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The membrane-bound basic carboxypeptidase from hog intestinal mucosa[1]

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Abstract

The carboxypeptidase activity occurring in hog intestinal mucosa is apparently due to two distinct enzymes which may be responsible for the release of basic COOH-terminal amino acids from short peptides. The plasma membrane-bound carboxypeptidase activity which occurs at neutral optimum pH levels was found to be enhanced by $CoCl_2$ and inhibited by guanidinoethylmercaptosuccinic acid, *o*-phenanthroline, ethylenediamine tetraacetic acid and cadmium acetate; whereas the soluble carboxypeptidase activity which occurs at an optimum pH level of 5.0 was not activated by $CoCl_2$ and only slightly inhibited by *o*-phenanthroline, ethylenediamine tetraacetic acid, $NiCl_2$ and $CdCl_2$. The latter activity was presumably due to lysosomal cathepsin B, which is known to be present in the soluble fraction of hog intestinal mucosa. Although the membrane-bound enzyme was evenly distributed along the small intestine, it was not anchored in the phospholipidic bilayer via a glycosyl-phosphatidylinositol moiety, as carboxypeptidase M from human placenta is. The enzyme was not solubilized by phosphatidylinositol-specific phospholipase C, but was solubilized to practically the same extent by several detergents. The purified trypsin-solubilized form is a glycoprotein with a molecular mass of 200 kDa, as determined by performing SDS–PAGE and gel filtration, which differs considerably from the molecular mass of human placental carboxypeptidase M (62 kDa). It was found to cleave lysyl bonds more rapidly than arginyl bonds, which is not so in the case of carboxypeptidase M, and immunoblotting analysis provided further evidence that hog intestinal and human placental membrane-bound carboxypeptidases do not bear much resemblance to each other. Since the latter enzyme has been called carboxypeptidase M, it is suggested that the former might be carboxypeptidase D, the recently described new member of the carboxypeptide B-type family.

Abbreviations: AMC, 4-methyl-7-coumarylamide; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulfonate; CMC, critical micellar concentration; CP, carboxypeptidase; DTT, dithiothreitol; EDTA, ethylenediamine tetraacetic acid; FPLC, fast protein liquid chromatography; GEMSA, guanidinoethylmercaptosuccinic acid; GPI, glycosyl-phosphatidylinositol; HEPES, 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid; HPLC, high-performance liquid chromatography; MDCK, Madin Darby canine kidney; NADPH, nicotinamide adenine dinucleotide phosphate; octyl-glucoside, *n*-octyl-β-D-glucopyranoside; PAGE, polyacrylamide gel electrophoresis; PCMS, *p*-chloromercuriphenyl sulfonate; PI-PLC, phosphatidylinositol-specific phospholipase C; PITC, phenylisothiocyanate; PMSF, phenylmethylsulfonyl fluoride; PTC, phenylthiocarbamyl; PVDF, polyvinylidene difluoride; RNA, ribonucleic acid; SDS, sodium dodecyl sulfate; TFA, trifluoroacetic acid; TLCK, N^{α}-*p*-tosyl-L-lysine chloromethylketone; Tris, tris(hydroxymethyl)aminomethane; Z, benzyloxycarbonyl

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[1] Enzymes: carboxypeptidase B (EC 3.4.17.2); carboxypeptidase D (EC 3.4.17.-); carboxypeptidase H (EC 3.4.17.10); carboxypeptidase M (EC 3.4.17.12); carboxypeptidase N (EC 3.4.17.3).

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1. Introduction

Basic carboxypeptidases are metallo-enzymes which cleave COOH-terminal arginine and lysine from peptides and proteins. They occur widely among mammals, and the secretory pancreatic carboxypeptidase B (CPB) is generally taken to be the most representative member of this class of enzymes [1]. Although pancreatic CPB is responsible for the intestinal digestion of food proteins and peptides, the other basic carboxypeptidases are not digestive enzymes but have regulatory effects on the activity of biological peptides such as anaphylatoxins, enkephalins and bradykinins [1]. Since all the mammalian basic carboxypeptidases have similar enzymatic activities, they may play different roles in vivo, depending on their localization and physical properties.

Human plasma CPN (which is also known as kininase I) is synthesized in the liver and released into the bloodstream in the form of a large tetrameric complex (280 kDa) containing two active subunits (48–55 kDa) and two inactive glycosylated subunits (83 kDa), the latter of which stabilize the active ones and keep them in the solubilized form [2,3]. Since CPN has mainly been found in circulating blood, its most likely function seems to be to protect the organism from the action of potent vasoactive and inflammatory peptides such as kinins and/or anaphylatoxins [1,4]. A plasma pro-CPB of hepatic origin which is structurally similar to pancreatic pro-CPB has recently been identified as a circulating protein, isolated from the blood, and found to form a non-covalent association with other plasma proteins [5,6]. Unlike pancreatic CPB, which has a purely digestive function, plasma CPB might be involved in fibrinolysis and has been defined as a thrombin activatable fibrinolysis inhibitor, or TAFI [7,8].

CPH, which is also known as CPE or enkephalin convertase, is located in the secretory granules of pancreatic islets, as well as in the adrenal and pituitary glands and brain [9–13], where it is present in both the soluble and membrane-bound forms [14,15]. This enzyme differs from the other basic carboxypeptidases in that it has an acidic optimum pH, which is consistent with the acidic interior of the granules [16,17]. CPH is probably involved in the removal of residual COOH-terminal arginine or lysine residues from peptidic prohormones during their processing in secretory granules [1,18]. Another recently discovered enzyme, CPD, which has similar characteristics but a different molecular mass, may also be involved in the processing of the peptides and proteins that transit via the secretory pathway [19,20].

CPM is a widely distributed membrane-bound basic carboxypeptidase which has been purified to homogeneity, cloned and sequenced from human placenta [21,22], where it is attached to the plasma membrane via a phosphatidylinositol glycan tail [23]. In view of its presence in plasma membranes and the fact that its optimal activity occurs at neutral pH levels, CPM might well participate in the local control of peptide hormone activity. Its proximity to plasma membrane receptors suggests that it may either inactivate or affect in some way the receptor specificity of peptide hormones released at local tissue sites [1].

A novel cDNA encoding CPZ has recently been identified based on its homology with known metallocarboxypeptidases [24]. CPZ was expressed in the baculovirus system and found to be more active at neutral pH levels than at pH 5.5 toward substrates with COOH-terminal basic amino acids [24]. Lastly, AEBP1 was identified as a transcription repressor showing some homologies with metallocarboxypeptidases. Although it lacks the residues on which carboxypeptidase activity is thought to depend, this enzymatic protein has been reported to cleave peptides containing a COOH-terminal arginine residue [25].

The increasing number of data becoming available nowadays on biologically active peptides has led to considerable attention being paid to mammalian basic carboxypeptidases. Little is known so far, however, about these non-digestive basic carboxypeptidases located in the intestinal tract, although it has been reported that most of the CPH activity in the rat intestine is membrane-bound, based on the finding that it is stimulated by $CoCl_2$ and inhibited by low concentrations of guanidinoethylmercaptosuccinic acid (GEMSA) at low pH levels [26]. CPM

has the same properties as CPH [27], and the presence of both enzymes has been briefly described in the dog small intestinal wall [28], but no further characterization has been performed. Here, we describe the purification procedure used and give some of the characteristics of the membrane-bound basic carboxypeptidase from hog intestine mucosa, an enzyme which definitely differs from the other known carboxypeptidases but which is similar in some respects to the newly described CPD, the mammalian homologous form of duck gp180 protein, a 180 kDa hepatitis B virus-binding glycoprotein.

2. Materials and methods

2.1. Materials

Porcine intestines were obtained from the Marseilles slaughterhouse. Hippuryl-L-arginine, trypsin, papain, subtilisin, as well as all the activators, inhibitors and detergents used here were purchased from Sigma Chemicals (St. Louis, MO, USA). Phospholipase from *Bacillus cereus* was from Boehringer (Mannheim, Germany). Arginine-Sepharose, phenyl-Sepharose, Sephacryl S200 HR were from Pharmacia Biotech (Uppsala, Sweden). The electrophoretic molecular mass markers were obtained from Bio-Rad Laboratories (Richmond, CA, USA). HCl, PITC and the amino acid analysis standards were purchased from Pierce (Rockford, IL, USA), while the polyvinylidene difluoride membrane was from Millipore (Bedford, MA, USA) and the nitrocellulose sheets (0.2 μm) from Schleicher and Schuell (Dassel, Germany). The rabbit polyclonal antiserum directed against human placental CPM and the IgG fraction of goat anti-rabbit serum conjugated with horseradish peroxidase were purchased from Tebu (Le Perray-en-Yvelines, France) and Sigma, respectively. Solvents (pure grade) were supplied by SDS (Peypin, France). All the other chemicals used were of reagent grade.

2.2. Enzyme and protein assays

All the enzyme activities were measured at 37°C. Basic carboxypeptidase (CP) activity was measured with hippuryl-L-Arg as the substrate. The activity was first quantified by performing an HPLC assay at 254 nm of the hippuric acid released. The enzyme was incubated with 1 mM hippuryl-L-Arg in a 0.1 M Tris–HCl buffer (pH 7.5) containing 0.14 M NaCl. The reaction was stopped by adding 20 μl of acetic acid to 200 μl of reaction mixture. After extraction with 200 μl of Cl_3CH, the aqueous phase was removed and filtered through Millipore filters (0.45 μm), and 15-μl samples were injected into the Vydac C18-RP column (0.4×25 cm, 5 μm) and separated at a flow rate of 1 ml/min on a 10-min linear gradient from 10% to 25% acetonitrile in 0.01% TFA containing H_2O. The basic CP activity was routinely determined spectrophotometrically during the purification steps with the same substrate, as previously described by Koheil and Forstner [29], and Skidgel and Erdös [30]. One unit of CP activity was defined as 1 μmol of hippuryl-L-Arg hydrolysed per minute.

The activity of aminopeptidase N and alkaline phosphatase, two microvillus membrane markers, was assayed as described by Louvard et al. [31], whereas (Na^+,K^+)-ATPase, which was used as the basolateral plasma membrane marker, was assayed using the method developed by Murer et al. [32]. The activity of NADPH-cytochrome *c* reductase, a microsomal contamination marker [33], was assayed as described by Sottocasa et al. [34], while cytochrome *c* oxidase, which was used as mitochondrial marker, was assayed using Cooperstein and Lazarow's method [35]. Acid phosphatase, a lysosome marker, was assayed according to Murer et al. [32], and cathepsin B activity was determined using the specific substrate Z-Arg-Arg-AMC [36] as described by Gabrijelcic et al. [37]. Proteins were determined as described by Bradford [38] with bovine serum albumin as the standard.

2.3. Preparation of mucosa and subcellular fractionation

Immediately after the hogs were killed, their small intestines were removed, washed with a 50 mM Tris–HCl buffer (pH 8.0) and placed at 4°C as soon as possible. The mucosa was scraped off with a microscope slide and kept at −20°C until being further processed. Crude brush-border membrane preparations were obtained as previously described by Schmitz et al. [39] and modified by Maury et al.

[40], with additional slight modifications. Hog intestinal mucosal scrapings (200 g) were homogenized in four times their mass of a 5 mM Tris–HCl buffer (pH 7.3) containing 0.25 M sucrose, 10 mM KCl and 1 mM $MgCl_2$ in a motor-driven Teflon–glass homogenizer, and filtered through a gauze. The resulting homogenate was submitted to differential centrifugation, and the final brush-border membrane-enriched pellet was resuspended in 100 ml of a 20 mM Tris–HCl buffer (pH 7.3) containing 0.14 M NaCl, 10 mM KCl and 1 mM $MgCl_2$. The other pellets were resuspended in 200 ml of the same buffer.

2.4. Effects of pH and chemicals

The pH–activity profiles were determined at 37°C with hippuryl-L-Arg as the substrate in the following two buffers: 0.1 M sodium acetate (pH 3.0 to 7.0) and 0.1 M Tris–HCl (pH 7.0 to 9.0) containing 0.14 M NaCl. The effects of a number of reagents on basic CP activities were investigated after a 15-min incubation period at 37°C in the buffers used for the enzyme activity determinations described above. The reaction was started by adding the hippuryl-L-Arg substrate and the relative activity was expressed as a percentage of the control value.

2.5. Distribution along the gastrointestinal tract

Small intestines, stomachs and colons were removed immediately after the hogs were killed and washed with a 50 mM Tris–HCl buffer (pH 8.0) and stored at 4°C. The intestines were further cut into 1-m pieces, their mucosa were scraped off with a microscope slide and crude brush-border membrane fractions were prepared as described above.

2.6. Solubilization with detergents, phospholipase C and proteases

Crude brush-border membrane preparations were washed five times with a 20 mM Tris–HCl buffer (pH 7.3) containing 2 M NaCl, 10 mM KCl and 1 mM $MgCl_2$. The final pellets were resuspended in the same buffer containing only 0.14 M NaCl, and the resulting suspensions (about 2 mg/ml of proteins) were incubated with various detergents under gentle shaking at 4°C for 60 min. Suspensions were then centrifuged at $105\,000 \times g$ for 30 min and the enzyme activities in the pellets and supernatants were measured. Solubilized activities were expressed as percentages of total activities.

Prior to the solubilization experiments with PI-PLC from *Bacillus cereus*, the final pellets were resuspended in a 0.1 M HEPES buffer (pH 8.0) containing 1 mM PMSF, 0.1 mM PCMS, 0.1 mM TLCK and 1 mM leupeptine in order to inhibit the action of any brush-border membrane proteases and proteases possibly contaminating the commercial PI-PLC preparation used. The resulting suspensions (about 2 mg/ml of proteins) were incubated at 37°C for 2 h with 1 U/ml of PI-PLC from *Bacillus cereus* (1 unit of PI-PLC cleaves 1 nmol of phosphatidylinositol per minute). We then proceeded as described in the case of detergents.

As regards the solubilization process used with proteases, the final pellets were resuspended in a 20 mM Tris–HCl buffer (pH 7.5) containing 0.25 M sucrose to obtain a 0.5 mg/ml protein solution. After the diluted membrane fractions had been incubated under gentle shaking at 4°C with increasing amounts of trypsin, papain and subtilisin, they were then processed as described in the case of detergents.

2.7. Purification of the soluble form of membrane-bound basic CP

Once the brush-border membranes had been incubated with 10% trypsin (w/w) at 4°C for 24 h, trypsin was inhibited with 4 mM PMSF. The suspension was then centrifuged at $105\,000 \times g$ for 30 min and the resulting supernatant concentrated and dialyzed against a 20 mM Tris–HCl buffer (pH 7.5) in an Amicon Concentrator with a PM-10 membrane. Solubilized CP was separated from the other proteins by performing chromatography using a Waters 650^E FPLC system, and 5-ml fractions were collected. The solubilized protein concentrate (about 50 ml) was first loaded on an arginine-Sepharose column (2.2×10 cm) pre-equilibrated with the dialysis buffer at a flow rate of 1 ml/min. After washing the column with the equilibration buffer, the proteins were eluted with a 90-min linear gradient from 0 to 0.15 M NaCl followed by a 30-min linear gradient from 0.15 to 0.5 M NaCl in the same buffer. The active fractions were pooled and dialyzed overnight against 5 l of a

20 mM Tris–HCl buffer (pH 7.5) containing 1 M $(NH_4)_2SO_4$. The dialyzed solution was then applied, at a flow rate of 1 ml/min, to a phenyl-Sepharose column (2.2×10 cm) pre-equilibrated with the dialysis buffer, which was subsequently washed with the equilibration buffer. The proteins were eluted with a 120-min linear gradient from 1 to 0 M $(NH_4)_2SO_4$ in the same buffer. The active fractions were pooled, dialyzed overnight against 10 l of a 20 mM Tris–HCl buffer (pH 7.5) containing 0.35 M NaCl and concentrated to 1 ml in an Amicon Concentrator equipped with a PM-10 membrane. The concentrate was further applied to a Sephacryl-S200 HR column (2.6×60 cm) pre-equilibrated with the dialysis buffer, at a flow rate of 1 ml/min. After eluting the active fractions with the same buffer, they were pooled, dialyzed overnight against 5 l of a 20 mM Tris–HCl buffer (pH 7.5) containing 0.14 M NaCl, and the protein material was concentrated to 2 ml in an Amicon Concentrator with a PM-10 membrane.

2.8. Polyacrylamide gel electrophoresis

SDS–PAGE was performed using Laemmli's method [41] with 10% slab gels. Native PAGE was also carried out in 10% slab gels as described by Gabriel [42]. Proteins were detected by performing Coomassie blue or silver staining, while glycosylated proteins were detected with Schiff reagent according to Zacharius et al. [43] with bovine serum albumin and fetuin from fetal calf serum as the standards.

2.9. Gel filtration on Sephacryl-S200 HR

The molecular mass of the native enzyme was estimated by performing gel filtration using a Sephacryl-S200 HR column (2.6×60 cm) under the same experimental conditions as for the purification procedure.

2.10. Amino acid analysis and automated Edman degradation

Proteins were electrotransferred from slab gels to PVDF membranes as described by Matsudaira [44], stained with Ponceau red and excised from the membrane for analysis. Amino acid compositions were determined using the Picotag procedure [45], after hydrolyzing the proteins with 6 N HCl at 110°C for 24 h and derivatizing the resulting amino acids with PITC before injecting them into the Picotag Waters column (0.39×30 cm). Edman degradation was carried out on an Applied Biosystems Sequencer Model 470 according to Hewik et al. [46]. Phenylthiohydantoin (PTH) identification was carried out by means of an Applied PTH column (0.21×22 cm, 5 μm).

2.11. Immunoblot analyses

Proteins were electro-transferred from polyacrylamide gels to nitrocellulose as described by Burnette [47]. After the transfer, Ponceau red protein staining and immunoprinting were carried out according to Coudrier et al. [48] as described by Gorvel et al. [49].

3. Results

3.1. Subcellular localization of hog intestinal basic CP activity and effects of the pH

Basic CP activity was detected in homogenates from hog intestinal mucosa assayed with hippuryl-

Table 1
Subcellular localization of basic CP activity in hog intestinal mucosa

Fraction	Enzymatic marker activities (%)	Subcellular localization	CP activity (%)
10 000×*g* pellet	Cytochrome *c* oxidase (73±7)	Mitochondrial fraction	0
$CaCl_2$ pellet	Cytochrome *c* reductase (78±3); Na^+/K^+ ATPase (73±5)	Microsomal fraction with basolateral membranes	0
105 000 g pellet	Aminopeptidase N (81±6)	Microvillar fraction	42±6
Final supernatant	Acid phosphatase (75±7)	Soluble fraction with lysosomal contamination	58±6

At each step in the subcellular fractionation procedure, the enzyme activities were measured in the pellet and the supernatant and expressed as a percentage of the total activity. Results are means based on three separate subcellular fractionations.

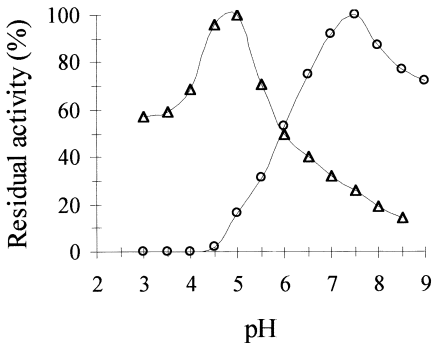

Fig. 1. Effects of pH on hog intestinal membrane-bound and soluble basic CP activities. pH–activity profiles were determined with hog intestinal microvillar membrane (○) and soluble (△) fractions using hippuryl-L-Arg as the substrate. Results are means based on three assays.

L-Arg at pH 7.5. Subcellular fractionation yielded a microvillar membrane fraction containing about 40% of the total basic CP activity and a soluble fraction containing the remaining 60% (Table 1). When the microvillar membrane fraction was washed with 2 M NaCl, no significant release of basic CP activity into the final supernatant was observed, which indicates that the enzyme was probably tightly bound to the intestinal cell membranes.

Two different pH activity profiles were obtained when the membrane-bound and soluble CP activities were tested with hippuryl-L-Arg at pH values ranging from 3.0 to 9.0 (Fig. 1). In the microvillar membrane fractions, the activity was highest at pH 7.5, and the enzyme was still 72% active at pH 9.0, whereas the activity dropped sharply at acidic pH levels and was practically nil below pH 4.5. These findings are in agreement with those obtained on CPM from human placenta and from MDCK cells [21,50]. Hog intestinal basic CP showed the highest level of activity at pH 5.0 with the soluble enzyme, and was still 57% active at pH 3.0, but its activity dropped sharply at basic pH levels. All these data suggested either that CPH may have been present, or that the lysosome did not remain intact during the subcellular fractionation, which would result in the soluble fractions being contaminated with lysosomal enzymes, which was apparently the case (Table 1). It was therefore concluded that the soluble CP activity showing an acidic optimum pH might be that of cathepsin B (also named lysosomal carboxypeptidase), which is not a metallocarboxypeptidase, but a cysteine proteinase [51]. This hypothesis was further confirmed upon testing the effects of some inhibitors and activators on the enzyme activity at acidic pH levels.

3.2. Effects of inhibitors and activators

As shown in Table 2, the membrane-bound CP activity was inhibited by *o*-phenanthroline and

Table 2
Effects of inhibitors and activators on hog intestinal membrane-bound and soluble basic CP activities

Chemical	mM	Relative activity (%)				
		Membrane fraction	CPM[a]	Soluble fraction	CPH[b]	Cathepsin B[c]
GEMSA	1	0	0			
$CoCl_2$	1	194 ± 46	150	98 ± 21	1090	95
o-Phenanthroline	0.1	8 ± 3	0	52 ± 4	7	86
EDTA	1	36 ± 11	–	88 ± 12	15	99
PMSF	1	100 ± 3	100			
PCMS	0.1	100 ± 5	100			
$HgCl_2$	10^{-3}	103 ± 3	–			
Cd acetate	0.1	74 ± 4	50			
$NiCl_2$	1			93 ± 17	200	78
$CdCl_2$	0.1			87 ± 10	12	79

Chemicals were incubated at 37°C for 15 min at pH 7.5 and pH 5.0 with hog intestinal microvillar membrane fractions and soluble fractions, respectively, before adding the substrate hippuryl-L-Arg. The activities are expressed as percentages of the control values, and the results are means of duplicate assays on two separate subcellular fractionations.

[a]On human placental CPM [21]

[b]On bovine adrenal enkephalin convertase (CPH) [9].

[c]On lysosomal carboxypeptidase (cathepsin B) [9].

EDTA, which strongly suggests that zinc or some other cation was required for the enzyme activity to occur, whereas the serine protease inhibitor PMSF, as well as PCMS, the sulfhydryl protease inhibitor which is known to be active on CPH, had no appreciable effects. The fact that no SH group was involved in the enzymatic activity was confirmed by the lack of effect of $HgCl_2$. As in the case of other mammalian basic CP, $CoCl_2$ increased the membrane-bound CP peptidase activity, while cadmium acetate was slightly inhibitory. As regards the soluble basic CP activity, no activation was observed with $CoCl_2$, the specific activator of basic carboxypeptidases, and only 50% inhibition occurred with *o*-phenanthroline, whereas EDTA and the two metal cations Ni^{2+} and Cd^{2+} were almost ineffective, in sharp contrast with what occurs in the case of CPH [9].

The subcellular localization, optimum pH and specific activation or inhibition by both CPM and hog intestinal membrane-bound CP reagents were very similar indeed (Table 2). It seems very likely that the soluble CP activity was mainly due to a lysosomal cathepsin, since this enzyme was also detected in the soluble fractions (data not shown) in the assay on cathepsin B activity with the specific substrate Z-Arg-Arg-AMC. However, the possibility that a soluble form of CPH or any other basic CP may have been present cannot be definitely ruled out.

3.3. Distribution of membrane-bound basic CP activity in the gastrointestinal tract

As shown in Fig. 2, basic CP activity was detected especially in the brush-border membrane preparations from hog small intestine. No significant difference between the duodenum, jejunum and ileum was observed, in agreement with the results obtained by Lynch et al. [26] on the localization of the membrane-bound form of CPH in the rat gastrointestinal tract. Since the methods used in the latter case were not specific to CPH, but can be used to detect all the basic CPs, the enzyme activity of the rat gastrointestinal tract may also involve CPM. Here we observed that, in the rat intestine, the specific activity of the microvillar basic CP (about 22.5 nmol/min.mg) does not differ significantly between the duodenum, jejunum and ileum (data not shown).

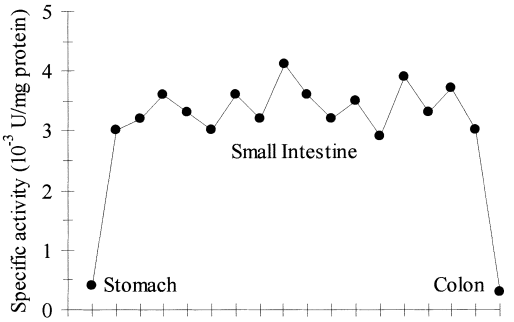

Fig. 2. Distribution of membrane-bound basic CP activity in hog gastrointestinal tract. CP activity was measured in the microvillar membrane fractions from hog stomach, intestine and colon with hippuryl-L-Arg as the substrate (1 enzymatic unit cleaves 1 μmol of hippuryl-L-Arg per min). Enzyme activity was measured along the small intestine in 1-m long sections. Results are means obtained on three animals.

3.4. Solubilization of membrane-bound basic CP

Since hog intestinal basic CP is apparently firmly attached to the microvillar plasma membrane, we compared its pattern of solubilization by detergents, phospholipase C and proteases in order to gain insights into the way in which the CP is anchored to the membrane.

3.4.1. Detergents

Fig. 3 clearly shows that the pattern of solubilization of the basic CP activity by a series of six detergents was comparable to that of aminopeptidase N, but differed greatly from that of alkaline phosphatase. The latter two enzymes are generally thought to be suitable markers for labeling proteins anchored by a hydrophobic amino acid residue sequence and by a GPI moiety, respectively, as pointed out by Hooper and Turner [52] in their study on pig kidney microvillar ectoenzymes. It is worth mentioning that only octyl-glucoside and CHAPS, two detergents with high CMC values, effectively released substantial amounts ($>60\%$) of GPI-anchored alkaline phosphatase activity, whereas all the detergents used solubilized 60–80% of the membrane-bound APN and basic CP activities.

3.4.2. Phospholipase C

When hog intestinal microvillar membrane fractions were incubated with PI-PLC from *Bacillus*

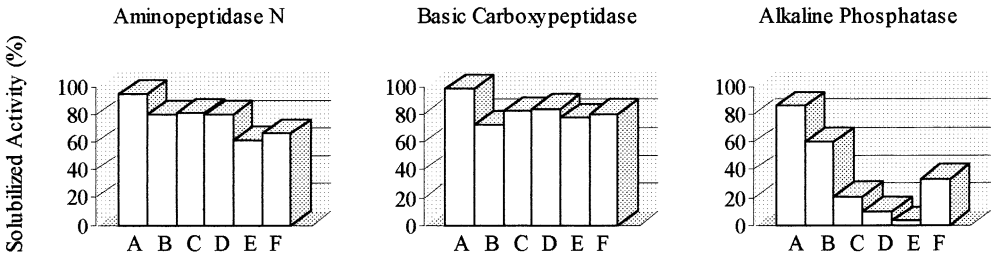

Fig. 3. Pattern of solubilization of hog intestinal membrane-bound ectoenzymes by a range of detergents. Hog intestinal microvillar membrane fractions (approx. 2 mg protein/ml) were incubated with the following detergents: 60 mM octyl-glucoside (A), 20 mM CHAPS (B), 9 mM sodium deoxycholate (C), 6 mM Nonidet P40 (D), 0.1% Triton X-100 (E) and 1% Triton X-100 (F). Results are means based on three separate experiments.

cereus, a specific release ($>50\%$) of alkaline phosphatase was observed, but neither aminopeptidase N nor basic CP were solubilized (data not shown). The presence of a mixture of protease inhibitors during the incubation of membrane fractions with PI-PLC, as indicated in Section 2, rules out the possibility that contaminating proteases may have been involved in the release of the GPI-anchored enzyme. This result therefore confirms the above conclusion that hog intestinal microvillar basic CP may not be bound to the membrane via a GPI tail like human placental CPM [23], but via a hydrophobic peptidic anchor. The same conclusion was previously reached by Hooper and Turner [53] in their study on microvillar basic CP from pig and human kidney.

3.4.3. Proteases

As shown in Fig. 4, when hog intestinal microvillar membrane fractions were incubated at 4°C with 5% trypsin (w/w), as much as 40% of the total basic CP activity was released after a 24-h treatment, and more than 80% with 10% trypsin (w/w). Aminopeptidase N amounted to 10% and 50%, respectively, whereas no alkaline phosphatase was released at all. Similar results were obtained with papain and subtilisin (data not shown).

After tryptic release of the soluble form of basic CP from its hydrophobic peptide anchor, the enzymatic activity remained intact. In addition, trypsin can be expected to cleave off only a relatively short peptide (about 20–30 amino acids) from the whole protein as observed in the case of CPM [50]. The tryptic release of a hydrophilic form of the basic CP from the microvillar plasma membrane was therefore taken as the starting point for the enzyme purification procedure.

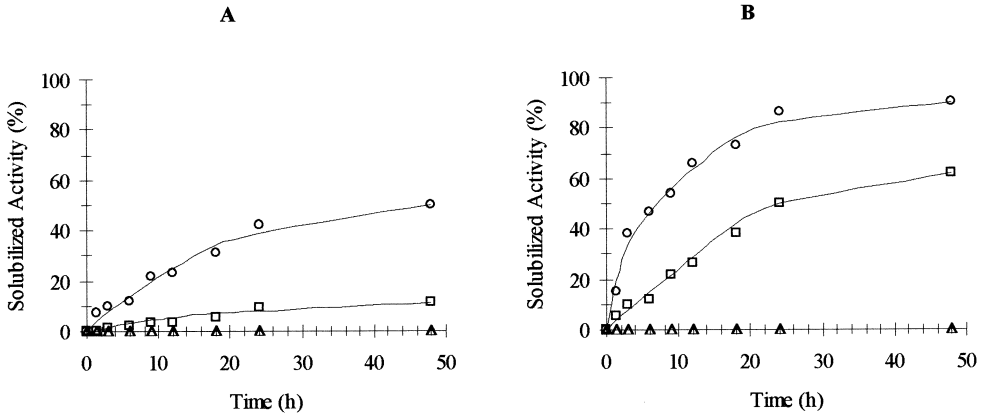

Fig. 4. Release of hog intestinal membrane-bound ectoenzymes by trypsin. Hog intestinal microvillar membrane fractions (0.5 mg protein/ml) were incubated at 4°C with trypsin as described in Section 2. Results are means of duplicate incubations and are expressed as a percentage of the control value: alkaline phosphatase (△), aminopeptidase N (□), and basic carboxypeptidase (○). (A) Solubilization with 5% trypsin (w/w); (B) solubilization with 10% trypsin (w/w).

Table 3
Purification of trypsin-solubilized hog intestinal membrane-bound basic CP

Purification step	Total activity (μmol/min)	Total protein (mg)	Specific activity (10^{-3} U/mg)	Purification (-fold)	Yield[a] (%)
Homogenate	5.0	9312	0.5	–	–
Brush border	1.9	640	3	15[b]	100
2 M NaCl washing	1.6	160	10	50	84
Trypsin solubilization	0.9	28	32	160	47
Arg-Sepharose	0.6	11	54	270	32
Phenyl-Sepharose	0.3	3	100	500	16
Sephacryl S200 HR	0,2	2	100	500	11

CP activity was measured with hippuryl-L-Arg as the substrate (1 enzymatic unit cleaves 1 μmol of hippuryl-L-Arg per min). Results of a typical experiment.
[a]Versus total activity.
[b]Based on total protein determination.

3.5. Purification of the trypsin-solubilized form of membrane-bound basic CP

Basic CP was purified from hog intestinal mucosa using a 6-step procedure including the preparation of microvillar plasmic membrane fraction, washing it with 2 M NaCl and treating it with trypsin to solubilize the enzyme, on which an affinity chromatography was subsequently performed on arginine-Sepharose, prior to hydrophobic chromatography on phenyl-Sepharose, and finally gel filtration on Sephacryl-S200 HR. As summarized in Table 3, in a typical experiment starting with 200 g of intestinal mucosa, the isolation procedure yielded about 2 mg of protein with a specific activity of 100 nmol/min per mg. The overall enzyme activity yield was 11% and a 500-fold purification of the enzyme was obtained. It is worth mentioning that the arginine-Sepharose-bound basic CP activity could not be specifically eluted from the column using the basic CP specific inhibitor GEMSA, which nevertheless completely inhibited the enzyme (data not shown). This might be due to the molecular mass of the protein which is about four times that of the other basic carboxypeptidases.

As shown in Fig. 5, the protein thus purified gave a single PAGE band under non-denaturing conditions, which indicates that the isolated protein might be homogeneous, but unfortunately two bands were observed in the presence of SDS. No further improvement of the purification level could be achieved although a number of other chromatographic procedures were tested.

3.6. Characterization of the trypsin-solubilized form of membrane-bound basic CP

Equal volumes of native enzyme and sweet potato β-amylase, the molecular mass of which is known to be about 200 kDa, were eluted from the Sephacryl-S200 HR column (data not shown). The molecular masses of the two protein bands separated by SDS–

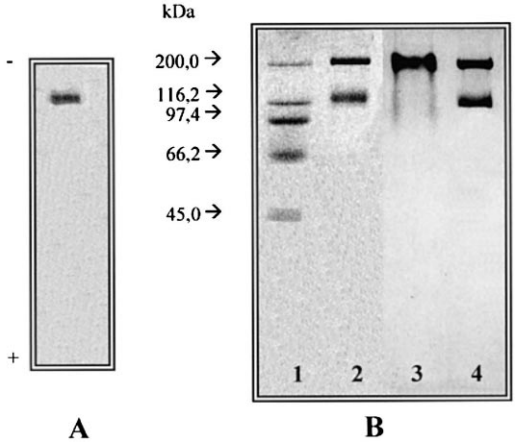

Fig. 5. Electrophoretic analysis of the purified trypsin-solubilized hog intestinal membrane-bound basic CP. Electrophoresis was carried out in 10% polyacrylamide gels. (A) PAGE of the purified enzyme (approximately 10 μg of protein) without SDS. (B) PAGE analysis in the presence of SDS under reducing conditions with heating (lane 2), non-reducing conditions without heating (lane 3), and non-reducing conditions with heating (lane 4). The following molecular mass markers (lane 1) were used: myosin (200 kDa), β-galactosidase (116.2 kDa), phosphorylase *B* (97.4 kDa), serum albumin (66.2 kDa) and ovalbumin (45 kDa).

PAGE under reducing conditions (Fig. 5, lane 2) were found to be 200 kDa and 120 kDa, respectively. When the SDS–PAGE was performed under non-reducing conditions, in the absence of DTT, and without any heating of the protein sample (Fig. 5, lane 3), a single band at 200 kDa was obtained on the contrary, which strongly suggests that the two protein units might be linked by one or more disulfide bonds. However, when the SDS–PAGE was carried out in the absence of DTT and heating the protein sample before running the electrophoresis (Fig. 5, lane 4), the 120 kDa protein was separated from the 200 kDa protein in the same way as under reducing and heating conditions. It is possible that the 120 kDa band may reflect the presence of a proteolytic fragment from the 200 kDa protein.

The sugar-specific pattern of slab gel staining obtained with periodate-Schiff's reagent clearly indicated that the two protein bands were glycosylated. Therefore, the native enzyme might be a 200 kDa glycoprotein, as compared with the molecular mass of CPM, which is 60 kDa [21]. The only basic carboxypeptidase which is known to be a single-chain glycoprotein with a molecular mass of 180 kDa is actually CPD, a carboxypeptidase which was recently characterized and found to have similar enzymatic properties to those of CPH [19]. Upon being subjected to SDS–PAGE, the purified membrane-bound and soluble forms of CPD from bovine pituitary gland showed a single 180 kDa band in the former case, and a 170 kDa and a 135 kDa band in the latter case [20]. The two polypeptide chains observed upon performing SDS–PAGE on the solubilized form of the basic CP purified from hog intestine were similar to those observed in the soluble form of CPD, and thus suggested that a membrane-bound CPD may also exist in the intestine.

The basic CP purified in this study was able to cleave hippuryl-L-Lys about 1.5-fold faster than hippuryl-L-Arg, contrary to what has been found to occur in the case of CPM [21], and its optimum pH was about 6.5 with the latter substrate. Since the enzyme was not detected when immunoblotting was performed with a polyclonal antiserum against human placental CPM, intestinal CP can definitely be said to be a structurally distinct protein from human placen-

Table 4
Comparative amino acid compositions of trypsin-solubilized hog intestinal membrane-bound basic CP and two other basic carboxypeptidases

Amino acids	120 kDa protein[a] (%)	200 kDa protein[a] (%)	CPM[b] (%)	CPD[c] (%)
Asx	4.2	12.3	12.6	11.2
Glx	5.4	9.7	8.6	9.8
Cys	0.4	0.5	1.6	1.4
Ser	10.8	9.2	6.3	7.9
Gly	11.9	9.7	6.1	7.9
His	3.1	3.1	3.7	3.3
Arg	5.4	5.1	3.5	4.6
Thr	6.5	6.1	4.2	6.5
Ala	7.7	7.2	4.2	6.0
Pro	8.1	6.1	6.8	5.8
Tyr	5.4	4.6	5.9	3.8
Val	7.7	6.7	6.8	6.8
Met	2.3	1.5	2.1	1.9
Ile	5.4	4.1	5.6	5.1
Leu	9.6	8.2	9.6	8.6
Phe	5.0	4.1	4.7	3.6
Lys	1.1	1.5	6.8	4.8
Trp	n.d.	n.d.	1.2	1.0

Results are expressed as a percentage of total residues determined. n.d., not determined.
[a]Mean value based on two analyses.
[b]Deduced from the cDNA nucleotide sequence of CPM [22].
[c]Deduced from the cDNA nucleotide sequence of CPD [54].

tal CPM-type enzyme. Since no NH_2-terminal residue was detected using the Applied Biosystems Automatic Sequencer, the protein N-terminus was taken to be blocked. The amino acid composition of the two solubilized forms of CP from intestine was determined and compared with those of human CPM and CPD (Table 4). The two proteins separated by SDS–PAGE showed similar amino acid contents, except for Asx and Glx, which suggests that the lower molecular mass form might be due to some proteolysis of the higher molecular mass form. The 200 kDa glycoprotein had roughly the same amino acid distribution as CPM and CPD, but its lysine content was not so high. However, although the amino acid compositions of hog intestinal CP, human CPM, and human CPD are comparable, the exact identity of the intestinal enzyme still needs to be confirmed.

4. Discussion

The COOH-terminal arginine and lysine from peptides and proteins may be cleaved by several carboxypeptidases, which show the same pattern of carboxypeptidase B activity, but have different molecular and enzymatic characteristics [1].

The results of the present study show that hog intestinal mucosa contains at least a membrane-bound carboxypeptidase capable of releasing basic COOH-terminal amino acids from short peptides. The trypsin-solubilized form of CP was isolated from the hog intestine, with a specific activity of 100 nmol/min per mg using hippuryl-L-Arg as the substrate. According to its subcellular localization and optimum pH, the enzyme activity greatly resembles that of CPM in the human placenta as well as that of MDCK cells [21,50]. The basic CP activity was equally distributed between the duodenum, jejunum and ileum, in agreement with the pattern of distribution of CPH in the rat gastrointestinal tract [26]. However, since the methods used by the latter authors were not really specific to CPH activity [27], the enzyme involved might have been CPM, and this might explain why CPH was sometimes found in areas apparently devoid of any endocrine function in the rat gastrointestinal tract. Although we observed that the ratio between intestinal membrane-bound and soluble CP activities was the same as that between membrane-bound and soluble CPH [26], it is worth noting that two distinct enzymes with different optimum pH values and behavior towards activators and inhibitors were found to exist in hog intestine. The soluble CP activity was probably due to cathepsin B, a cysteine enzyme of lysosomal origin with several endo- and exopeptidase activities [51,55] usually found in the gastroduodenal mucosa [56,57]. This enzyme, which is not a metallocarboxypeptidase, was not further characterized in this study.

Hog intestinal basic CP did not appear to have the characteristics of a GPI-anchored enzyme, judging from its pattern of solubilization by a range of detergents, in addition to the fact that it was not released from microvillar membrane preparations in response to PI-PLC. Consequently, this cell-surface peptidase is probably anchored by a sequence of hydrophobic amino acid residues to the lipid bilayer. The basic carboxypeptidases present in human and hog kidney microvillar membranes are known to have the same pattern of solubilization by detergents and to resist solubilization by PI-PLC [53], whereas CPM from human placenta and that from MDCK cells are anchored via a GPI tail [23,50]. The discovery in human placenta of a membrane-bound carboxypeptidase which was not GPI-anchored and had different characteristics from those of CPM [23] may support the idea that membrane-bound CPM is not the sole microvillar carboxypeptidase. Since Hooper and Turner [53] described the solubilization of basic CP from the rat kidney without further characterizing the enzyme, it is therefore possible that the microvillar membrane-bound carboxypeptidase is another member of the B-type carboxypeptidase family. A placental enzyme distinct from CPM was recently partially purified and denoted CPD [58], a basic carboxypeptidase which was still unknown when the present work was carried out.

Although the basic CP from hog intestinal mucosa has some properties in common with CPM, it is definitely a distinct enzyme, since CPM is a GPI-anchored single-chain 60 kDa glycoprotein, whereas hog intestinal CP is apparently a single-chain 200 kDa glycoprotein which is anchored to the membrane by a number of hydrophobic amino acid residues. Moreover, intestinal CP cleaved lysyl bonds

faster than arginyl bonds, contrary to CPM, and immunoblotting analysis provided further evidence that hog intestinal CP was different from CPM. The molecular mass of the intestinal CP (200 kDa) was not very different from that of the membrane-bound CPD (180 kDa), which was first described in the bovine pituitary gland as a CPH-like enzyme [19,20]. CPD is a widely distributed enzyme [20,58] which has recently been partly purified from human placental microvilli [58], indicating that CPM is not the sole microvillar carboxypeptidase of the B-type. The optimum pH of the purified intestinal CP was actually between 6.0 and 7.0, which is similar to that of human placental CPD (5.5–6.5).

The sole membrane-bound CP which was isolated and characterized from hog intestine in the present study was identified as a new member of the B-type metallocarboxypeptidases, and is consequently different from CPB, CPN, pCPB, CPH and CPM. It appeared to be similar to the newly described CPD, which is the mammalian homologue of duck gp180, a 180 kDa hepatitis B virus-binding glycoprotein which was recently cloned and sequenced [59]. It is worth noting that unlike bovine CPD, the N-terminus of which was determined chemically [19,20], no NH_2-terminal residue could be detected when NH_2-terminal Edman degradation was performed on the gp180 protein purified from duck liver [59]. Although CPD occurs in a wide range of tissues, this is the first time a CPD-like enzyme is purified from intestinal tissue. Further characterization of the structural, catalytic and immunological properties of hog intestinal CP is still necessary to definitely confirm that this enzyme is a CPD.

As the various carboxypeptidases described so far have the same catalytic properties, their specific biological function must depend mainly on their site of action, which is generally directly related to their physical state (e.g., solubilized vs membrane-bound). It is therefore widely recognized that the pancreatic CPB present in the intestine in the form of a soluble enzyme contributes along with other digestive enzymes to degrading food proteins and peptides, whereas CPH in the secretory granules of neuroendocrine tissues might be involved in the maturation and sorting of peptidic hormones [60,61]. Two other carboxypeptidases present in most mammalian tissues and organs, namely CPN, which circulates in the blood, and CPM, which is bound to the plasma membrane of several cells, play key physiological roles, consisting of regulating the activity of various peptides. These two enzymes in particular have been described as kininases [62], although their exact function has not yet been established. The function of CPD is still unknown, but the recent finding that it is located mainly in the trans-Golgi network is consistent with the idea that it is involved in the processing of a broad range of proteins that transit along the secretory pathway, playing a similar role to that of CPH in prohormone processing in the regulated secretory pathway [63]. In addition, the finding that CPD is recycled from the cell surface to the trans-Golgi network constitutes important evidence supporting the suggestion that it may act as a receptor for hepatitis B virus [63]. Another metal containing peptidase, aminopeptidase N, has been found to be a coronavirus receptor [64,65]. The question as to whether the intestinal membrane-bound basic CP has a specific and exclusive degradative role completing that of pancreatic CPB, or is involved independently or concomitantly in the regulation of some of the active biological peptides present in the luminal secretions of the intestine, as well as in the immunological response of the intestinal mucosa to the presence of exogenous peptidic antigens, still remains to be elucidated.

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