow maximally at very low concentrations (4 to 10 nM) of 5-methyltetrahydrofolate or 5-formyltetrahydrofolate, even after continuous and long-term growth in standard medium containing high levels (2.2 μM) of the less effective transport substrate, folate (28a, 33).

L1210 cells contain a second transport system for folate compounds that accommodates folate, 5-formyltetrahydrofolate, and methotrexate with about equal effectiveness (K_t range = 3–10 μM) and shows a large and distinct enhancement with decreasing pH (28a). Transport of methotrexate via this second system (at pH 7.4) occurs at about 2% the rate via the reduced-folate carrier, whereas it contributes a much greater fraction (10%) of the total uptake of folate. Anion inhibitors of the reduced-folate transport system (thiamine pyrophosphate and bromosulfophthalein) and the *N*-hydroxysuccinimide ester of methotrexate do not inhibit this second uptake route. L1210/R81 cells, which are resistant to methotrexate because of a transport defect (54), lack the reduced-folate transport system and rely solely on this alternative system for the acquisition of folate compounds (28a). This second route could not be detected in CCRF-CEM cells. A third uptake system for folate compounds has been identified in L1210 sublines adapted to low levels of folate or 5-formyltetrahydrofolate (33, 40). Transport of folate compounds in these cells occurs via a high-affinity folate-binding protein (mFBP), the levels of which have increased by more than 100-fold relative to the parental cells. A similar mutant that produces very high amounts of a mFBP has also been isolated from CCRF-CEM cells (41).

Methotrexate and other folate compounds exit L1210 cells via at least three carrier-mediated transport systems (27, 29, 37). One of these routes has been

identified as efflux via the reduced-folate carrier system, whereas the other two routes are unidirectional outward and show a high dependence on metabolic energy. Direct binding measurements have not been performed on the components of the unidirectional systems, although inhibitor sensitivity studies have revealed that these efflux systems for folate compounds can also mediate the unidirectional efflux of cholate (30), and perhaps also of urate (30), cyclic AMP (29, 63), and oxidized glutathione (48). The proposed function for these efflux systems has been to extrude large organic anions that might otherwise become toxic if allowed to accumulate in cells (29, 30). A similar extruding mechanism for large and potentially toxic neutral and cationic compounds and a corresponding binding protein (p-glycoprotein) have been reported in intestinal mucosal cells, renal proximal tubules, and various other normal tissues (24, 47). In tumor cells, overproduction of the latter efflux protein leads to multidrug resistance (8, 24).

Transport in the Small Intestine

The small intestine represents the site of entry of essential folate compounds in mammals (9, 64). Dietary folates, however, are usually polyglutamylated and must undergo conjugase digestion to monoglutamate forms prior to absorption by the enterocytes of adults (62, 64). Transport of folate compounds in the small intestine has been measured using various mammalian sources and experimental techniques (9, 64, 67, 70, 71, 74, 81). The results vary but generally describe a principal carrier-mediated transport system that accommodates monoglutamate forms of various folate compounds about equally, exhibits half-maximal transport rates at substrate concentrations between 0.5 and 5 μM, and has a pH optimum (5.5–6.0) that approximates the physiological pH of the lumen of the small intestine. The mechanism of transport appears to involve H^+/folate cotransport, and H^+-gradients probably act as the inward driving force (70, 74). A second, nonsaturable uptake component is also present and shows little dependence on pH (64, 67). The pH-dependent transport system is more active in the ileum than in the jejunum, whereas both of these segments of the small intestine show equal activity for the nonsaturable component (64). The nonsaturable component may correspond to a conductive pathway that takes up the negatively charged folate compound with a cation (98), be an organic-anion transport system with a high V_{max} and a low substrate specificity, or reflect only passive diffusion. Transport of folate compounds out of the enterocyte via the basolateral membrane occurs by a carrier-mediated process that resembles the high-affinity uptake system on the brush-border membrane (71). Studies with porcine intestinal brush-border membranes (62) identified a binding component that appears to mediate the saturable, pH-dependent transport of folate compounds. The binder was present at 0.24 pmol/mg protein, exhibited an

affinity for folate (K_d = 0.08 μM) that was similar to the K_t for folate transport in the small intestine of rabbit (K_t = 0.19 μM) (74) and rat (K_t = 0.42 μM) (81), and was inhibited by 5-methyltetrahydrofolate (K_i = 0.8 μM). Covalent labeling with activated folate compounds has also been achieved (61). mFBP has been identified in membranes derived from small intestine (79), but its low level and the presence of other more-active uptake processes suggest that FBPs do not serve an important role in the intestinal uptake of folates. The broad specificity of intestinal folate transport systems is consistent with the accumulation of various monoglutamate forms of folate compounds that are metabolized, primarily within the enterocyte, to 5-methyltetrahydrofolate and then are passed on to the liver for tissue distribution. Congenital defects in the absorption of folate by humans results in folate deficiency, which can be circumvented by large doses of folate (19, 50), but the defective step in the overall absorption process has not been identified.

Transport in the Liver

The liver represents a major site for the storage and processing of folate compounds for distribution to other mammalian tissues. Folate compounds received by the liver from the portal vein or from the general circulation are taken up, metabolized to coenzyme forms (primarily 5-methyltetrahydrofolate), and then released back into the circulation or diverted into the bile. The amount of 5-methyltetrahydrofolate shunted to the bile is substantial, as demonstrated by perfusion studies with rat liver (85). Hepatocytes isolated from rat liver provide evidence for two transport components for 5-methyltetrahydrofolate (39). Studies have identified a high-affinity system that functioned half-maximally at 0.89 μM 5-methyltetrahydrofolate, was dependent on Na^+, and was inhibited by methotrexate with a K_i of 5.5 μM. The second component was also dependent on Na^+ but it did not exhibit saturation kinetics and was not inhibited by high concentrations of folate compounds. The saturable and nonsaturable components contribute about equally to total uptake at 1 μM 5-methyltetrahydrofolate. Methotrexate transport by hepatocytes (23) also occurred via saturable and nonsaturable components, but folate compounds did not inhibit the high-affinity route for methotrexate, which suggests that the uptake systems for methotrexate and 5-methyltetrahydrofolate may be distinct. Bile acids, bromosulfophthalein, and rose bengal inhibited both the saturable and nonsaturable uptake routes for methotrexate (22). Possibly both of these routes are carrier-mediated, and methotrexate may be transported via systems whose primary substrates are bile acids or organic anions. mFBP has been identified in hepatocyte membranes (14), but the low amount argues against a major role for mFBP in the uptake of folate compounds by the liver.

SUMMARY

Folate-binding proteins of three major classes have been observed in various bodily fluids and in the plasma membrane and cytoplasm of normal and neoplastic cells. A major class, the high-affinity folate-binding proteins, show a preferential and tight binding of folic acid relative to reduced folates and methotrexate and consist of water-soluble and membrane-associated forms. Soluble forms of the high-affinity binders are present in serum and milk and in the growth medium of certain cultured cell lines, whereas membrane-associated forms are observed on the surface of various cells and tissues. The binders in serum have no clearly defined function, whereas the milk binders serve to accumulate and stabilize reduced-folate compounds in milk and they may also facilitate the absorption of folates by the intestinal mucosa of neonates. Membrane-bound forms of high-affinity folate-binding proteins mediate the transport of folate compounds across plasma membranes and appear to utilize endocytosis as the transport mechanism. Membrane-associated high-affinity binding proteins contain covalently bound phospholipids and hydophobic C-terminal amino acid sequences that are absent in the soluble forms. The remaining protein portions of these binders show considerable sequence homology. The second class is composed of folate-binding proteins that reside solely in the plasma membrane and are structurally and mechanistically distinct from the high-affinity binders. These proteins function in transport, exhibit varied substrate specificities that accommodate reduced-folate compounds with equal or higher affinity than folate, and do not utilize endocytosis as the mechanism for substrate internalization. The third class of folate-binding proteins consists of enzymes that reside in the cytoplasm of cells.

ACKNOWLEDGMENTS

This work was supported by NIH research grant CA23970.

Literature Cited

1. Abrahamson, D. R., Powers, A., Rodewald, R. 1979. Intestinal absorption of immune complexes by neonatal rats: a route of antigen transfer from mother to young. *Science* 206:567–69
2. Antony, A. C., Kane, M. A., Portillo, R. M., Elwood, P. C., Kolhouse, J. F. 1985. Studies of the role of a particulate folate binding protein in the uptake of 5-methyltetrahydrofolate by cultured human KB cells. *J. Biol. Chem.* 260:14911–17
3. Antony, A. C., Utley, C., Marcell, P. D., Kolhouse, J. F. 1982. Isolation, characterization, and comparison of the solubilized particulate and soluble folate binding proteins from human milk. *J. Biol. Chem.* 257:10081–89
4. Antony, A. C., Utley, C., Van Horne, K. C., Kolhouse, J. F. 1981. Isolation and characterization of a folate receptor from human placenta. *J. Biol. Chem.* 256:9684–92
5. Antony, A. C., Verma, R. S. 1989.

Hydrophobic erythrocyte folate binding proteins are converted to hydrophilic forms by trypsin in vitro. *Biochim. Biophys. Acta* 979:62–68

6. Antony, A. C., Verma, R. S., Unune, A. R., LaRosa, J. A. 1989. Identification of a Mg^{2+}-dependent protease in human placenta which cleaves hydrophobic folate binding proteins to hydrophilic forms. *J. Biol. Chem.* 264:1911–14
7. Areekul, S., Vongyuthitham, M., Yamarat, P., Sunthornthum, S. 1978. Folic acid and folic acid binding proteins in milk. *J. Med. Assoc. Thailand* 61:81–86
8. Beck, W. T. 1987. The cell biology of multiple drug resistance. *Biochem. Pharmacol.* 36:2879–87

8a. Blakley, R. L., Whitehead, V. M., eds. 1986. *Folates and Pterins,* Vol. 3: *Nutritional, Pharmacological, and Physiological Aspects*. New York: Wiley

9. Bowman, B. B., McCormick, D. B., Rosenberg, I. H. 1989. Epithelial transport of water-soluble vitamins. *Annu. Rev. Nutr.* 9:187–99
10. Brown, C. M., Smith, A. AM., Picciano, M. F. 1986. Forms of human milk folacin and variation patterns. *J. Pediatr. Gastroenterol. Nutr.* 5:278–82
11. Colman, N., Herbert, V. 1976. Total folate binding capacity of normal human plasma and variations in uremia, cirrhosis, and pregnancy. *Blood* 48:911–12
12. Colman, N., Herbert, V. 1980. Folate binding proteins. *Annu. Rev. Med.* 31:433–39
13. Corrocher, R., Abramson, R. G., King, V. F., Schreiber, C., Dikman, S., Waxman, S. 1985. Differential binding of folates by rat cortex brush border and basolateral membrane preparations. *Proc. Soc. Exp. Biol. Med.* 178:73–84
14. da Costa, M., Rothenberg, S. P. 1988. Characterization of the folate binding protein associated with the plasma membrane of rat liver. *Biochim. Biophys. Acta* 939:533–41
15. da Costa, M., Sharon, M. 1980. The synthesis of folate binding proteins in lymphocytes during transformation. *Br. J. Haematol.* 46:575–79
16. Davis, R. E., Nichol, D. J. 1988. Folic acid. *Int. J. Biochem.* 20:133–39
17. Elwood, P. C. 1989. Molecular cloning and characterization of the human folate binding protein cDNA from placenta and malignant tissue culture (KB) cells. *J. Biol. Chem.* 264:14893–14901
18. Elwood, P. C., Kane, M. A., Portillo, R. M., Kolhouse, J. F. 1986. The isolation, characterization, and comparison of the membrane-associated and soluble folate binding proteins from human KB cells. *J. Biol. Chem.* 261:15416–23
19. Erbe, R. W. 1975. Inborn errors of folate metabolism. *New Engl. J. Med.* 293:807–12
20. Fernandez-Costa, F., Metz, J. 1979. Role of serum folate binders in the delivery of folates to tissues and to the fetus. *Br. J. Haematol.* 41:335–42
21. Ford, J. E. 1974. Some observations on the possible nutritional significance of vitamin B_{12} and folate binding proteins in milk. *Br. J. Nutr.* 31:243–57
22. Gewirtz, D. A., Randolph, J. K., Goldman, I. D. 1980. Potent bile acid and organic anion inhibition of methotrexate uptake and accumulation in the freshly isolated rat hepatocyte. *Cancer Res.* 40:1852–57
23. Gewirtz, D. A., White, J. C., Randolph, J. K., Goldman, I. D. 1980. Transport, binding, and polyglutamation of methotrexate in freshly isolated rat hepatocytes. *Cancer Res.* 40:573–78
24. Gottesman, M. M., Pastan, I. 1988. The multidrug transporter, a double-edged sword. *J. Biol. Chem.* 263:12163–66
25. Green, T., Ford, H. C. 1984. Human placental microvilli contain high-affinity binding sites for folate. *Biochem. J.* 218:75–80
26. Hansen, S. I., Holm, J., Lyngbye, J., Pedersen, T. G., Svendsen, I. 1983. Dependence of aggregation and ligand affinity on the concentration of the folate binding protein from cows milk. *Arch. Biochem. Biophys.* 226:636–42
27. Henderson, G. B. 1986. Transport of folate compounds into cells. See Ref. 8a, pp. 207–50
28. Henderson, G. B., Grzelakowska-Sztabert, B., Zevely, E. M., Huennekens, F. M. 1980. Binding properties of the 5-methyltetrahydrofolate/methotrexate transport system in L1210 cells. *Arch. Biochem. Biophys.* 202:144–49

28a. Henderson, G. B., Strauss, B. P. 1990. Characteristics of a novel transport system for folate compounds in wild-type and methotrexate-resistant L1210 cells. *Cancer Res.* In press

29. Henderson, G. B., Tsuji, J. M. 1987. Methotrexate efflux in L1210 cells: Kinetic and specificity properties of the efflux system sensitive to bromosulfophthalein and its possible identity with a system which mediates the efflux of 3',5'-cyclic AMP. *J. Biol. Chem.* 262:13571–78
30. Henderson, G. B., Tsuji, J. M. 1990.

Identification of cholate as a shared substrate for the unidirectional efflux systems for methotrexate in L1210 mouse cells. *Biochim. Biophys. Acta.* 1051:60–70

31. Henderson, G. B., Tsuji, J. M., Kumar, H. P. 1986. Characterization of the individual transport routes that mediate the influx and efflux of methotrexate in CCRF-CEM human lymphoblastic cells. *Cancer Res.* 46:1633–38
32. Henderson, G. B., Tsuji, J. M., Kumar, H. P. 1987. Transport of folate compounds by leukemic cells: Evidence for a single influx carrier for methotrexate, 5-methyltetrahydrofolate, and folate in CCRF-CEM human lymphoblasts. *Biochem. Pharmacol.* 36:3007–14
33. Henderson, G. B., Tsuji, J. M., Kumar, H. P. 1988. Mediated uptake of folate by a high-affinity binding protein in sublines of L1210 cells adapted to nanomolar concentrations of folate. *J. Membr. Biol.* 101:247–58
34. Henderson, G. B., Zevely, E. M. 1983. Structural requirements for anions substrates of the methotrexate transport system in L1210 cells. *Arch. Biochem. Biophys.* 221:438–46
35. Henderson, G. B., Zevely, E. M. 1984. Transport routes utilized by L1210 cells for the influx and efflux of methotrexate. *J. Biol. Chem.* 259:1526–31
36. Henderson, G. B., Zevely, E. M. 1984. Affinity labeling of the 5-methyltetrahydrofolate/methotrexate transport protein of L1210 cells by treatment with an N-hydroxysuccinimide ester of [^{3}H]methotrexate. *J. Biol. Chem.* 259:4558–62
37. Henderson, G. B., Zevely, E. M. 1985. Inhibitory effects of probenecid on the individual transport routes which mediate the influx and efflux of methotrexate in L1210 cells. *Biochem. Pharmacol.* 34:1725–29
38. Henderson, G. B., Zevely, E. M. 1985. Characterization of the multiple transport routes for methotrexate in L1210 cells using phthalate as a model anion substrate. *J. Membr. Biol.* 85:263–68
39. Horn, D. W., Briggs, W. T., Wagner, C. 1978. Transport of 5-methyltetrahydrofolate and folic acid in freshly isolated hepatocytes. *J. Biol. Chem.* 253:3529–35
40. Jansen, G., Kathmann, I., Rademaker, B. C., Braakhuis, B. J. M., Westerhof, G. R., et al. 1989. Expression of a folate binding protein in L1210 cells grown in low folate medium. *Cancer Res.* 49:1959–63
41. Jansen, G., Westerhof, G. R., Kathmann, I., Rademaker, B. C., Rijksen, G., Schornagel, J. H. 1989. Identification of a membrane-associated folate binding protein in human leukemic CCRF-CEM cells with transport-related methotrexate resistance. *Cancer Res.* 49:2455–59
42. Kamen, B. A., Capdevila, A. 1986. Receptor-mediated folate accumulation is regulated by the cellular folate content. *Proc. Natl. Acad. Sci. USA* 83:5983–87
43. Kamen, B. A., Caston, D. J. 1975. Purification of folate binding factor in normal umbilical cord serum. *Proc. Natl. Acad. Sci. USA* 72:4261–64
44. Kamen, B. A., Wang, M. T., Streckfuss, A. J., Peryea, X., Anderson, R. G. W. 1988. Delivery of folates to the cytoplasm of MA104 cells is mediated by a surface membrane receptor that recycles. *J. Biol. Chem.* 263:13602–9
45. Kane, M. A., Elwood, P. C., Portillo, R. M., Antony, A. C., Kolhouse, J. F. 1986. The interrelationship of the soluble and membrane associated folate binding proteins in human KB cells. *J. Biol. Chem.* 261:15625–31
46. Kane, M. A., Portillo, R. M., Elwood, P. C., Antony, A. C., Kolhouse, J. F. 1986. The influence of extracellular folate concentration on methotrexate uptake by human KB cells. *J. Biol. Chem.* 261:44–49

46a. Kisliuk, R. L., Brown, G. M., eds. 1979. *Chemistry and Biology of Pteridines*. New York: Elsevier

47. Klohs, W. D., Steinkampf, R. W. 1988. Possible link between the intrinsic drug resistance of colon tumors and a detoxification mechanism in intestinal cells. *Cancer Res.* 48:3025–30
48. Kondo, T., Dale, G. L., Beutler, E. 1980. Glutathione transport by inside-out vesicles from human erythrocytes. *Proc. Natl. Acad. Sci. USA* 77:6359–62
49. Lacey, S. W., Sanders, J. M., Rothberg, K. G., Anderson, R. G. W., Kamen, B. A. 1989. Complementary DNA for the folate binding protein correctly predicts anchoring to the membrane by glycosyl-phosphatidylinositol. *J. Clin. Invest.* 84:715–20
50. Lanzkowsky, P., Erlandson, M. E., Bezan, A. I. 1969. Isolated defect of folate acid absorption associated with mental retardation and cerebral calcification. *Blood* 34:452–65
51. Laskowski, M., Laskowski, M. 1951. Crystalline trypsin inhibitor from colostrum. *J. Biol. Chem.* 190:563–73
52. Luhrs, C. A., Pitiranggon, P., da Costa, M., Rothenberg, S. P., Slomiany, B. L., et al. 1987. Purified membrane and soluble folate binding proteins from cul-

tured KB cells have similar amino acid compositions and molecular weights but differ in fatty acid acylation. *Proc. Natl. Acad. Sci. USA* 84:6546–49

52a. Luhrs, C. A., Slomiany, B. L. 1989. A human membrane-associated folate binding protein is anchored by a glycosylphosphatidylinositol tail. *J. Biol. Chem.* 264:21446–49

53. Mason, J. B., Selhub, J. 1988. Folate binding protein and the absorption of folic acid in the small intestine of the suckling rat. *Am. J. Clin. Nutr.* 48:620–25

54. McCormick, J. I., Susten, S. S., Freisheim, J. H. 1981. Characterization of the methotrexate transport defect in a resistant L1210 lymphoma cell line. *Arch. Biochem. Biophys.* 212:311–18

55. McHugh, M., Cheng, Y-C. 1979. Demonstration of a high-affinity folate binder in human cell membranes and its characterization in cultured human KB cells. *J. Biol. Chem.* 254:11312–18

56. Metz, J. 1970. Folate deficiency conditioned by lactation. *Am. J. Clin. Nutr.* 23:843–47

57. Metz, J., Zalusky, R., Herbert, V. 1968. Folic acid binding by serum and milk. *Am. J. Clin. Nutr.* 21:289–97

58. Picciano, M. F. 1985. Analysis of water-soluble vitamins in human milk: folicin and vitamin B_{12}. In *Human Lactation: Milk Components and Methodologies,* ed. R. G. Jensen, M. C. Neville, pp. 143–51. New York: Plenum

59. Price, E. M., Ratnam, M., Rodeman, K. M., Freisheim, J. H. 1988. Characterization of the methotrexate transport pathway in murine L1210 leukemia cells: involvement of a membrane receptor and a cytoplasmic protein. *Biochemistry* 27:7853–58

60. Ratnam, M., Marquardt, H., Duhring, J. L., Freisheim, J. H. 1989. Homologous membrane folate binding proteins in human placenta: Cloning and sequence of a cDNA. *Biochemistry* 28:8249–54

61. Reisenauer, A. M. 1988. Affinity labeling of the intestinal folate binding protein. *Gastroenterology* 94:A372

62. Reisenauer, A. M., Chandler, C. J., Halstead, C. H. 1986. Folate binding and hydrolysis by pig intestinal brush-border membranes. *Am. J. Physiol.* 251:G481–86

63. Rindler, M. J., Bashor, M. M., Spitzer, N., Saier, M. H. 1978. Regulation of adenosine 3',5'-monophosphate efflux from animal cells. *J. Biol. Chem.* 253:5431–36

64. Rosenberg, I. H. 1986. Intestinal absorption of folates. See Ref. 8a, pp. 147–76

65. Rubinoff, M., Abramson, R., Schreiber, C., Waxman, S. 1981. Effect of folate binding protein on the plasma transport and tissue distribution of folic acid. *Acta Haematol.* 65:145–52

66. Rubinoff, M., Schreiber, C., Waxman, S. 1977. The isolation and characterization of the folate binding protein from goat milk. *FEBS Lett.* 75:244–48

67. Russell, R. D., Dhar, J. G., Dutta, S. K., Rosenberg, I. H. 1979. Influence of intraluminal pH on folate absorption: studies in control subjects and in patients with pancreatic insufficiency. *J. Lab. Clin. Med.* 93:428–36

68. Sadasivan, E., da Costa, M., Rothenberg, S. P., Brink, L. 1987. Purification, properties, and immunological characterization of folate binding proteins from human leukemia cells. *Biochim. Biophys. Acta* 925:36-47

69. Sadasivan, E., Rothenberg, S. P. 1989. The complete amino acid sequence of a human folate binding protein from KB cells determined from the cDNA. *J. Biol. Chem.* 264:5806–11

70. Said, H. M., Ghishan, F. K., Redha, R. 1987. Folate transport by human intestinal brush-border membrane vesicles. *Am. J. Physiol.* 252:G229–36

71. Said, H. M., Redha, R. 1987. A carrier-mediated transport for folate in basolateral membrane vesicles of rat small intestine. *Biochem. J.* 247:141–46

72. Salter, D. N., Mowlem, A. 1983. Neonatal role of milk folate binding protein: studies on the course of digestion of goats milk binder in the 6 day-old kid. *Br. J. Nutr.* 50:589–96

73. Salter, D. N., Scott, K. J., Slade, H., Andrews, P. 1981. The preparation and properties of folate binding protein from cow's milk. *Biochem. J.* 193:469–76

74. Schron, C. M., Washington, C., Blitzer, B. L. 1985. The transmembrane pH gradient drives uphill folate transport in rabbit jejunum. *J. Clin. Invest.* 76:2030–33

75. Schuetz, J. D., Matherly, L. H., Westin, E. H., Goldman, I. D. 1988. Evidence for a functional defect in the translocation of the methotrexate transport carrier in a methotrexate-resistant murine L1210 leukemia cell line. *J. Biol. Chem.* 263:9840–47

76. Selhub, J., Arnold, R., Smith, A. M., Picciano, M. F. 1984. Milk folate binding protein (FBP): A secretory protein for folate? *Nutr. Res.* 4:181–87

77. Selhub, J., Emmanouel, D., Stravropoulos, T., Arnold, R. 1987. Renal fo-

late absorption and the kidney folate binding protein. I. Urinary clearance studies. *Am. J. Physiol.* 252:F750–56
78. Selhub, J., Franklin, W. A. 1984. The folate binding protein of rat kidney: Purification, properties, and cellular distribution. *J. Biol. Chem.* 259:6601–6
79. Selhub, J., Gay, A. G., Rosenberg, I. H. 1979. Folate binding activity in epithelial brush border membranes. A system for investigating membrane-associated folate binding proteins. See Ref. 46a, pp. 593–96
80. Selhub, J., Nakamura, S., Carone, F. A. 1987. Renal folate absorption and the kidney folate binding protein: II. Microinfusion studies. *Am. J. Physiol.* 252:F757–60
81. Selhub, J., Rosenberg, I. H. 1981. Folate transport in isolated brush border membrane vesicles from rat intestine. *J. Biol. Chem.* 256:4489–93
82. Sirotnak, F. M. 1980. Correlates of folate analog transport, pharmacokinetics, and selective antitumor action. *Pharmacol. Ther.* 8:71–103
83. Sirotnak, F. M., Goutas, L. J., Mines, L. S. 1985. Extent of requirement for folate transport by L1210 cells for growth and leukemogenesis in vivo. *Cancer Res.* 45:4732–34
84. Smith, A. M., Picciano, M. F., Deering, R. H. 1983. Folate supplementation during lactation: maternal folate status, human milk folate content, and their relationship to infant folate status. *J. Pediatr. Gastroenterol. Nutr.* 2:622–28
85. Strum, W. B., Liem, H. H., Muller-Eberhard, U. 1979. Enterhepatic circulation of folates: II. Hepatic uptake, intracellular accumulation, and bilary excretion. See Ref. 46a, pp. 615–18
86. Suleiman, A. S., Spector, R. 1981. Purification and characterization of a folate binding protein from porcine choroid plexus. *Arch. Biochem. Biophys.* 208:87–94
87. Svendsen, I., Hansen, S. I., Holm, J., Lyngbye, J. 1982. Amino acid sequence homology between human and bovine low molecular weight folate binding protein isolated from milk. *Carlsberg Res. Commun.* 47:371–76
88. Svendsen, I., Hansen, S. I., Holm, J., Lyngbye, J. 1984. The complete amino acid sequence of the folate binding protein from cow's milk. *Carlberg. Res. Commun.* 49:123–31
89. Sweiry, J. H., Yudilevich, D. L. 1985. Transport of folates at maternal and fetal sides of the placenta: lack of inhibition by methotrexate. *Biochim. Biophys. Acta* 821:497–501
90. Sweiry, J. H., Yudilevich, D. L. 1988. Characterization of folate uptake in guinea pig placenta. *Am. J. Physiol.* 254:C735–43
91. Wagner, C. 1985. Folate binding proteins. *Nutr. Rev.* 43:293–99
92. Wagner, C. 1986. Proteins binding pterins and folates. See Ref. 8a, pp. 251–95
93. Waxman, S., Schreiber, C. 1973. Characteristics of folic acid binding protein in folate-deficient serum. *Blood* 42:291–301
94. Waxman, S., Schreiber, C. 1975. The purification and characterization of the low molecular weight human folate binding protein using affinity chromatography. *Biochemistry* 14:5422–28
95. Weiner, M. L. 1988. Intestinal transport of some macromolecules in food. *Food Chem. Toxicol.* 26:867–80
96. Yang, C-H., Sirotnak, F. M., Mines, L. S. 1988. Further studies on a novel class of genetic variants of the L1210 cell with increased folate analogue transport inward. *J. Biol. Chem.* 263:9703–9
97. Zheng, D. B., Lim, H. M., Pene, J. J., White, H. B. 1988. Chicken riboflavin binding protein: cDNA sequence and homology with milk folate binding protein. *J. Biol. Chem.* 263:11126–29
98. Zimmerman, J., Selhub, J., Rosenberg, I. H. 1986. Role of sodium ion in transport of folic acid in the small intestine. *Am. J. Physiol.* 251:G218–22