Folylpoly-γ-glutamate Carboxypeptidase from Pig Jejunum

MOLECULAR CHARACTERIZATION AND RELATION TO GLUTAMATE CARBOXYPEPTIDASE II*

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Jejunal folylpoly-γ-glutamate carboxypeptidase hydrolyzes dietary folates prior to their intestinal absorption. The complete folylpoly-γ-glutamate carboxypeptidase cDNA was isolated from a pig jejunal cDNA library using an amplified homologous probe incorporating primer sequences from prostate-specific membrane antigen, a protein capable of folate hydrolysis. The cDNA encodes a 751-amino acid polypeptide homologous to prostate-specific membrane antigen and rat brain N-acetylated α-linked acidic dipeptidase. PC3 transfecant membranes exhibited activities of folylpoly-γ-carboxypeptidase and N-acetylated α-linked acidic dipeptidase, while immunoblots using monoclonal antibody to native folylpoly-γ-glutamate carboxypeptidase identified a glycoprotein at 120 kDa and a polypeptide at 84 kDa. The kinetics of native folylpoly-γ-carboxypeptidase were expressed in membranes of PC3 cells transfected with either pig folylpoly-γ-carboxypeptidase or human prostate-specific membrane antigen. Folylpoly-γ-carboxypeptidase transcripts were identified at 2.8 kilobase pairs in human and pig jejunum, human and rat brain, and human prostate cancer LNCaP cells. Thus, pig folylpoly-γ-carboxypeptidase, rat N-acetylated α-linked acidic dipeptidase, and human prostate-specific membrane antigen appear to represent varied expressions of the same gene in different species and tissues. The discovery of the jejunal folylpoly-γ-carboxypeptidase gene provides a framework for future studies on relationships among these proteins and on the molecular regulation of intestinal folate absorption.

Dietary folates, a heterogeneous mixture of folylpoly-γ-glutamates, are absorbed by a two-stage process of progressive hydrolysis at the jejunal brush border membrane followed by transport of monoglutamyl folate derivatives across the intestinal mucosa (1). Previously, our laboratory (2) purified folylpoly-γ-glutamate carboxypeptidase (FGCP)† from human jejunal brush-border membranes as a zinc-activated exopeptidase that releases terminal glutamates sequentially and is stable at pH greater than 6.5. We identified a separate intracellular lysosomal carboxypeptidase in human jejunal mucosa that cleaves folylpoly-γ-glutamates with an endopeptidase mode of action at a pH optimum of 4.5 and that is distinguished from membranous FGCP by its complete inhibition by p-hydroxymercuribenzoate (3). Subsequent experiments detected the two separate folate hydrolases in intracellular and brush-border membrane fractions of pig jejunal mucosa, each with properties identical to those found in human jejunum (4). A monoclonal antibody Mab-3 to the purified pig jejunal brush-border FGCP detected a 120-kDa subunit protein that was localized by immunoreactivity to the jejunal brush-border site of in vivo hydrolysis of folylpoly-γ-glutamates (5).

Attempts at molecular characterization of pig jejunal FGCP were facilitated by the recent and serendipitous descriptions of the molecular properties of two other proteins, human prostate-specific membrane antigen (PSM) and rat brain N-acetylated α-linked acidic dipeptidase (NAALADase). The cDNAs encoding these two proteins demonstrate 87% nucleotide and 85% amino acid sequence identity (6–8) and appear to be homologues of the same enzyme. Previously, we (8, 9) showed that PC3 cells transfected with either of these cDNAs exhibit N-acetylaspartylglutamate (NAAG)-hydrolyzing activity characteristic of NAALADase. Others found that PC3 cells transfected with the human PSM cDNA are capable of hydrolysis of folylpoly-γ-glutamate (10) with an exopeptidase activity mechanism similar to that previously described for human jejunal FGCP (2). The discovery that the hydrolysis of both NAAG and folylpoly-γ-glutamate can be attributed to the same molecule (PSM) led to the recommendation that human PSM and rat brain NAALADase be identified under a single IUBMB-approved name (11), subsequently designated glutamate carboxypeptidase II (GCP II; EC 3.4.17.21).

The goals of the present study were to characterize the molecular structure of pig jejunal FGCP while exploring its potential genetic and biological similarities to human PSM and rat NAALADase. We found extensive molecular homology and overlapping catalytic capabilities among pig FGCP, human PSM, and rat NAALADase, consistent with the concept that the three proteins represent varied expressions of the same gene in different species and tissues. The original discovery of the pig FGCP gene provides a molecular framework for future studies on the biological relationships among these proteins and on the integration of jejunal folate hydrolysis within the overall process of the intestinal absorption of dietary folates.

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§ The abbreviations used are: FGCP, folylpoly-γ-glutamate carboxypeptidase; NAALADase, N-acetylated α-linked acidic dipeptidase; PSM, prostate-specific membrane antigen; NAAG, N-acetylated aspartylglutamate; GCP II, glutamate carboxypeptidase II; I100, ileal 100-kDa protein; DPP IV, dipeptidyl peptidase IV; GH, glutamate hydrolase; RFC, reduced folate carrier protein; FBP, folate-binding protein; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; bp, base pairs; kb, kilobase pairs.

¶ This paper is available on line at http://www.jbc.org
Pig Jejunal Folypoly-γ-glutamate Carboxypeptidase

**EXPERIMENTAL PROCEDURES**

**Reagents**—The SuperScript preamplification system was purchased from Life Technologies, Inc. Tag DNA polymerase was purchased from Sigma. [α-32P]dCTP (3000 mCi/mmole) and [α-32P]dATP (1000 mCi/mmole) were purchased from Amersham Pharmacia Biotech. A cDNA probe for human actin was obtained from CLONTech (Palo Alto, CA). N-Acetylasparyl-[3,4,5-H]glutamate (41.5 Ci/mmole) and α-[32P]dATP (6000 Ci/mmol) were obtained from New England Nuclear. Peptide nucleic acids (PNA) were purchased from Oligo-2 (Carlsbad, CA) (10) and was used for custom construction of a pig jejunal cDNA library.

**Animal and Human Tissues**—Fresh jejunal and ileal mucosal scrapings were obtained from market pigs within 5 min of killing at the University of California (Davis, CA) slaughterhouse and were immediately washed in ice-cold saline, then frozen in liquid nitrogen, and stored at −70 °C. They were then used for the preparation of brush-border membranes that were purified >20-fold according to appropriate marker enzymes and our previously described procedure (5). For subsequent RNA and poly(A+) RNA preparations, portions of pig liver; renal cortex; and duodenal, jejunal, and ileal mucosa were frozen in liquid nitrogen and stored at −70 °C. Human jejunal segments of ~2-cm length were obtained from operating room patients following elective gastric bypass surgery with gastrectojunal anastomosis, according to acceptable use exemption from the University of California Davis Human Subjects Committee. Segments were opened longitudinally and were washed immediately in ice-cold 4 m guanidium thiocyanate prior to freezing in liquid nitrogen and storage at −70 °C.

**Cell Lines**—Tumor cell lines were obtained from the American Type Culture Collection (Rockville, MD). Cells were grown in MEM supplemented with 2% glutamine, 10% fetal bovine serum, 50 units/ml penicillin G, and 50 µg/ml streptomycin; LNCaP cells were cultured in RPMI supplemented with nonessential amino acids, 5% fetal bovine serum, 50 units/ml penicillin G, and 50 µg/ml streptomycin. All media reagents were obtained from Life Technologies.

**Peptide Microsequencing**—As described previously, FCGP was purified from pig jejunal brush-border membranes, and the major subunit protein was identified at 120 kDa by denaturing 6% polyacrylamide gel electrophoresis and immunoblot with Mab-3 monoclonal antibody (5). A parallel gel was stained with Coomassie Blue, and the single 120-kDa band was electroeluted using the Amicon Centrulut system (12). A peptide digest was prepared by overnight incubation of the eluate with a 50-fold molar excess of cyanogen bromide in 70% formic acid. The peptide digest was prepared by overnight incubation of the eluate with a 50-fold molar excess of cyanogen bromide in 70% formic acid. The peptide digest was lyophilized and then blotted to ProBlott membranes (Applied Biosystems, Foster City, CA). A 50-fold molar excess of cyanogen bromide in 70% formic acid. The peptide digest was prepared by overnight incubation of the eluate with a 50-fold molar excess of cyanogen bromide in 70% formic acid. The peptide digest was lyophilized and then blotted to ProBlott membranes (Applied Biosystems, Foster City, CA). Peptide sequencing followed the Edman reaction, and amino acids were identified by high performance liquid chromatography (12).

**Two peptide sequences contained the sequences** KIILARYGKSI and LTKELK, respectively (10). The corresponding PSM nucleotide sequences encoding KIILARYGKSI and LTKELK in the amino acid sequence of human PSM, which were obtained from Oxford Glyco Sciences (Bedford, MA). All other reagents were obtained from Sigma, Fisher, and various other commercial sources.

**Preparation and Expression of the Closed Enzyme**—A construct of the cDNA of FCGP was prepared by HindIII and XbaI excision from the mammalian expression vector pcDNA3 (Invitrogen). One hundred-mm dishes of PC3 cells were transfected with 25 µg of supercoiled plasmid DNA containing the cDNA of pig FCGP or human PSM (construct PSMa2) (9) using the calcium phosphate-mediated method in 50 µl Hepes buffer, pH 7.05 (19). Mock transfected PC3 cells served as controls. Cells were harvested 72 h post-transfection for enzymatic assays by scraping them into 50 ml Tris-HCl buffer (pH 7.4 at 37 °C). Membranes were prepared from the transfected and control PC3 cells by brief sonication followed by centrifugation (35,000 × g) for 30 min. The membrane pellets were then solubilized by sonication into 50 ml Triton X-100. The protein concentration of the solubilized membrane was determined using the enhanced protocol BCA assay (Pierce) or Bio-Rad kit.

**Enzyme Activities**—The hydrolysis of NAAG was measured in purified pig jejunal and ileal brush-border membranes and in transfected and mock transfected PC3 cell membranes by radiolabeled NAAG and measured in purified pig jejunal and ileal brush-border membranes and in transfected and mock transfected PC3 cell membranes by radiolabeled NAAG and hydrolysate. The hydrolysis of NAAG was measured in purified pig jejunal and ileal brush-border membranes and in transfected and mock transfected PC3 cell membranes by radioenzymatic assay, whereby hydrolysis was quantitated via scintillation spectrometry of [3H]glutamate produced from radiolabeled substrate after separation of substrate and product by ion exchange chromatography (20). Assays were initiated by the addition of labeled NAAG at a concentration of 2.5 nM.

Folate hydrolysis was measured in membranes from PSM and FCGP transfected and mock transfected PC3 cells using substrate folyly-Glu-Glu-γ-[14C]Glu and a modification (5) of the method of Krumdieck and Baugh (21) in which terminal [14C]Glu is counted in a liquid scintillation counter after charcoal precipitation of unreacted substrate. Duplicate reactions used 12 µl substrate in 50 µl 3,5-dimethylglutarate buffer containing 0.1 µM ZnCl2 and 0.1 µM DTT. Initial rate dependence and the inhibitory effect of 0.5 mM p-hydroxymercuribenzoate in membranes from each cell preparation. Subsequently, kinetic properties were compared in membranes from purified pig jejunal brush borders and from FCGP and PSM transfected by measurements over a range of substrate concentrations at pH 6.5.

**Immunoblot**—Membranes from the PC3 cells that were transfected with the cDNA of either human PSM or pig FCGP or that were mock transfected were solubilized in 0.1% Triton X-100. Membrane proteins from the FCGP transfected were deglycosylated under denaturing conditions using peptide-N-glycosidase F according to the manufacturer's protocol. Solubilized membrane proteins and a sample of purified native pig jejunal brush-border FCGP (5) were electrophoresed in parallel on 8% SDS-polyacrylamide gels (22), followed by transfer to polyvinylidene difluoride membranes (Millipore Corp., Marlborough, MA). Protein bands were identified using the monoclonal antibody Mab-3 to native pig jejunal brush-border membranes and to localize FCGP in pig intestine immunohistochemically (5).

**Northern Blots**—Total RNA was extracted from rat brain, LNCaP cells, and pig and human jejunal mucosa (13). Poly(A)+ RNA was prepared from pig liver and kidney and duodenal, jejunal, and ileal mucosa (16). Human brain poly(A)+ RNA was obtained from CLONTECH Inc. (Palo Alto, CA). A 2.4-kb EagI–NdeI fragment of FCGP was
FIG. 1. Nucleotide and amino acid sequences of pig FGCP. Amino acid sequences that correspond with 100 and 83% identity to two peptide sequences from native pig jejunal brush-border FGCP are shown in boldface type. There are 146 bp in the 5'-untranslated region, 2253 bp translating to 751 amino acids in the open reading frame, and 133 bp in the 3'-end. The putative transmembrane domain (11) is underlined, and the 3'-terminal polyadenylation signal is double underlined. Flanking residues Arg 16 and Arg 17 are conserved at the N-terminal side of the leucine-rich hydrophobic transmembrane domain between residues 20 and 43. The putative catalytic domain (11) is composed of residues 275–588. There are 12 glycosylation sites (*stars*), of which 10 are conserved in the human PSM sequence (6) and 9 are conserved in the rat NAALADase sequence (7, 8). Zinc-binding residues are conserved at His 378, Asp 388, Glu 426, Asp 454, and His 554 (*closed circles*). Four positively charged residues predicted to be involved in substrate binding are conserved at Arg 464, Lys 501, Arg 537, and Lys 546 (*open triangles*).
purified and 32P-labeled for subsequent probing of Northern blots. Pig tissue samples were also probed with a 32P-labeled fragment of human actin cDNA as a positive internal control. After electrophoretic separation in 1.2% agarose, 2.2 M formaldehyde gels and transfer to nylon membranes (Schleicher & Schuell), RNA species were identified by hybridization to cDNA probes as detected autoradiographically (23).

RESULTS

Molecular Sequence of Pig Jejunal FGCP—The complete nucleotide and deduced amino acid sequences of the cDNA of pig FGCP are shown in Fig. 1. The deduced amino acid sequences KILIARYGKIP and MYSLVYNLTKELQ correspond with 100 and 85% identities to two amino acid sequences, KILIARYGKIP and MYILVYLTKELQ, that were identified in the peptide digest of the native purified enzyme. The complete cDNA of FGCP is composed of 2532 bases: 146 in the 5‘-untranslated region, 2253 in the open reading frame that encodes 751 amino acids, and 133 in the 3‘-untranslated region. The nucleotide and deduced amino acid sequences of pig FGCP were compared with those of human PSM (6) and rat NAALADase (7, 8). Within the open reading frame, the nucleotide identities between pig FGCP and human PSM and rat brain NAALADase were 88 and 83% respectively, while there was very little similarity in the 5‘-untranslated region. The amino acid sequence of pig FGCP was 92% similar and 91% identical to that of human PSM and was 87% similar and 85% identical to that of rat NAALADase (Table I). Structural comparisons followed the recent Rawlings and Barrett analysis of human PSM, and NAALADase (24) of pig jejunal FGCP was identical to those of rat ileal I100 (29), and human dipetidyl peptidase IV (30). All five putative catalytic zinc binding residues (11) were conserved between pig jejunal FGCP and rat ileal I100 at His397, Asp358, Glu426, Asp454, and His554, while only one zinc binding residue at Glu426 was conserved in dipetidyl peptidase IV. Among the putative substrate binding basic amino acids (11) that were conserved in FGCP, PSM, and NAALADase, only Arg464 was conserved in I100, and only Arg537 was conserved in dipetidyl peptidase IV. Several amino acids typical of a serine carboxypeptidase mechanism (29) were conserved further downstream, including Ser252 in all three proteins and Asp287 and His300 in FGCP and I100. Structural similarities between FGCP and selected other proteins relevant to folate hydrolysis and transport were also investigated. Human glutamate hydrolase (an intracellular peptidase capable of folylpoly-g-glutamate hydrolysis (31)) and two proteins involved in the transport of monoglutamyl folates (the mouse reduced folate carrier protein (RFC) (32) and pig folate-binding protein (FBP) (33)) showed only weak similarities to short regions at the N- or C-terminal ends outside of the catalytic region of FGCP.

Enzyme Activities—As depicted in Fig. 2, NAALADase-specific activity was 16-fold greater in pig jejunal brush-border membranes than in ileal brush-border membranes. NAALADase was abundant in membranes from PC3 cells transfected with the cDNA of pig jejunal FGCP but was absent from control PC3 cells. Previously characterized inhibitors (9, 20) nearly eliminated NAALADase activity in jejunal brush-border membranes and in FGCP-transfectant membranes but had minimal effect on NAALADase activity in ileal brush-border membranes. As depicted in Fig. 3 (left panel), FGCP activity in PC3 transfectant membranes was maximal at pH 6.5 and was not inhibited by the addition of p-hydroxymercuribenzoate to the reaction mixture. FGCP activity with an identical pH profile and lack of p-hydroxymercuribenzoate inhibition was found in PC3 cells transfected with the cDNA of PSM (not shown). By contrast, folate hydrolysis was much less in membranes of

| Protein       | Reference | GenBank™ accession No. | FGCP region | Similarity | Identity |
|---------------|-----------|------------------------|-------------|------------|----------|
| Human PSM     | 6         | M99487                 | 1–751       | 92         | 91       |
| Rat NAALADase | 7         | U75973                 | 1–751       | 88         | 83       |
| Rat NAALADase | 8         | AF040256               | 1–751       | 88         | 83       |
| Human transferrin receptor | 26      | M11507                 | 9–747       | 44         | 31       |
| V. proteolyticus | 27      | S24314                 | 180–647     | 43         | 33       |
| S. griseus    | 28        | S66427                 | 357–555     | 45         | 36       |
| Rat I100      | 29        | AF009921               | 20–750      | 50         | 41       |
| Human DPP IV  | 30        | M80536                 | 259–711     | 41         | 29       |
| Human GH      | 31        | U55206                 | 521–706     | 42         | 28       |
| Mouse RFC     | 32        | L23755                 | 507–708     | 35         | 27       |
| Pig FBP       | 33        | U89949                 | 4–182       | 38         | 29       |

Homologies with Other Relevant Proteins—The BESTFIT computer program was used to analyze regional amino acid sequence homologies between pig FGCP and selected structurally and functionally related proteins (Table I). In addition to extensive sequence similarities and identities among FGCP, PSM, and NAALADase, FGCP exhibited similarities with three other M28 family members: human transferrin receptor (26) and aminopeptidases from Vibrio proteolyticus (27) and Streptomyces griseus (28). Rat I100, a recently characterized ileal peptidase with type II structure (29), also shares extensive amino acid similarity with FGCP, whereas there was less sequence similarity between FGCP and human dipetidyl peptidase IV, an enzyme that appears to be functionally related to I100 (30). The PILEUP program was used to clarify amino acid alignments within the putative catalytic regions of FGCP, rat ileal I100 (29), and human dipetidyl peptidase IV (30). All five putative catalytic zinc binding residues (11) were conserved between pig jejunal FGCP and rat ileal I100 at His378, Asp388, Glu426, Asp454, and His554, while only one zinc binding residue at Glu426 was conserved in dipetidyl peptidase IV. Among the putative substrate binding basic amino acids (11) that were conserved in FGCP, PSM, and NAALADase, only Arg464 was conserved in I100, and only Arg537 was conserved in dipetidyl peptidase IV. Several amino acids typical of a serine carboxypeptidase mechanism (29) were conserved further downstream, including Ser252 in all three proteins and Asp287 and His300 in FGCP and I100. Structural similarities between FGCP and selected other proteins relevant to folate hydrolysis and transport were also investigated. Human glutamate hydrolase (an intracellular peptidase capable of folylpoly-g-glutamate hydrolysis (31)) and two proteins involved in the transport of monoglutamyl folates (the mouse reduced folate carrier protein (RFC) (32) and pig folate-binding protein (FBP) (33)) showed only weak similarities to short regions at the N- or C-terminal ends outside of the catalytic region of FGCP.
mock transfected PC3 cells and exhibited a different optimal pH 4.5 with complete inhibition by p-hydroxymercuribenzoate.

The kinetic characteristics of FGCP activity were compared in membranes from FGCP and PSM transfectants and in purified pig jejunal brush borders. As shown in Fig. 3 (right panel) and summarized in Table II, \( K_m \) and \( V_{\text{max}} \) values were similar in all three samples and were consistent with the kinetic profile of purified pig jejunal brush-border FGCP (4).

**Immunoblots**—Fig. 4 compares the immunoreactivities of the monoclonal antibody Mab-3 (5) with purified native pig FGCP, with pig FGCP transfectant membranes before and after treatment with peptide-N-deglycosidase F, and with human PSM transfected membranes. Mab-3 detected the native pig FGCP and the pig FGCP transfectant glycoprotein at the identical size of 120 kDa and detected the deglycosylated polypeptide at 84 kDa but did not react with the human PSM transfected membranes or with mock transfected control membranes.

**Northern Blots**—The cDNA of pig FGCP showed a strong hybridization signal at 2.8 kb in pig duodenum and jejunum and a faint signal in pig kidney, while no signal was detected in pig liver or ileum (Fig. 5). A band of similar size was identified in RNA extracts from pig and human jejunal mucosa. A positive actin signal was present in all samples. Several bands of hybridization appeared in RNA samples from rat and human brain and the LNCaP prostate carcinoma cell line (Fig. 6). Bands of roughly equal intensity were observed in rat brain at approximately 3.9, 2.95, and 2.85 kDa, while a predominant species of 2.8 kb was found in human brain and in the human LNCaP prostate cancer cell line.

**DISCUSSION**

The present study has achieved the original molecular characterization of FGCP from pig jejunal mucosa. The authenticity of the pig FGCP cDNA sequence and its specific functional expression was established by (a) the incorporation of two native peptide sequences into the deduced amino acid sequence (Fig. 1), (b) the reproduction of the activity profile and kinetics of native pig FGCP (2, 4) in FGCP transfectant membranes (Fig. 3), (c) the immunoblot identification of the FGCP transcript by monoclonal antibody to native pig FGCP at the identical 120-kDa molecular size of the purified native enzyme (Ref. 5; Fig. 4) and identification of the deglycosylated polypeptide at the 84-kDa molecular size predicted by the amino acid sequence (Fig. 1), and (d) the identification of FGCP transcripts at 2.8 kb in pig jejunal mucosa and their absence in pig ileal mucosa (Fig. 5), consistent with the established intestinal distribution of the activity and immunoreactivity of the native enzyme (5). The additional presence of similar FGCP transcripts in pig and human jejunal mucosa (Fig. 5) suggests that the same gene expresses FGCP in human and pig jejunal brush-border membranes (2, 5).

The present experiments complete a circle of evidence for extensive molecular homologies among pig FGCP, human PSM, and rat NAALADase. The findings of 83–91% amino acid sequence identities between pig FGCP and each of the other sequences (Fig. 1; Table I) is in keeping with prior reports on the extensive amino acid identities between human PSM and rat NAALADase (6–9, 11) and is consistent with the concept that all three proteins represent species-specific homologues of the same gene. While the amino acid sequence of each protein predicts a polypeptide molecular size of 84 kDa (Fig. 1; Refs. 6–8), the presence of 12 glycosylation sites accounts for the greater 120-kDa molecular size of native (5) or transfectant FGCP (Fig. 4) compared with the reported molecular sizes of 100 kDa for PSM with 10 glycosylation sites (6) and of 94 kDa for NAALADase with nine glycosylation sites (7, 8, 34). While the epitope for our monoclonal antibody to native pig FGCP is unknown, incomplete amino acid sequence identities and differences in glycosylation between pig FGCP and human PSM could account for the lack of antibody cross-reactivity with PSM in transfected membranes (Fig. 4). Prior findings of NAALADase transcripts at 2.8 kb in rat kidney (7, 8) are extended by the detection of a weak FGCP hybridization signal at 2.8 kb in pig kidney poly(A') RNA (Fig. 5), while the prior findings of PSM-like transcripts and immunoreactivity in human small intestine (35–37) are complemented by the detection of the FGCP hybridization signal at 2.8 kb in pig duodenal and jejunal poly(A') RNA and in human jejunal RNA (Fig. 5). The tissue distribution and predominant size of FGCP-like transcripts in rat and human brain and LNCaP cells (Fig. 6) is similar to other descriptions of the distribution and sizes of PSM and NAALADase transcripts in these tissues (6–9, 38). The previous finding of NAALADase activity in membranes of LNCaP cells and PSM transfectants (9) is complemented by finding NAALADase activity in pig jejunal brush-border membranes and in FGCP transfectant membranes (Fig. 2).
The extensive amino acid identities, common structural motifs, member of the M28 family of peptidases (11) (EC 3.4.17.21). brain NAALADase as GCP II, a single type II glycoprotein PSM transfectant membranes (Fig. 3; Table II).

catalytic kinetic properties of purified native FGCP in FGCP or tamates (10) is confirmed and extended by finding nearly iden-

tical protein bands were absent from membranes of PSM transfectants was identical to that of FGCP transfectants (not shown). FGCP activity was optimal in FGCP transfectant membranes at pH 6.0 

A recent analysis classified human prostate PSM and rat GCP II gene (Fig. 1). GCP II and two prototypical bacterial aminopeptidases V. proteolyticus (27) and S. griseus (28) are members of the M28 peptidase family by virtue of homologous catalytic domains, which appear to bind two co-catalytic zinc

observation that membranes of LNCaP cells or PSM transfectants were capable of progressive hydrolysis of folypoly-

FIG. 3. Folate hydrolysis by membranes from native pig jejunal brush borders, mock transfected PC3 cells, and PC3 cells transfected with the cDNA of FGCP or PSM. Reaction mixtures consisted of 12 μM substrate folyl-γ-Glu-γ-[14C]Glu in 33 mM 3,3-dimethylglutarate buffer containing 0.1 mM zinc acetate and 0.67 mM NaCl in the final concentration. Left panel, Effect of varied buffer pH on folate hydrolysis by membranes from mock transfected and FGCP-transfected PC3 cells. FGCP activity was optimal in FGCP transfectant membranes at pH 6.0 (closed circles), in contrast to lesser folate hydrolysis in mock transfected PC3 cell membranes at optimal pH 4.0 (closed boxes). The addition of 0.5 mM ρ-hydroxymercuribenzoate in the final concentration had no effect on FGCP activity in FGCP transfectant membranes (open circles) but resulted in complete inhibition of folate hydrolysis in control PC3 cell membranes (open boxes). The FGCP activity profile of membranes of PSM transfectants was identical to that of FGCP transfectants (not shown). Right panel, kinetics of FGCP activity in membranes from pig jejunal brush borders and PC3 cells transfected with the cDNA of FGCP or PSM. Lineweaver-Burk plots of kinetics at pH 6.5 over a range of folyl-

Left panel

FGCP kinetics in native pig and transflectant cell membranes

A summary of activity constants (K_m) and maximal activities (V_max) of FGCP in membranes from purified pig jejunal brush borders, PC3 cells transfected with the cDNA of FGCP or PSM, and previously reported purified native pig jejunal FGCP (4). Kinetic data were obtained from studies that used a range of concentrations of substrate folyl-γ-Glu-γ-[14C]Glu at pH 6.5 and conditions as described under “Experimental Procedures,” followed by Lineweaver-Burk analysis of the results as shown in Fig. 5.

Table II

| Source                        | K_m (μM) | V_max (nmol · mg⁻¹ · min⁻¹) |
|-------------------------------|----------|----------------------------|
| Pig jejunal brush border membrane | 3.9      | 338                        |
| FGCP transflectant membrane     | 5.8      | 858                        |
| PSM transflectant membrane      | 1.4      | 152                        |
| Purified pig jejunal FGCP (4)   | 1.7      | 540                        |

FIG. 4. Immunoblots showing the reaction of monoclonal antibody to native pig FGCP (5) to transflectant membrane proteins. Seven μg of solubilized membrane protein was added to each lane. An identical protein band was identified at 120 kDa in purified native pig FGCP (lane 1) and in membranes from the FGCP transfectant (lane 2), while the deglycosylated FGCP polypeptide appeared at 84 kDa (lane 3). Protein bands were absent from membranes of PSM transfectants (lane 4) and mock transfected PC3 cells (lane 5).

FIG. 5. Northern hybridization of 32P-labeled pig FGCP cDNA and human β-actin to pig and human tissues. Left panel, a band of hybridization at 2.8 kb was prominent in poly(A⁺) RNA from pig duodenal and jejunal mucosa (lanes 3 and 4), present in kidney (lane 2), and absent from liver (lane 1) and ileal mucosa (lane 5). Right panel, bands of hybridization of similar intensities were found at 2.8 kb in total RNA from pig (lane 1) and human jejunal mucosa (lane 2). Control hybridization to actin is shown at 2.0 kb.

FIG. 6. Northern hybridization of 32P-labeled pig FGCP cDNA to brain and prostate RNAs. Samples contained different amounts of total RNA in rat brain (10 mg) and LNCaP cells (5 mg) and poly(A⁺) RNA in human brain (2 mg). A longer exposure was required to develop the signal from rat brain. Bands of hybridization were observed in rat brain RNA at 3.9, 2.95, and 2.8 kb (lane 1). A predominant hybridization signal appeared at 2.8 kb in LNCaP cell RNA (lane 2) and in human brain poly(A⁺) RNA (lane 3).
Our original studies identified an initial stage of jejunal hydrolase (EC 3.4.19.9) (Table I; Ref. 31).

These relationships prompted our evaluation of potential structural similarities among FGCP, I100, and dipeptidyl peptidase IV. The conservation of all five zinc-binding residues suggests that FGCP and I100 share the same catalytic mechanism. On the other hand, an alternative potential serine carboxypeptidase mechanism (29) is suggested by conservation of Ser^312 in all three sequences.

While pig FGCP, rat NAALADase, and human PSM may represent different species-specific expressions of same GCP II gene, their functions appear to differ according to the tissue in which the gene is expressed. Thus, GCP II may function as FGCP in the jejunum by cleaving γ-linked glutamyl residues sequentially from dietary folypoly-γ-glutamates prior to the intestinal transport of folic acid (1, 2, 4, 5) and as NAALADase in the brain to release α-linked glutamate from NAAG to regulate subsequent neurotransmission (8, 9). These different functions may reflect tissue differences in available substrate, since NAAG is concentrated at neuronal synapses (8), while folypoly-γ-glutamates are concentrated as dietary components at the brush-border surface of the proximal small intestine (1).

The present study offers molecular clarity to the mechanism of folate absorption at the intestinal brush-border membrane. Our original studies identified an initial stage of jejunal hydrolysis of dietary folypoly-γ-glutamates that precedes the intestinal uptake of the folic acid product (1). We identified and characterized FGCP as a zinc-dependent exopeptidase that is active at a neutral pH optimum in human and pig jejunal brush-border membrane fractions (2, 4) and that was localized to the ileal brush-border membrane by the monclonal antibody Mab-3 to the purified enzyme (5). These observations are extended by the present molecular characterization of FGCP as a type II protein of the M28 peptidase family with a zinc-binding motif, for which the transcripts are expressed in proximal but not distal pig small intestine (Fig. 5). The finding of a different activity profile of folate hydrolysis by mock transfected PC3 cells including an acid pH optimum and complete p-hydroxymercuribenzoate inhibition (Fig. 3) is consistent with our prior definition of the characteristics of a separate lysosomal endopeptidase that provides intracellular folate hydrolysis in human and pig jejunal mucosa (3, 4). The recently described PSM' splice variant (42) cannot provide the separate profile of folate hydrolysis found in mock transfected PC3 cells (Fig. 3), since no genetically similar species is expressed in native PC3 cells (6, 9). Alternatively, the second folate hydrolizing activity in mock translated PC3 cell membranes (Fig. 3) and in the lysosomal fraction of jejunal mucosa (3) may be attributed to the recently described and genetically dissimilar glutamate hydrolase (EC 3.4.19.9) (Table I; Ref. 31).

The present studies provide a molecular framework for future studies on the regulation of FGCP by conditions known to affect intestinal folate absorption and on the relationship of FGCP to RFC and FBP, two proteins involved in membrane transport of monoglutamyl folates (Table 1). The cDNA sequence of mouse and human RFC have been defined, and its intestinal transcription and functional capability for transport of monoglutamyl folate in cell transfectants has been proven (32, 43, 44). The alternate receptor FBP has been characterized at the molecular level in pig liver, but its transcripts and activity are absent from the jejunum (33).2 The present study shows that FGCP is genetically distinct from both RFC and FBP, since their amino acid sequences are minimally represented in FGCP (Table I). In summary, the available data indicate that the intestinal absorption of dietary folypoly-γ-glutamates is achieved by a two-step process of progressive hydrolysis of γ-linked glutamyl residues by FGCP at the jejunal brush-border membrane, releasing folic acid and other monoglutamyl folate derivatives for subsequent membrane transport by genetically distinct RFC. The integration of folate hydrolysis by jejunal FGCP and folic acid transport by intestinal RFC in the overall process of folate absorption has yet to be defined. These studies are now feasible due to the molecular identification of FGCP.

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