58K, a Microtubule-binding Golgi Protein, Is a Formiminotransferase Cyclodeaminase*

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58K was previously identified as a rat liver protein that binds microtubules *in vitro* and is associated with the cytoplasmic surface of the Golgi apparatus *in vivo* (Bloom, G. S., and Brashear, T. A. (1989) *J. Biol. Chem.* 264, 16083–16092). We now report that 58K is a formiminotransferase cyclodeaminase (FTCD), a bifunctional enzyme that catalyzes two consecutive steps in the modification of tetrahydrofolate to 5,10-methenyl tetrahydrofolate. Comparative immunoblotting using several monoclonal antibodies made against 58K and a polyclonal antibody made against a chicken liver protein (p60) with similar properties (Hennig, D., Scales, S. J., Moreau, A., Murley, L. L., De Mey, J., and Kreis, T. E. (1998) *J. Biol. Chem.* 273, 19602–19611) demonstrated precise co-purification of protein recognized by all antibodies through multiple fractionation steps, including gel filtration and ion exchange chromatography, and sucrose gradient ultracentrifugation. Eight peptides derived from 58K showed high sequence identity to amino acid sequences predicted by full length cDNA for p60 and porcine liver FTCD. Furthermore, purified 58K was associated with formiminotransferase and cyclodeaminase activities. Based on these collective results, 58K was concluded to be a rat liver version of FTCD. Microtubules assembled from brain tubulin, but not from liver tubulin, were able to bind rat liver FTCD. Binding to brain microtubules is suspected to occur via polyglutamates that are added post-translationally to tubulin in brain, which was shown to contain very low levels of FTCD, but not to tubulin in liver, which was determined to be the richest tissue source, by far, of FTCD. The physiological significance of the microtubule binding activity of FTCD is thus called into question, but an association of FTCD with the Golgi apparatus has now been established.

A powerful approach for identifying factors that function in concert with microtubules (MTs)[1] has been to seek proteins that bind to MTs in preparations of cytosol. This strategy was originally applied to mammalian brain cytosol, leading to the discovery of numerous MT-associated proteins (MAPs) (3–6), which are structural components of MTs and regulate their assembly. Modifications of this general approach also led to the discovery of brain versions of kinesin (7, 8) and cytoplasmic dynein (9), the first two MT motor proteins to have been found outside of cilia and flagella.

To broaden the search for MAPs, molecular motors and other factors that may interact with MTs, several groups have made use of cytosol isolated from pure cell populations or non-neural tissues as potential sources of new MT-binding proteins (10–17). In one such case, our laboratory discovered a novel MT-binding protein, 58K, in preparations of rat liver cytosol (1). Curiously, 58K was localized by immunofluorescence to the Golgi apparatus in cultured hepatoma cells and was present on purified liver Golgi membranes as a peripheral membrane protein (1). 58K was also detected by both Western blotting[2] and immunofluorescence microscopy (18, 19) in a wide variety of cultured mammalian cell types. As was found for hepatoma cells, immunofluorescence labeling of other cell types with anti-58K antibodies yielded bright staining of the Golgi complex.

Although the localization of a MAP-like protein on Golgi membranes was initially surprising, it was not unprecedented (20) and offered potential insight into the intriguing relationship between MTs and the Golgi complex in interphase cells. The Golgi complex in nondividing cells typically comprises an interconnected network of stacked cisternae (21), which are located either near the nucleus (22), or in some types of polarized epithelial cells, adjacent to the apical surface (23, 24). Exposure of interphase cells to MT depolymerizing drugs, such as colcemid or nocodazole, causes the Golgi apparatus to break apart into scores of fragments that are widely distributed throughout the cytoplasm (25, 26). Similar effects on the Golgi apparatus have been observed in cells exposed to Taxol (25, 27), a drug which stimulates MT assembly, and as a by-product, disrupts the normal organization of MTs. It is evident, therefore, that the structural integrity and normal localization of the Golgi apparatus in interphase cells depends upon the presence and normal organization of MTs. At the time that 58K was initially characterized, the dependence of the Golgi on MTs was well known, but poorly understood mechanistically. We therefore decided to investigate the hypothesis that 58K anchors Golgi membranes to MTs and helps to maintain the structural unity of the Golgi and its proper positioning within the cell (1).

New evidence derived from biochemical, immunological, molecular biological, and enzymological studies indicates that 58K is a version of formiminotransferase cyclodeaminase (FTCD), a bifunctional enzyme which catalyzes two consecutive steps in the modification of tetrahydrofolate (THF) to 5,10-methenyl-THF (28, 29). We thus refer to 58K as rat liver FTCD. MTs

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[1] The abbreviations used are: MT, microtubule; FTCD, formiminotransferase cyclodeaminase; MAP, microtubule-associated protein; PAGE, polyacrylamide gel electrophoresis; THF, tetrahydrofolate; PIPES, 1,4-piperazinediethanesulfonic acid.

[2] A.-M. Bashour and G. S. Bloom, unpublished results.

assembled from brain tubulin, but not from liver tubulin, were able to bind avidly to rat liver FTCD, although Western blotting of rat tissue extracts revealed that FTCD is far more abundant in liver than in all other tissues examined, including brain. Considering that FTCD is virtually liver-specific, but does not bind to liver MTs, the ability of FTCD to bind brain MTs is unlikely to be physiologically relevant. Although the hypothesis that 58K (FTCD) links Golgi membranes to MTs *in vivo* must now be regarded with skepticism, an unexpected association of FTCD with the Golgi apparatus has been revealed. It is noteworthy that analogous results were obtained independently in another laboratory for a chicken liver version of FTCD, and are reported in Henning *et al.* (2).

EXPERIMENTAL PROCEDURES

Materials—Monoclonal antibodies made against rat liver 58K (FTCD) have been previously described (1) and were prepared in our laboratory as either ascites fluids or hybridoma-conditioned tissue culture media. Polyclonal antisera made against chicken (2) and porcine liver (30) FTCD were generously provided by Drs. Thomas Kreis of the l'Université de Genève and Robert E. MacKenzie of McGill University, respectively. Affinity-purified antibodies directed against mouse or rabbit IgG and labeled with horseradish peroxidase were purchased from Kierkegaard and Perry. Polyvinylidine difluoride blotting membranes were aquired from Bio-Rad. Luminol chemiluminescence reagents were purchased from Pierce and used according to the vendor's instructions. Superose 6 chromatography medium and a Resource Q column were purchased from Pharmacia LKB Biotechnology, Inc. Buffers, salts, and other reagents were purchased from Sigma. Taxol was provided by Nancita R. Lomax of the National Cancer Institute. Aquacide II was purchased from Calbiochem.

Purification of 58K (FTCD)—Our previously described method for purification of 58K (FTCD) from rat liver (1) was modified by substituting Superose 6 for the gel filtration medium that was used earlier, and in some cases by the addition of two more steps after gel filtration, which originally was the final purification step. Concentrated gel filtration fractions were further fractionated on a 1-ml Resource Q anion exchange column using a 30-ml gradient of 0.5–1 M KCl in PEM buffer (0.1 M PIPES, pH 6.62, 1 mM EGTA, 1 mM $MgSO_4$). Fractions enriched for 58K were then concentrated by dialysis *versus* Aquacide II, layered on top of a 2-ml gradient of 5–20% sucrose, and centrifuged at 4 °C for 4 h at 55,000 rpm ($118,000 \times g_{max}$) in a Beckman Optima TLX 120 table top ultracentrifuge using a TLS-55 swinging bucket rotor. Samples of 50 μl each were collected using a Densiflow IIc fractionator (Searle Diagnostics). Analysis of fractions at each step of purification was accomplished by SDS-polyacrylamide gel electrophoresis (PAGE) using 9% polyacrylamide gels and a Tris-glycine buffer system (31), and chemiluminescent Western blotting with monoclonal antibodies to 58K (1), or polyclonal antibodies to chicken (2) or porcine liver (30) FTCD. Eletrophoretic transfer of protein from polyacrylamide gels to polyvinylidene difluoride membranes was performed as described earlier (32) with the following modifications. The transfer buffer was 12.5 mM Tris-OH at pH 8.2–8.6, 96 mM glycine, 0.01% sodium dodecyl sulfate, 20% methanol (by volume); the gel was preincubated with transfer buffer for 15 min; and transfer was for 1 h at 100 V.

Production, Purification, and Sequencing of 58K (FTCD) Peptides—Approximately 400 pmol of rat liver 58K purified through the gel filtration step (see above) were subjected to SDS-PAGE using a 7.5% polyacrylamide gel, transferred to nitrocellulose, and digested with trypsin (33). Resulting peptides were fractionated by two sequential reverse phase chromatography steps using an Applied Biosystems model 130A high performance liquid chromatography and a Brownlee model RP300 2.1 × 100-mm C8 column. The first column run employed a 0–50% gradient of acetonitrile in 0.1% trifluoroacetic acid, and selected peaks were resolved by a second run in which the gradient was 0–50% acetonitrile in 0.1% ammonium acetate. Peak fractions from the second C8 column run were sequenced using an Applied Biosystems model 470A sequencer after being collected on Whatman GF/C paper and subsequent reduction and alkylation of cysteines (34).

Enzymatic Activities of Rat Liver 58K (FTCD)—The formiminotransferase activity of FTCD is responsible for conversion of THF to 5-formimino-THF, which is converted by the cyclodeaminase activity of FTCD to 5,10-methenyl-THF (28). For the study described here, purified rat liver 58K was assayed for both activities by Dr. Laura Lea Murley in the laboratory of Dr. Robert E. MacKenzie at McGill University, exactly as described previously (29). Formiminotransferase

assays, which were performed in a manner that allowed quantitation of enzyme activity, measured the overall 58K-dependent conversion of THF to 5,10-methenyl-THF. This was accomplished by first incubating THF with 58K for 3 min at 30°, yielding the primary reaction product, 5-formimino-THF, some of which was converted to 5,10-methenyl-THF by the cyclodeaminase activity of 58K. The reaction mixture was then incubated for 55 s at 100 °C to allow thermally driven conversion of the remaining 5-formimino-THF to 5,10-methenyl-THF (29). Thus, although 5,10-methenyl-THF was produced by a combination of cyclodeaminase activity and heating, its yield in each assay was exactly equal to the yield of 5-formimino-THF, the product of the formiminotransferase assay. Cyclodeaminase assays detected direct conversion of 5-formimino-THF to 5,10-methenyl-THF (29), using a substrate concentration well below the K_m for recombinant porcine FTCD. The cyclodeaminase assays were thus reliable for qualitative assessment of enzyme activity, but not for rigorous quantitation.

Binding of 58K (FTCD) in Liver Cytosol to Liver or Brain MTs—Bovine brain tubulin was purified as described earlier (35), and rat liver tubulin was generously supplied by Dr. Tony Frankfurter of the University of Virginia. Both types of tubulin were polymerized into MTs by addition of equimolar Taxol, and a 5-min incubation at 37°. For the MT binding assays, rat liver cytosol (1) was supplemented with rat liver or bovine brain MTs to a final concentration of 0.5 mg/ml polymerized tubulin. After a 30-min incubation on ice, the 0.5-ml samples were centrifuged for 30 min at 40,000 rpm ($86,553 \times g_{max}$) at 4 °C in a Beckman Optima TLX-120 table top ultracentrifuge using a TLA 100.3 rotor. The resulting pellets were resuspended in PEM buffer to volume, and each of the 0.5-ml supernatants and pellets was then supplemented with 125 μl of 5× sample buffer for SDS-PAGE. Samples of 3 μl each were then analyzed by SDS-PAGE and Western blotting, as described above. The 58K-2 antibody (1) was used for the immunoblotting step. Chemiluminescent immunoblots were digitally photographed using a Dage-MTI CCD-72 camera as an input device for an Apple Power Macintosh 7600 computer. NIH Image software was used for image capturing and for quantitation of background corrected bands.

Tissue Distribution of 58K (FTCD)—Several organs (see Fig. 4) were dissected from a freshly sacrificed rat, homogenized in 1 ml of buffer per g of tissue, and centrifuged at 48,000 rpm ($100,000 \times g_{max}$) for 30 min at 4 °C in a Beckman TK120.2 rotor and a Beckman Optima TLX 120 table top ultracentrifuge. The homogenization buffer was 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 2 mM $CaCl_2$, supplemented with protease inhibitors (1 μg/ml each of leupeptin, chymostatin, and aprotinin). Protein concentrations of the resulting cytosolic extracts were determined by a colorimetric assay (36) using bovine serum albumin as a standard. A 50-μg aliquot of each extract was then analyzed by Western blotting as described above, using a mixture of the 58K-2, 58K-4, 58K-7, and 58K-12 antibodies (1).

RESULTS

58K Is an FTCD—As described in a companion study (2), an M_r 60,000 chicken liver protein (p60) with properties similar to rat liver 58K was isolated and characterized independently in another laboratory. p60 binds MTs assembled from brain tubulin and is localized on Golgi membranes and post-Golgi vesicles as a peripheral membrane protein. After learning about these properties of p60,[3] the similarities between it and 58K prompted us to investigate whether the two were species-specific versions of the same or similar proteins.

Several monoclonal antibodies made against purified 58K (1) and a polyclonal antibody made against p60 (2) were used for comparative Western blotting of various fractionation steps for the purification of 58K from rat liver. The first step (not shown) was to supplement rat liver cytosol with MTs assembled with Taxol from brain tubulin, centrifuge the MTs, and elute non-tubulin proteins from the MTs by washing them with a high salt containing buffer (1). The non-tubulin proteins were then fractionated by gel filtration (Superose 6) chromatography, and peak fractions of 58K were identified by Western blotting with anti-58K monoclonal antibodies. Subsequent fractionation steps, which were also monitored by Western blotting using the monoclonal antibodies, included ion exchange (Resource Q)

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[3] T. E. Kreis, personal communication.

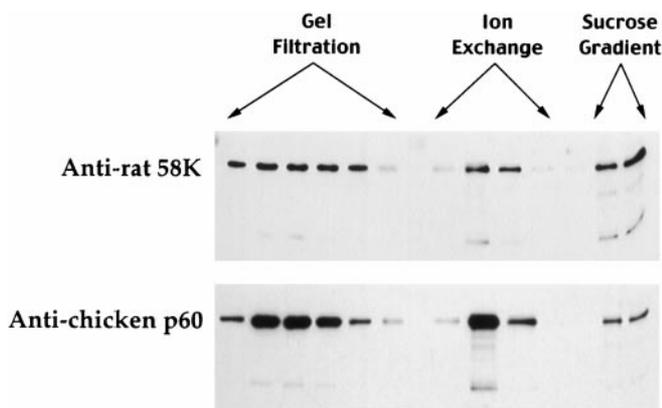*FTCD is a Golgi Protein That Binds Microtubules*

Fig. 1. **Immunoreactivity of rat 58K with an antibody to chicken p60.** MT-binding proteins isolated from rat liver were resolved by sequential gel filtration (Superose 6) and ion exchange (Resource Q) chromatography, followed by sucrose gradient ultracentrifugation. Fractions from each step were then analyzed by Western blotting using a monoclonal antibody against 58K (anti-58K-2) (1) and polyclonal anti-chicken p60 (no. 569) (2). Note that the two immunoblots yielded analogous results.

chromatography followed by sucrose gradient ultracentrifugation. As shown by Western blotting in Fig. 1, protein labeled by the anti-58K-2 monoclonal antibody fractionated identically to protein recognized by the antibody (no. 569) to p60 throughout the gel filtration, ion exchange, and sucrose gradient steps. Comparable results were obtained when other monoclonal antibodies to 58K were substituted for 58K-2 (not shown). Although not evident on Fig. 1, protein recognized by the anti-58K antibodies had the same electrophoretic mobility as protein that was recognized by the antibody to p60.

As part of our overall strategy for characterizing 58K, we initially intended to clone and sequence cDNA for the protein. Accordingly, rat liver cDNA libraries were screened with monoclonal antibodies to 58K, as well as with several oligonucleotides whose designs were based on amino acid sequences of multiple peptide fragments that we generated from purified 58K, and subsequently sequenced. Before much progress was made, p60 was fully sequenced (2)[3] and found to include highly conserved versions of all eight peptides that we had obtained for 58K, and which together comprised 124 amino acid residues. Rat liver 58K and p60 were therefore closely related proteins, in agreement with the Western blotting results shown in Fig. 1.

Immediately after discovering the structural similarity of 58K and p60, a search of the DNA and protein data bases failed to reveal any entries with sequences comparable to those of the 58K peptides or full-length p60. Eventually, however, a protein with high identity appeared in on-line data bases. The protein was porcine liver FTCD (30), and like p60, it was predicted to comprise 541 amino acid residues. Fig. 2 illustrates an amino acid sequence alignment of the eight sequenced peptides derived from 58K and corresponding regions of p60 and porcine FTCD. Seven positions within the 58K peptides yielded strong signals in the sequencing reactions for two amino acids. The final position in peptide 1 was either alanine (2 pmol) or lysine (0.5 pmol), and the first position in peptide 5 was either cysteine (5 pmol) or leucine (4 pmol). The greatest amount of ambiguity was in peptide 3, in which position 1 was alanine (16 pmol) or leucine (10 pmol), position two was glutamic acid (24 pmol) or proline (16 pmol), position four was valine (8 pmol) or threonine (3 pmol), position five was proline (4 pmol)) or isoleucine (3 pmol), and position 10 was glutamic acid (2 pmol) or serine (0.5 pmol). Optimal alignments of the 58K peptides to chicken p60 and porcine FTCD did not depend upon which

amino acids at any of the ambiguous positions were used. The 58K peptides were distributed throughout a region that began at amino acid residue 48, and ended at residue 413 of porcine FTCD and p60, which corresponds to nearly 70% of their full length sequences. The 124 amino acid residues within the 58K peptides were 85 and 66% identical to the equivalent residues in porcine FTCD and p60, respectively (ambiguous positions in 58K peptides were counted as a match if either of the 2 amino acids were identical to the corresponding residue in porcine FTCD or p60).

Consistent with the molecular data, purified rat liver 58K was associated with both formiminotransferase and cyclodeaminase activities, which together form the enzymatic signature of porcine liver FTCD (28, 29). Formiminotransferase activity, which was measured by assaying conversion of THF to 5,10-methenyl-THF (see "Experimental Procedures"), was 7 μmol/min/mg of 58K. This value is ~20% of the specific activity typically observed for porcine liver FTCD (29). 58K was also found to catalyze conversion of 5-formimino-THF to 5,10-methenyl-THF, and thus to be associated with the signature activity of a cyclodeaminase. Because cyclodeaminase assays were performed using substrate at a concentration well below the K_m for recombinant porcine FTCD, however, reliable quantitation of the activity was not possible. Based on the evidence presented to this point, we now consider 58K and p60 to be rat and chicken versions, respectively, of FTCD. In light of the fact that FTCD from two vertebrate species, pig and chicken, has been cloned and fully sequenced, we have suspended efforts to complete the cloning and sequencing of rat FTCD.

FTCD Binds to MTs Polymerized from Brain, but Not Liver Tubulin—The finding that 58K is equivalent to a previously described bifunctional enzyme raised doubts about the hypothesis that the protein serves yet a third role *in vivo*: to anchor Golgi membranes to MTs. If that were the case, we expected that FTCD would bind to liver MTs, because liver is the only tissue from which native FTCD has been purified (1, 2, 28, 29). All of our previous data regarding the MT binding activity of rat liver FTCD had made use of MTs assembled with taxol from purified bovine brain tubulin (1). To test the ability of FTCD to bind liver MTs, rat liver cytosol was supplemented with MTs assembled with taxol from either rat liver or bovine brain tubulin, or as a control, with buffer. After a 30-min incubation on ice, the samples were centrifuged, and the pellets were then resuspended to volume. The amounts of FTCD in equal volume samples of each supernatant and pellet were then determined by quantitative immunoblotting using the anti-58K-2 monoclonal antibody. As illustrated in Fig. 3, 60% of the FTCD sedimented in the absence of MTs, and thus represents background. Presumably, the large amount of FTCD that sedimented under these conditions reflected the large size of the native protein, which is an ~480,000-kDa homooctomer (37), the small volume of the sample that was centrifuged, and the specific centrifugation protocol, which was optimized for effectively pelleting MTs. No increase in pelleted FTCD was observed in the presence of liver MTs. Indeed, slightly less FTCD (51%) was found in the liver MT pellet. In the presence of brain MTs, however, 89% of the FTCD was found in the pellet, a sharp increase over the amount of FTCD found in the control and liver MT pellets. Taken together, these results demonstrate that the affinity of FTCD for liver MTs is negligible, despite the fact that the protein binds readily to brain MTs.

FTCD Is Highly Enriched in Liver—The surprising discovery that FTCD does not bind liver MTs prompted us to compare the level of FTCD in liver with its levels in brain and other tissues. Accordingly, 27-μg aliquots of cytosol obtained from each of several rat tissues were analyzed by Western blotting using a

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                         K
❶ VYTFVGQPECVVEGALSA      rat 58K        LAEELNVPVYLYGEAAQMPSPR ❷
  VYTFVGTPEAVVEGALSA      chicken p60    LSEELGVPVYLYGEAAROESRR
  VYTFVGRPEDVVEGALNA      porcine FTCD   LAEELGVPVYLYGEAARTAGRQ
  48               65                    115                136


        LP TI     S
     ❸ AESVPDFGPE          rat 58K        AQELNLPVVGSQ ❹
       PEWVPDFGPP          chicken p60    AEALKLPVVGSQ
       AEWAPDFGPS          porcine FTCD   AQELSLPVVGSQ
       156      165                       257      268


        L
     ❺ CFVLEEEHR           rat 58K        LGLDSLAPFDPK ❻
       LFILEEEHK           chicken p60    LGLDSLSPFNPR
       LFLLQDEHR           porcine FTCD   LGLDSLAPFKPK
       291     299                        307      318


  ❼ IIEYLVPDSGPEQSLLDASLR   rat 58K        LIPPFHAASAQLTSLVDADA ❽
    IIEYLVQAGQEDKGLVTKPLG   chicken p60    VIPPLHQAMDELVAMVDADS
    IIEYLVPEAGPEQSLLHKPLR   porcine FTCD   LIPPFHAASAKLTSLVDADA
    321            341                     394            413
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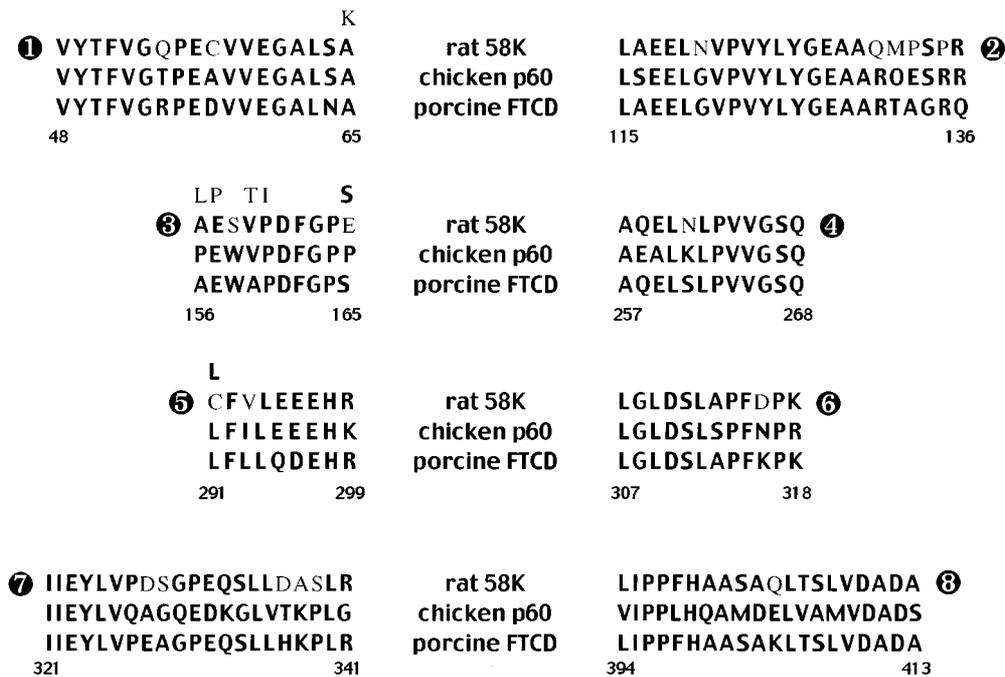
FIG. 2. **Alignment of rat 58K peptides to predicted amino acid sequences of chicken p60 and porcine liver FTCD.** Eight tryptic peptide fragments of 58K were sequenced, and conserved versions of each were found within the full-length sequence of chicken p60 (2). Subsequently, both rat 58K and chicken p60 were found to have high sequence identity with porcine liver FTCD (30). An alignment of the 58K peptides, and corresponding regions within chicken p60 and porcine FTCD are shown here. Note that seven positions within the 58K peptides were ambiguous, and each yielded strong signals for two amino acids. At each of these positions, the amino acid that produced the strongest signal is shown within the full peptide sequence, directly below the potential alternative amino acid. The number of picomoles of each amino acid that were detected at the ambiguous positions is stated under "Results." Rat 58K residues that are different from corresponding residues in both chicken p60 and porcine FTCD are indicated by the contrasting font. The numbers below each set of sequences refer to positions of the first and last residue within the predicted full-length sequences of both chicken p60 and porcine FTCD.
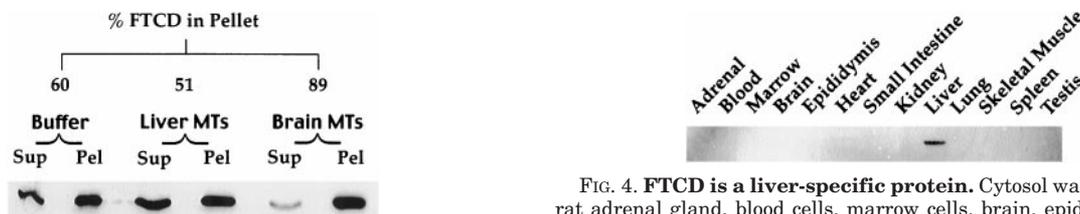

FIG. 3. **Rat liver FTCD binds to MTs assembled from brain, but not liver tubulin.** Rat liver cytosol was supplemented with MTs assembled with taxol from either rat liver or bovine brain tubulin, or as a control, with buffer. After a 30-min incubation on ice, the samples were centrifuged, and the pellets were then resuspended to volume. The amounts of FTCD in equal volume samples of each supernatant and pellet were then determined by quantitative immunoblotting using the anti-58K-2 monoclonal antibody. Note that the buffer (control) and liver MT pellets contained comparable levels of FTCD (60 and 51%, respectively), and that substantially more FTCD (89%) pelleted in the presence of brain MTs. FTCD therefore binds to brain, but not liver, MTs.

FIG. 4. **FTCD is a liver-specific protein.** Cytosol was isolated from rat adrenal gland, blood cells, marrow cells, brain, epididymis, heart, small intestine, kidney, liver, lung, skeletal muscle, spleen, and testis. Aliquots containing 27 μg of protein each were then analyzed by immunoblotting using a mixture of the 58K-2, 58K-4, 58K-7, and 58K-12 monoclonal antibodies (1), which are now known to be directed against FTCD. Note that FTCD was abundant in liver, but was undetectable in the other samples. At higher protein loads and longer exposures, very low levels of FTCD were detected in tissues other than liver and in cultured mammalian cells (not shown). In practical terms, therefore, FTCD is a liver-specific protein.

mixture of the 58K-2, 58K-4, 58K-7, and 58K-12 antibodies (1). As shown in Fig. 4, FTCD was readily detected in liver, but under the conditions of this experiment was undetectable in the adrenal gland, blood cells, marrow cells, brain, epididymis, heart, small intestine, kidney, liver, lung, skeletal muscle, spleen, and testis. Although higher protein loads and longer exposures of chemiluminescent blots enabled faint detection of FTCD in tissues other than liver (not shown), these results establish that liver is an especially rich source of FTCD.

DISCUSSION

Based on several criteria, 58K has been shown to be an FTCD. By immunoblotting, antibodies made against chicken (Fig. 1) or porcine (not shown) liver FTCD labeled protein that co-migrated with 58K in SDS-PAGE, and precisely co-fractionated with 58K during its purification from rat liver cytosol. In addition, several monoclonal anti-58K antibodies reacted with purified native or recombinant porcine liver FTCD (not shown). High sequence identity was observed between eight peptide fragments of 58K, and the full-length sequences of porcine and chicken liver FTCD (Fig. 2). Finally, both enzyme activities known to be associated with porcine liver FTCD, formiminotransferase and cyclodeaminase (28, 29), were also associated with purified 58K. Until more is known about the structure of FTCD genes and flanking regions of genomic DNA, it will not be possible to judge whether the rat, chicken, and porcine proteins discussed here represent species-specific products of equivalent, as opposed to closely related, genes. Likewise, ambiguities in the amino acid sequence of rat FTCD at several positions (see Fig. 2) raise the possibility that multiple FTCD isoforms are encoded by multiple genes within a single animal species. Regardless, in light of the new evidence about rat liver 58K, its classification as an FTCD seems amply justified.

Although 58K was associated with formiminotransferase ac-

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tivity, the measured activity was only ~20% of that reported for recombinant porcine FTCD (30). The following considerations, individually or collectively, might account for this discrepancy. First, no effort was made to optimize recovery of the enzyme activities of 58K, which was purified by a method (see "Experimental Procedures") that was radically different than the procedure used to purify recombinant porcine FTCD (30). Next, isoelectric focusing gel electrophoresis resolved purified 58K into eight closely spaced variants that collectively were recognized by antibodies to rat 58K, chicken p60, and porcine FTCD (data not shown). The electrophoretic diversity of 58K could reflect the presence of multiple, distinct translation products, post-translational modifications, or both, although no evidence that might discriminate among these possibilities is presently available. Regardless, by comparison to recombinant porcine FTCD, the purified 58K that was used for enzyme assays was very diverse in molecular terms, and by extension, perhaps in enzymatic properties as well. Finally, species-specific differences in enzymatic efficiency might explain, at least in part, the low formiminotransferase activity of rat liver 58K relative to that of recombinant porcine FTCD.

Before 58K was shown to be an FTCD, studies of the protein demonstrated that it could bind MTs *in vitro*, was localized by immunofluorescence to the Golgi apparatus of cultured cells, and was a cytoplasmically oriented, peripheral membrane protein of isolated Golgi membranes (1, 18, 19). In light of the ability of 58K to bind MTs and Golgi membranes, and the fact that a properly localized, structurally intact Golgi requires MTs to be present and normally organized (25–27), we postulated that 58K anchors Golgi membranes to MTs (1). New evidence presented here, however, strongly suggests that 58K, now realized to be an FTCD, does not cross-link Golgi membranes to MTs *in vivo*.

The pertinent evidence is that FTCD binds to MTs assembled from brain, but not liver tubulin (Fig. 3). Thus, FTCD is unlikely to bind MTs in the only tissue in which it is abundant, namely liver (Fig. 4). There is one obvious difference between brain and liver tubulin that might account for the preferential binding of FTCD to brain MTs. Located near the C termini of both α-tubulins and β-tubulins are glutamic acid residues to which polyglutamic acid side chains of variable length can be added post-translationally *in vivo* (38, 39). In brain, approximately half of the α-tubulin (39), and a lesser, but substantial, amount of the β-tubulin (38) are polyglutamylated. In other tissues, including liver, polyglutamylated tubulin has also been detected, but at dramatically lower levels and only on β-tubulins (38).

Why might FTCD preferentially bind MTs assembled from polyglutamylated, as compared with unmodified tubulin? FTCD is a homooctomeric enzyme (37), and each molecule comprises 4 binding sites for the polyglutamate moiety of THF (40). Indeed, affinity chromatography using a polyglutamate-Affi-Gel column has been used as a final enrichment step for purification of recombinant porcine liver FTCD (30). We postulate, therefore, that the polyglutamates which are uniquely abundant on brain tubulin enable MTs assembled from brain, but not liver, tubulin to bind avidly to FTCD. The formal proof of this hypothesis ultimately must rest on MT binding studies using tubulin whose polyglutamylation state can be altered experimentally. Unfortunately, such experiments are not presently possible, because none of the enzymes responsible for post-translationally adding glutamates to tubulin or subsequently removing them are reported to have been discovered.

It may seem puzzling that FTCD appears by immunofluorescence microscopy to be localized predominantly on the Golgi and post-Golgi vesicles (1, 2, 18, 19), but is routinely purified

from cytosol, rather than from membranes (1, 2, 28, 29). One potential explanation for this apparent anomaly is that both soluble and membrane-associated pools of FTCD exist, but FTCD is far more concentrated on membranes than in the soluble phase of cytoplasm. Bearing in mind that immunofluorescence reports relative concentrations of accessible epitopes, it is possible that the majority of FTCD is soluble, but at a concentration near or below the level of detectability by immunofluorescence. Furthermore, because FTCD is a cytoplasmically oriented peripheral membrane protein that binds membranes by relatively weak ionic interactions (1, 2), it might be easily solubilized from membrane surfaces by the homogenization and extraction procedures that routinely are used to prepare cytosol as a first step in the purification of FTCD. An analogous situation exists for the MT motor protein, kinesin. Immunofluorescence and other *in situ* localization methods indicate that ~30–90% of the cellular pool of kinesin is associated with membrane surfaces *in vivo* (41–44), even though kinesin is purified from cytosol (8).

While emphasizing that FTCD is unlikely to bind MTs *in vivo*, the results shown here and in a companion study about chicken FTCD (2) also raise the question of why FTCD is associated with the Golgi. FTCD catalyzes two consecutive reactions: transfer of a single carbon from formiminoglutamate to THF, followed by conversion of the product, 5-formimino-THF, to 5,10-methenyl-THF plus ammonia (28). Several metabolically relevant purposes are served by these reactions (45). First, formiminoglutamate is a product of the histidine metabolism pathway, and its modification by FTCD completes the multistep conversion of histidine to glutamic acid. Next, 5,10-methenyl-THF, the end product of FTCD catalysis, can be converted directly to active coenzymes, such as 5-formyl-THF and 10-formyl-THF. Finally, 5,10-methenyl-THF also lies directly in the pathways for synthesis of several nucleotides and amino acids, as well as other compounds, including *S*-adenosylmethionine and formylmethionine tRNA.

The two reactions catalyzed by FTCD have classically been considered to occur *in vivo* within the soluble phase of cytoplasm. The finding that FTCD is localized, at least in part, on the Golgi apparatus and post-Golgi vesicles (1, 2) raises the possibility that its site of action is actually on membrane surfaces. Liver apparently contains the highest level of folates of all tissues (46), and acquires its folates from plasma as complexes of folate binding protein and 5-methyl-THF (46, 47). Folate binding protein is a soluble, desialylated version of the membrane-associated, glycosylphosphatidylinositol-anchored folate receptor, and like other asialoglycoproteins, is believed to enter cells by receptor-mediated endocytosis through coated pits (48). After 5-methyl-THF enters hepatocytes, it transits to the cytoplasm from the luminal space of membrane-bounded compartments, presumably late endosomes and lysosomes. Because these late endocytotic compartments are often localized very close to the Golgi complex, concentrating FTCD on the outer surface of the Golgi may enhance the rate of productive collisions between FTCD and 5-methyl-THF. An appealing feature of this model is that it does not exclude the possibility that a soluble pool of FTCD is also capable of modifying 5-methyl-THF.

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