Identification of membrane dipeptidase as a major glycosyl-phosphatidylinositol-anchored protein of the pancreatic zymogen granule membrane, and evidence for its release by phospholipase A

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Membrane dipeptidase (EC 3.4.13.19) enzyme activity that is inhibited by cilastatin has been detected in pancreatic zymogen granule membranes of human, porcine and rat origin. Immunoelectrophoretic blot analysis of human and porcine pancreatic zymogen granule membranes with polyclonal antisera raised against the corresponding kidney membrane dipeptidase revealed that the enzyme is a disulphide-linked homodimer of subunit mass 61 kDa in the human and 45 kDa in the pig. Although membrane dipeptidase was, along with glycoprotein-2, one of the only two major components of carbonate high pH-washed membranes, no enzyme activity or immunoreactivity was detected in the zymogen granule contents. Digestion with bacterial phosphatidylinositol-specific phospholipase C (PI-PLC), and subsequent recognition by antibodies specific for the cross-reacting determinant, revealed that membrane dipeptidase in human and porcine pancreatic zymogen granule membranes is glycosyl-phosphatidylinositol-anchored. Membrane dipeptidase was released from the pancreatic zymogen granule membranes by an endogenous hydrolase, and the released form migrated as a disulphide-linked dimer on SDS/PAGE under non-reducing conditions. Under reducing conditions it migrated with the same apparent molecular mass as the membrane-bound form, and was still a substrate for bacterial PI-PLC. Treatment of kidney microvillar membranes with phospholipase A_2 resulted in the release of membrane dipeptidase in a form that demonstrated electrophoretic and cilastatin–Sepharose binding properties identical to those of the endogenously released form of the enzyme from zymogen granule membranes. These results indicate that the glycosyl-phosphatidylinositol anchor on the pancreatic membrane dipeptidase is cleaved by an endogenous hydrolase, probably a phospholipase A, and that this cleavage may promote the release of the protein from the membrane.

INTRODUCTION

The surfaces of a wide variety of mammalian cell types are endowed with members of a group of enzymes that are involved in the metabolism of a range of bioactive peptides such as hormones and neuropeptides [1]. These ectopeptidases are integral proteins of the plasma membrane that are asymmetrically oriented on the apical membrane of, for example, epithelial cells, with their catalytic site exposed at the extracytoplasmic surface. Members of this group of enzymes include angiotensin-converting enzyme (EC 3.4.15.1), neprilysin (EC 3.4.24.11), dipeptidyl peptidase IV (EC 3.4.14.5) and a number of aminopeptidases. Membrane dipeptidase (MDP; EC 3.4.13.19; dehydropeptidase-I; renal or microsomal dipeptidase) is one such ectopeptidase, which is found predominantly in the brush-border membrane of the kidney and also in the lungs (for a review, see [2]). MDP is a zinc metalloenzyme capable of hydrolysing a wide range of dipeptides, including those with a D-amino acid in the C-terminal position [3,4]. The enzyme may have a role in the metabolism of glutathione and leukotriene D_4 [5,6], and is the only known mammalian enzyme to exhibit β-lactamase activity [7]. This β-lactamase activity of MDP has led to the development of potent and selective inhibitors of the enzyme, of which the most extensively characterized is the reversible competitive inhibitor cilastatin [8].

Porcine MDP was the first mammalian peptidase that was found to be anchored in the lipid bilayer by a covalently attached glycosyl-phosphatidylinositol (GPI) moiety [9]. Subsequently, human MDP was also shown to be GPI-anchored [10], and the anchors on both porcine and human MDP have been extensively characterized in terms of their hydrolyses by bacterial phosphatidylinositol-specific phospholipase C (PI-PLC) and a plasma phospholipase D [11–14]. Recently, the complete structure of the GPI anchor on porcine MDP has been determined and its site of attachment in the polypeptide chain identified [15]. The glycan core structure of the GPI anchor on human MDP was also determined, representing the first inter-species comparison of the GPI anchors on the same protein [15]. In addition, polyclonal antisera have been generated against the cross-reacting determinant (CRD) on both porcine and human MDP [16,17], and these antisera have been used widely to demonstrate the presence of a GPI anchor on proteins following cleavage by phospholipase C.

The primary function of pancreatic acinar cells is the synthesis and regulated secretion of digestive enzymes, which are stored as proenzymes or zymogens in granules located towards the apical end of the cell. Both nervous (cholinergic) and hormonal (cholecystokinin and secretin) stimuli induce large-scale exocytosis of zymogen granules upon ingestion of food. The proteins within the zymogen granule membrane (ZGM) therefore probably play an important role in granule formation and/or secretion. The protein composition of ZGMs from different

Abbreviations used: CRD, cross-reacting determinant; DipF, di-isopropylfluorophosphate; GP-2, glycoprotein-2; GPI, glycosyl-phosphatidylinositol; MDP, membrane dipeptidase; PI-PLC, phosphatidylinositol-specific phospholipase C; PLA, phospholipase A; PSA, porcine serum albumin; octyl glucoside, n-octyl β-D-glucopyranoside; ZGM, zymogen granule membrane.

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species, including pig and rat, is relatively simple, consisting of 8–10 different proteins as revealed by SDS/PAGE [18]. The most abundant of these, glycoprotein-2 (GP-2), which is GPI-anchored [19], was the first of these proteins to be cloned and sequenced [20,21]. Another of the ZGM proteins, GP-3, has also been cloned [22] and localized to the inner surface of the ZGM [23]. Although the functions of these ZGM proteins are unknown, GP-2 and GP-3 show sequence similarity to phospholipase A_2 (PLA_2) and triacylglycerol lipases respectively [22,24], suggesting a possible involvement in lipid hydrolysis and/or binding. However, a recent study failed to demonstrate that either of these proteins had phospholipase activity (at least under the assay conditions employed), but identified a carboxyl ester hydrolase as a phospholipid-hydrolysing enzyme of the ZGM [25].

In the present study we show that MDP, along with γ-glutamyl transpeptidase (EC 2.3.2.2) [26], is the only ecto-peptidase present to any significant extent in the pancreatic ZGM. Furthermore we show that MDP is an abundant protein of the ZGM and that it is anchored by a GPI moiety. We also provide evidence indicating that the GPI anchor on pancreatic MDP is cleaved by an endogenous hydrolase, possibly a PLA, and that this cleavage may promote the release of the protein from the ZGM and/or the plasma membrane.

EXPERIMENTAL

Materials

Cilastatin was a gift from Merck, Sharp and Dohme Research Laboratories (Rahway, NJ, U.S.A.). *Bacillus thuringiensis* PI-PLC was a gift from Dr. M. G. Low (Columbia University, New York, NY, U.S.A.). Porcine pancreatic PLA_2 (EC 3.1.1.4), n-octyl β-D-glucopyranoside (octyl glucoside), di-isopropyl-fluorophosphate (DipF), Gly-D-Phe, Gly-Pro-Hyp, benzoyl-Gly-His-Leu, [D-Ala^2,Leu^5]enkephalin, Asp-Phe, Ala *p*-nitroanilide, α-Glu *p*-nitroanilide, Gly-Pro *p*-nitroanilide and γ-Glu *p*-nitroanilide were from Sigma (Poole, Dorset, U.K.). Microvillar membranes were prepared from porcine kidney cortex by the method of Booth and Kenny [27]. Cilastatin–Sepharose was prepared as described previously [11]. MDP was purified from porcine or human kidney cortex, following solubilization with PI-PLC, by affinity chromatography on cilastatin–Sepharose [11,13]. Protein was determined using the bicinchoninic acid method [28] modified for use in 96-well microtitre plates [29] with BSA as standard.

Preparation of pancreatic ZGMs and granule contents

Zymogen granules were prepared from pigs by a previously published procedure [18] by a series of differential centrifugation steps in sucrose. The purified granules were then lysed by alkaline treatment in 20 mM Hepes, 100 mM KCl, 0.5 mM PMSF, pH 8.0. The released zymogens (called the granule contents) were separated from the membranes by layering the granule lysate over a step gradient of 0.3 and 1.0 M sucrose [30]. After centrifugation, the granule contents were collected on top of the gradient and the membranes floated on the 0.3 M/1.0 M sucrose interface. The latter were resuspended in 0.25 M NaBr to release any adsorbed zymogens.

Enzyme assays

MDP enzymic activity was determined with Gly-D-Phe (1 mM) as substrate in 0.1 M Tris/HCl, pH 8.0. Reactions were terminated by heating at 100 °C for 4 min, and the substrate and reaction products were resolved and quantified by reverse-phase HPLC [9]. X-Pro aminopeptidase, X-Trp aminopeptidase, angiotensin-converting enzyme and neprilysin were assayed with Gly-Pro-Hyp, Asp-Phe, benzoyl-Gly-His-Leu and [D-Ala^2,Leu^5]enkephalin respectively as substrate, and the substrate and reaction products were resolved and quantified by reverse-phase HPLC [10,31–33]. Glutamyl aminopeptidase, membrane alanyl aminopeptidase, dipeptidyl peptidase IV and γ-glutamyl transpeptidase were assayed with α-Glu *p*-nitroanilide, Ala *p*-nitroanilide, Gly-Pro *p*-nitroanilide and γ-Glu *p*-nitroanilide respectively as substrate, and the released *p*-nitroaniline was quantified spectrophotometrically in 96-well plates [29]. The specificity of the hydrolysis of each substrate was assessed by the inclusion of the appropriate inhibitor.

SDS/PAGE and immunoelectrophoretic blot analysis

SDS/PAGE was performed with a 7–17 % (w/v) polyacrylamide gradient as described previously [34]. Reducing and non-reducing conditions were achieved by respectively including or omitting dithiothreitol in the sample loading buffer. Immuno-electrophoretic blot analysis was carried out with Immobilon P (PVDF) membranes as described previously [32]. Polyclonal antisera against porcine or human kidney MDP and porcine GP-2 were generated as described previously [16,35]. Anti-CRD antibodies were isolated from the bulk of the anti-MDP antiserum by fractionation on a column of the immobilized soluble form of the trypanosome variant surface glycoprotein [16,17]. Bound antibody was detected using peroxidase-conjugated secondary antibody in conjunction with the ECL (Amersham) detection method.

Incubation of membranes with phospholipases or detergent, and temperature-induced phase separation in Triton X-114

Triton X-114 was precondensed before use [36]. Membrane samples were incubated either alone (endogenous release), or in the presence of *B. thuringiensis* PI-PLC (0.1 unit) or porcine pancreatic PLA_2 (40 units) in 50 mM Hepes, pH 7.4, at 37 °C. The reactions were terminated by placing the tubes in ice, before phase separation in Triton X-114 [37]. An equal volume of 10 mM Tris/HCl, 0.15 M NaCl, 2 % (v/v) Triton X-114, pH 7.4, was added to each sample and then incubated on ice for 5 min. The samples were then incubated at 30 °C for 3 min before centrifugation at 3000 ***g*** for 3 min. The detergent-poor phase was removed to a clean tube, and the detergent-rich phase was made up to the same volume as the detergent-poor phase with 10 mM Tris/HCl, 0.15 M NaCl, pH 7.4. Membrane samples were separately solubilized by the addition of octyl glucoside to a final concentration of 60 mM and then incubated with mixing at 4 °C.

Atropine infusion into pigs

Large White hogs (approx. 50 kg live weight) were fitted with two permanent cannulae under halothane anaesthesia as previously described [38]. One cannula was inserted into the pancreatic duct, while the other was positioned in the duodenum for restitution of the pancreatic juice. A silastic catheter was positioned in the jugular vein for perfusion of secretin and atropine. After an overnight fast with free access to water, cannulated pigs were perfused at 10 ml/h with saline containing 0.5 % porcine serum albumin (saline-PSA) for a period of 1 h. Stimulation was then initiated by intravenous perfusion of secretin dissolved in saline-PSA at 36 pmol/h per kg for 4 h. Atropine dissolved in saline-PSA was infused intravenously 1 h after the beginning of the secretin stimulation at a dose of 50 μg/h per kg for 3 h.

Secretions were collected continuously; 25% of the volume was retained and pooled in 30-min fractions. The catheters for pancreatic juice collection and return were connected to an apparatus which immediately reintroduced the secreted pancreatic juice into the duodenum [39] in order to maintain the feedback inhibition produced by the continuous flow of pancreatic secretions into the intestine. We thank Dr. Tristan Corring (INRA, Jouy-en-Josas, France) for performing the surgery on the pigs and for the collection of the pancreatic juice from the conscious animals.

RESULTS AND DISCUSSION

MDP is an abundant enzyme of pancreatic ZGMs

Porcine pancreatic ZGMs were assayed for a number of ectopeptidases that are abundant in the kidney microvillar membrane (Table 1). A significant amount of MDP enzymic activity towards Gly-D-Phe, which was inhibitable by cilastatin, was detected in the porcine pancreatic ZGM. γ-Glutamyl transpeptidase was also present in the porcine pancreatic ZGM, consistent with previous results [26]. However, the activities of a number of other ectopeptidases that are abundant in the kidney microvillar membrane either were not detected (glutamyl aminopeptidase, X-Pro aminopeptidase, X-Trp aminopeptidase, angiotensin-converting enzyme and dipeptidyl peptidase IV) or were present at very low levels (membrane alanyl aminopeptidase and neprilysin) (Table 1). MDP enzymic activity was also detected in pancreatic ZGMs from human and rat (Table 2). In the porcine and human pancreatic ZGMs, the specific activity of MDP was 2.3- and 3.8-fold higher respectively than in the porcine kidney microvillar membrane (Table 2).

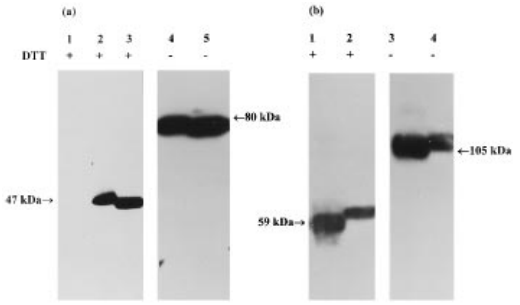

Figure 1 Immunoelectrophoretic blot of pancreatic ZGMs and granule contents with anti-MDP antiserum

Pancreatic ZGM or granule contents were subjected to SDS/PAGE under reducing or non-reducing conditions and then subjected to immunoelectrophoretic blot analysis as described in the Experimental section with (**a**) a polyclonal antiserum raised to MDP purified from porcine kidney [11] or (**b**) a polyclonal antiserum raised to MDP purified from human kidney [17]. (**a**) Lane 1, porcine pancreatic zymogen granule contents (50 μg of protein); lanes 2 and 4, affinity-purified porcine kidney MDP (0.1 μg of protein); lanes 3 and 5, porcine pancreatic ZGM (25 μg of protein). (**b**) Lanes 1 and 3, affinity-purified human kidney MDP (0.25 μg of protein); lanes 2 and 4, human pancreatic ZGM (25 μg of protein).

Table 1 Distribution of peptidases in the porcine pancreatic ZGM as compared with the kidney microvillar membrane

Enzymes were assayed as described in the Experimental section. Results are the means of duplicate determinations. n.d., no activity detected.

Peptidase	Specific activity (nmol/min per mg)	
	Pancreatic ZGMs	Kidney microvillar membranes
MDP (EC 3.4.13.19)	1138.9	488.6
Membrane alanyl aminopeptidase (EC 3.4.11.2)	13.6	645.5
Glutamyl aminopeptidase (EC 3.4.11.7)	n.d.	89.3
X-Pro aminopeptidase (EC 3.4.11.9)	n.d.	68.5
X-Trp aminopeptidase (EC 3.4.11.16)	n.d.	135.6
Dipeptidyl peptidase IV (EC 3.4.14.5)	n.d.	567.0
Angiotensin-converting enzyme (EC 3.4.15.1)	n.d.	115.3
Neprilysin (EC 3.4.24.11)	0.9	851.8
γ-Glutamyl transpeptidase (EC 2.3.2.2)	24.2	42.6

Table 2 Specific activity of MDP in porcine, human and rat pancreatic zymogen granules

Specific activity is defined as the MDP activity inhibitable by 0.1 mM cilastatin (assayed as described in the Experimental section). In the absence of cilastatin, maximally 5% substrate breakdown was observed with any of the samples. Results are either the means of duplicate determinations or the means $\pm$ S.E.M. of triplicate determinations.

Source	Species	Specific activity (nmol of D-Phe/min per mg)	Relative activity (%)
Kidney microvilli	Pig	517.1 $\pm$ 51.4	100.0
ZGM	Pig	1214.6 $\pm$ 65.6	234.9
	Human	1960.7 $\pm$ 128.3	379.2
	Rat	291.9	56.4
Zymogen granule content	Pig	0.6	0.1
	Human	0.1	0.02
	Rat	0.7	0.1

Immunoelectrophoretic blot analysis of the pancreatic ZGM and granule contents was performed with antisera that recognize the protein portion of either porcine or human MDP (Figure 1). In the porcine pancreatic ZGM a single polypeptide band of approx. 45 kDa was recognized by the anti-MDP antibody under reducing conditions, with a single polypeptide band of $\sim$ 80 kDa being recognized under non-reducing conditions (Figure 1a). Immunoelectrophoretic blot analysis of human pancreatic ZGMs with an antiserum raised against the human kidney dipeptidase revealed the presence of a single polypeptide band of $\sim$ 61 kDa under reducing conditions and of $\sim$ 110 kDa under non-reducing conditions (Figure 1b). The polypeptides recognized by the anti-MDP antibodies in the human and porcine pancreatic ZGMs were of approximately the same molecular mass as MDP purified from human (59 kDa) or porcine (47 kDa) kidney, and also behaved as disulphide-linked dimers under non-reducing conditions [12,13]. The difference in size between porcine and human MDPs has been shown to be due exclusively to more extensive N-linked glycosylation of the human protein [13,40]. The slight difference in size between MDP in kidney and pancreas from the same species is probably due to tissue-specific differences in the nature and extent of N-linked glycosylation. Thus, on the basis of its ability to hydrolyse the dipeptide Gly-D-Phe, its inhibition by cilastatin, its recognition by specific polyclonal antisera, its molecular mass and its existence as a disulphide-linked dimer, we conclude that MDP is present in pancreatic ZGM.

When porcine pancreatic ZGMs were washed with 0.1 M Na_2CO_3, pH 11.0, only two proteins were subsequently detected on SDS/PAGE analysis of the washed membrane pellet (Figure

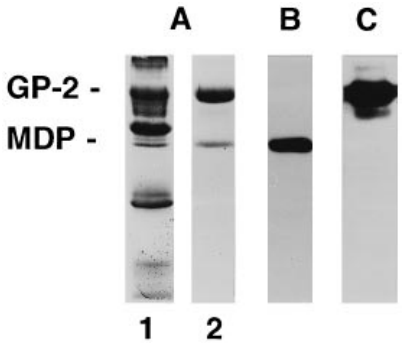

Figure 2 Na_2CO_3 extraction of porcine ZGMs

Porcine pancreatic ZGMs were subjected to SDS/PAGE under reducing conditions and then either stained for protein or subjected to immunoelectrophoretic blot analysis as described in the Experimental section. (**A**) Purified ZGM (lane 1) and membranes following extraction with 100 mM Na_2CO_3, pH 11.0 (lane 2), stained with Coomassie Blue. (**B**) Na_2CO_3-extracted membranes immunoblotted with a polyclonal antiserum raised to MDP purified from porcine kidney [11]. (**C**) Na_2CO_3-extracted membranes immunoblotted with a polyclonal antiserum raised to GP-2 [35].

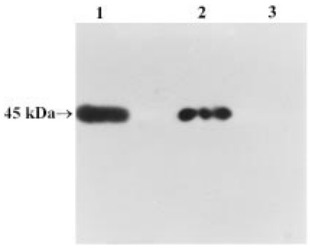

Figure 3 Immunoelectrophoretic blot with anti-CRD antiserum

The samples were electrophoresed under reducing conditions and subjected to immunoelectrophoretic blot analysis with an antiserum that is specific for the CRD epitope on the GPI anchor [16], as described in the Experimental section. Lane 1, porcine pancreatic ZGMs were incubated in the presence of 0.1 unit of *B. thuringiensis* PI-PLC at 37 °C for 2 h. At the end of the incubation the sample was subjected to temperature-induced phase separation in Triton X-114. The resulting detergent-poor phase was incubated with cilastatin–Sepharose for 18 h at 4 °C in 50 mM Tris/HCl, 0.5 M NaCl, pH 7.6. After extensive washing, bound material was eluted from the cilastatin–Sepharose by boiling for 4 min in the presence of SDS/PAGE loading buffer. Lane 2, PI-PLC-cleaved, affinity-purified porcine kidney MDP (5 μg of protein); lane 3, as lane 2, except that the sample was treated with 1 M HCl for 30 min prior to electrophoresis.

Table 3 Release of MDP from pancreatic ZGMs by phospholipases and an endogenous hydrolase

Pancreatic ZGMs or kidney microvillar membranes were incubated as indicated and then subjected to temperature-induced phase separation in Triton X-114 as described in the Experimental section. The resulting detergent-rich and detergent-poor phases were assayed for MDP activity, and the activity in the detergent-poor phase was expressed as a percentage of the total activity recovered in both phases. The results are the means of duplicate incubations and are representative of at least three such experiments.

	Activity in detergent-poor phase (% of total activity)		
Incubation conditions	Pig pancreatic ZGMs	Human pancreatic ZGMs	Pig kidney microvillar membranes
4 °C, 6 h	24.6	16.7	17.0
37 °C, 6 h	69.8	38.5	16.5
37 °C, 6 h, +0.1 mM DipF	19.5	–	–
37 °C, 2 h, +0.1 unit of PI-PLC	87.7	82.2	85.1
37 °C, 4 h, +40 units of PLA_2	–	–	50.7

2A). Immunoelectrophoretic blot analysis revealed that the protein of ~45 kDa was recognized by the antiserum raised against affinity-purified porcine kidney MDP (Figure 2B), indicating that MDP is one of the two major components of the Na_2CO_3-washed pancreatic ZGM. The larger polypeptide of ~92 kDa is the other major GPI-anchored protein of the ZGM, GP-2 (Figure 2C) [18]. This makes the exocrine pancreatic secretory granule membrane quite peculiar, as it is almost exclusively constituted of GPI-anchored integral membrane proteins. Although considerable levels of MDP enzymic activity were detected in the pancreatic ZGM, no enzymic activity (Table 2), even after extensive dialysis against a Zn^{2+}-containing buffer, or immunoreactivity (Figure 1) were detected in the zymogen granule contents, indicating that MDP is not a soluble component of the granule.

A previous report has described the purification from human pancreas of a zinc metallodipeptidase, using the substrate L-Leu-L-Leu to monitor activity [41]. Although at the time activity towards the more selective substrates of MDP containing a D-amino acid in the C-terminal position, e.g. Gly-D-Phe, and inhibition by the specific inhibitor cilastatin were not investigated, the observations that the pancreatic dipeptidase consisted of two subunits each of 68 kDa [41] and was able to hydrolyse leukotriene D_4 [42] are consistent with it being MDP. More recently, Northern blot analysis has revealed weak expression of MDP in rat pancreas [43], consistent with the results reported herein. However, none of these studies examined the subcellular distribution of the enzyme.

MDP in pancreatic ZGMs is GPI-anchored

The presence of a GPI anchor on MDP in pancreatic ZGMs was assessed by digestion with *B. thuringiensis* PI-PLC followed by temperature-induced phase separation in Triton X-114 (see Table 3). More than 80% of the MDP was released into the detergent-poor phase following treatment of either porcine or human pancreatic ZGMs with PI-PLC. Confirmation of the presence of a GPI anchor on porcine pancreatic zymogen granule MDP was obtained by immunoelectrophoretic blot analysis of the protein with an anti-CRD antiserum following digestion with PI-PLC (Figure 3). The PI-PLC-released form of porcine pancreatic zymogen granule MDP (Figure 3, lane 1) and porcine kidney MDP that had been purified following PI-PLC-induced release from the membrane (Figure 3, lane 2) were recognized by the anti-CRD antiserum, indicating that the GPI anchor had been cleaved to reveal the inositol 1,2-cyclic monophosphate epitope [16,17]. The specificity of the recognition by this antibody for the CRD was demonstrated by the loss of recognition of porcine kidney MDP that had been treated with 1 M HCl (which selectively decyclizes the inositol 1,2-cyclic monophosphate epitope) prior to electrophoresis (Figure 3, lane 3). Thus, like the enzyme in kidney, pancreatic MDP is also anchored by a GPI moiety.

MDP is released from the ZGM by an endogenous hydrolase

When porcine or human pancreatic ZGM was incubated at 37 °C in the absence of added PI-PLC, MDP was released in a time-dependent manner into the detergent-poor phase following temperature-induced phase separation in Triton X-114 (Table 3

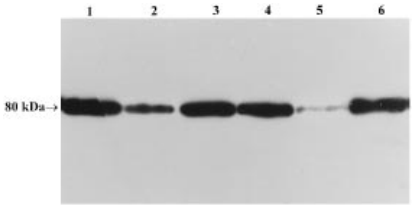

Figure 4 Immunoelectrophoretic blot under non-reducing conditions of ZGMs with anti-MDP antiserum

Porcine pancreatic ZGMs were incubated in the absence or presence of 0.1 unit of *B. thuringiensis* PI-PLC, and then subjected to temperature-induced phase separation in Triton X-114 as described in the Experimental section. The resulting phases were electrophoresed on SDS/PAGE under non-reducing conditions and then subjected to immunoelectrophoretic blot analysis with an antiserum raised against porcine kidney MDP. Lanes 1 and 2, detergent-rich and detergent-poor phases respectively at zero time; lanes 3 and 4, detergent-rich and detergent-poor phases respectively after a 4 h incubation at 37 °C in the absence of PI-PLC; lanes 5 and 6, detergent-rich and detergent-poor phases respectively after a 1 h incubation at 37 °C in the presence of PI-PLC.

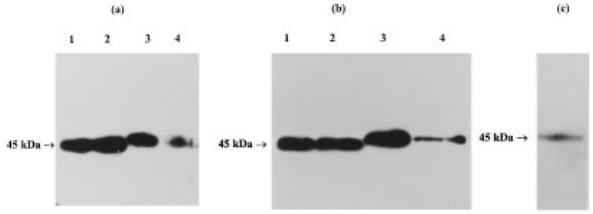

Figure 5 Immunoelectrophoretic blot under reducing conditions of ZGMs and kidney microvillar membranes with anti-MDP antiserum

Porcine pancreatic ZGMs and kidney microvillar membranes were incubated either on their own, or with 0.1 unit of *B. thuringiensis* PI-PLC or 40 units of PLA_2, and then subjected to temperature-induced phase separation in Triton X-114 as described in the Experimental section. The resulting phases were electrophoresed on SDS/PAGE under reducing conditions and then subjected to immunoelectrophoretic blot analysis with an antiserum raised against porcine kidney MDP. (**a**) Pancreatic ZGM: lane 1, detergent-rich phase at zero time; lane 2, detergent-poor phase after a 6 h incubation at 37 °C in the absence of PI-PLC; lane 3, detergent-poor phase after a 1 h incubation at 37 °C in the presence of PI-PLC; lane 4, as lane 2, but detergent-poor phase further incubated with PI-PLC for 1 h at 37 °C prior to electrophoresis. (**b**) Kidney microvillar membranes: lane 1, detergent-rich phase at zero time; lane 2, detergent-poor phase after a 4 h incubation at 37 °C in the presence of 40 units of PLA_2; lane 3, detergent-poor phase after a 1 h incubation at 37 °C in the presence of PI-PLC; lane 4, as lane 2, but detergent-poor phase further incubated with PI-PLC for 1 h at 37 °C prior to electrophoresis. (**c**) Immunoelectrophoretic blot analysis with an antiserum that is specific for the CRD epitope on the GPI anchor [16] of the detergent-poor phase resulting from endogenous release from porcine pancreatic ZGMs (equivalent to lane 2 in **a**) further incubated in the presence of 0.1 unit of *B. thuringiensis* PI-PLC at 37 °C for 2 h. The sample was then incubated with cilastatin–Sepharose for 18 h at 4 °C in 50 mM Tris/HCl, 0.5 M NaCl, pH 7.6. After extensive washing, bound material was eluted from the cilastatin–Sepharose by boiling for 4 min in the presence of SDS/PAGE loading buffer.

and Figure 4). In contrast, negligible release occurred at 4 °C (Table 3 and Figure 4), and the release at 37 °C was completely inhibited by prior incubation of the membranes with 0.1 mM DipF (Table 3), indicating that this is an enzymic process. In contrast, no release of MDP was observed from porcine kidney microvillar membranes in the absence of exogenously added PI-PLC (Table 3), consistent with previous results [9,14]. Analysis of the endogenously released form of pancreatic ZGM MDP on SDS/PAGE under non-reducing conditions followed by immunoelectrophoretic blot analysis revealed that it was still a disulphide-linked dimer (Figure 4). The single interchain disulphide bond in porcine kidney MDP has recently been identified by site-directed mutagenesis and expression as involving solely Cys-361 [44], and the site of GPI anchor addition has been identified as Ser-368 [15]. Thus the observation that the endogenously released form of pancreatic ZGM MDP is a disulphide-linked dimer indicates that the protein has been cleaved C-terminal to Cys-361, by either a proteinase or a phospholipase.

Under reducing conditions, the PI-PLC-cleaved forms of pancreatic ZGM and kidney microvillar membrane MDP migrated on SDS/PAGE with greater apparent molecular masses than the membrane-bound forms (Figures 5a and 5b). This difference in migration of the PI-PLC-released and membrane-bound forms of MDP has previously been shown for the enzyme purified from porcine kidney [11,12]. Certain other GPI-anchored proteins display this phenomenon on SDS/PAGE [45,46], presumably due to anomalous binding of SDS to the hydrophobic fatty acid chains. In contrast, the endogenously released form of pancreatic ZGM MDP migrated on SDS/PAGE under reducing conditions with the same apparent molecular mass as the membrane-bound form (compare lanes 1 and 2 in Figure 5a), suggesting incomplete removal of the fatty acid chains from the GPI anchor. This assumption was confirmed by a shift in the apparent molecular mass of MDP on SDS/PAGE to that of the PI-PLC-released form upon subsequent incubation of the detergent-poor phase of the endogenously released ZGM form with PI-PLC (Figure 5a, lane 4). Confirmation of cleavage by PI-PLC was shown by the subsequent recognition of this form of pancreatic MDP by the anti-CRD antiserum (Figure 5c). This indicates that the endogenously released form of pancreatic MDP is still a substrate for PI-PLC, suggesting that it still has either an intact diacylglycerol moiety (a less likely probability due to the clear change of behaviour upon Triton X-114 phase separation) or, more likely, a partially cleaved anchor, i.e. a lysophospholipid. In support of this, the lyso-alkyl-PI moieties of the *Leishmania major* and *L. donovani* GPI anchors have been shown to be cleaved by *Staphylococcus aureus* PI-PLC [47,48]. In addition, both *B. thuringiensis* and *S. aureus* PI-PLCs hydrolyse lyso-PI [49,50] and, as these enzymes require the presence of at least one fatty acid group attached to the glycerol [51], this provides additional evidence that the GPI anchor on the endogenously released form of pancreatic MDP has at least one of the fatty acid chains intact. Further evidence for the presence of at least part of the hydrophobic moiety on the GPI anchor of endogenously released pancreatic MDP comes from the observation that this form of the enzyme did not bind efficiently to cilastatin–Sepharose, in a similar manner as the form solubilized with octyl glucoside, whereas the PI-PLC-cleaved form bound completely (Table 4). For a number of years we have consistently observed that, following solubilization from the kidney membrane with detergent, the amphipathic form of MDP, which still contains the intact hydrophobic fatty acid chains, does not bind efficiently to cilastatin–Sepharose [12] (Table 4), although the reason for this is not yet known.

Release of MDP from the membrane by PLA

Further information on the identity of the endogenous hydrolase responsible for the release of MDP from pancreatic ZGMs comes from the observation that PLA_2 isolated from porcine pancreas releases MDP from the kidney microvillar membrane (from which there is no endogenous release of MDP; Table 3) in a form that also does not bind efficiently to cilastatin–Sepharose (Table 4) and displays identical electrophoretic behaviour on SDS/PAGE (Figure 5b), migrating with the same apparent

Table 4 Differential binding of the various solubilized forms of MDP to cilastatin–Sepharose

Porcine pancreatic ZGMs or kidney microvillar membranes were incubated either on their own for 6 h at 37 °C (endogenous release), or in the presence of 0.1 unit of *B. thuringiensis* PI-PLC for 2 h at 37 °C, 40 units of PLA_2 for 4 h at 37 °C or 60 mM octyl glucoside for 2 h at 4 °C. At the end of the incubation the samples, other than the octyl glucoside-solubilized membranes (which were incubated with cilastatin–Sepharose directly), were subjected to temperature-induced phase separation in Triton X-114. The resulting detergent-poor phase was incubated with cilastatin–Sepharose for 18 h at 4 °C in 50 mM Tris/HCl, 0.5 M NaCl, pH 7.6. The cilastatin–Sepharose was removed by centrifugation and the activity of MDP in the resulting supernatant, and in the detergent-poor phase before incubation with cilastatin–Sepharose, was determined as described in the Experimental section. The results are the means of duplicate determinations and are representative of two such experiments.

Membranes	Release/ solubilization method	MDP activity (nmol of D-Phe/30 min) Before precipitation with cilastatin–Sepharose	After precipitation with cilastatin–Sepharose	Binding to cilastatin–Sepharose (%)
Pancreatic ZGMs	PI-PLC	838.0	0.0	100.0
	Endogenous	131.6	93.7	28.8
	Octyl glucoside	413.7	276.3	33.2
Kidney microvillar	PI-PLC	649.5	0.0	100.0
	PLA_2	175.5	101.5	42.2
	Octyl glucoside	201.5	85.4	57.6

molecular mass as the membrane-bound form, and subsequently migrating with a greater apparent molecular mass upon cleavage with PI-PLC. Thus it would appear that MDP in pancreatic ZGMs is not cleaved by a protease, but rather by the action of a PLA, resulting in its release from the membrane in a form that is still a substrate (at least *in vitro*) for PI-PLC. We have shown recently that MDP purified from porcine kidney has exclusively diacyl-PI (predominantly distearoyl-PI, with a minor amount of stearoyl-palmitoyl-PI) in its GPI anchor, rather than the alkyl-acyl-PI that is more common in mammalian GPI anchors [15]. Thus, if porcine pancreatic MDP also has diacyl-PI in its anchor, it would be susceptible to cleavage by either PLA_1 or PLA_2 activities. In this context, the diacyl (dimyristoyl)-PI anchor of the *Trypanosoma brucei* variant surface glycoprotein has been shown to be cleaved by *Crotalus atrox* PLA_2 [52], and PLA_2 from *Crotalus adamanteus* has been used to remove the fatty acid from the 2-position on the glycerol of GPI anchors prior to its analysis [53]. So, although it is well documented that GPI anchors can be hydrolysed by exogenously added PLA_2, there has been no report, to our knowledge, of the cleavage and subsequent release of a GPI-anchored protein by an endogenous PLA_1/PLA_2 as indicated in this study for pancreatic MDP.

It has been shown that carboxyl ester hydrolase exists in a membrane-associated form in rat pancreatic ZGMs and that it has Ca^{2+}-independent PLA_1 activity [25]. Like carboxyl ester hydrolase, the endogenous hydrolase described in the present study is inhibited by DipF (Table 3), and the PLA_1 action of carboxyl ester hydrolase on the GPI anchor of MDP would be consistent with the observations in Figure 5. The co-localization in the ZGM of carboxyl ester hydrolase with MDP and GP-2 would clearly suggest that the former could act on the two GPI-anchored proteins to partially cleave their anchors, thereby promoting their release from the membrane.

Functional role of MDP in the pancreas

As demonstrated in the present study for MDP, GP-2 has also been shown to be released in a time- and temperature-dependent manner from the ZGM by an endogenous hydrolase [19,20]. The observation that the released form of GP-2 exhibited nearly identical immunochemical and electrophoretic properties to the membrane-bound form and was not recognized by an anti-CRD antiserum led to the conclusion that it was not released by proteolysis, but more likely by a phospholipase. Although proteolysis has recently been suggested as the mechanism whereby transfected pancreatic GP-2 is released from the apical surface of MDCK cells [54], this mechanism still has to be confirmed in pancreatic tissue. However, the results of the present study clearly show that proteolysis is not involved in the release of MDP from the pancreatic ZGM. The similarity between the level of enzymic activity and the structure of MDP between the pig and the human is worth noting. The amount of MDP in the pig ZGM is more like that in the human ZGM than in the rat membrane. Together with the observation that the structure of the GPI anchor on pig renal MDP is very similar to that on human MDP [15], these results argue for the pig being an excellent model for the human situation at the molecular as well as the physiological level [55].

The possible physiological role(s) of MDP in pancreatic ZGMs is not readily apparent. As its name implies, the catalytic activity

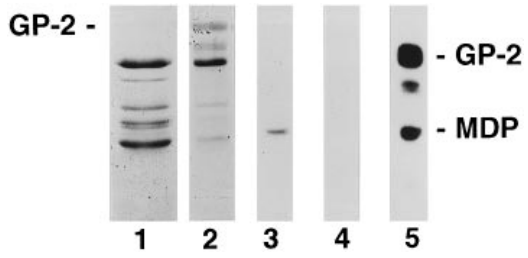

Figure 6 Pancreatic secretion of MDP and GP-2 by the conscious pig under atropine infusion

Electrophoresis on SDS/PAGE under reducing conditions of pancreatic secretions collected during 4 h of continuous intravenous infusion into pigs of 36 pmol of secretin/h per kg. After the first 1 h of secretin infusion, atropine at 50 mg/h per kg was also infused intravenously [56]. Lane 1, proteins secreted under secretin perfusion only; lane 2, proteins secreted during the addition of atropine to the secretin infusion. Both lanes are stained with Coomassie Blue. The pooled samples from the atropine block were subjected to immunoelectrophoretic blot analysis with an antiserum raised against porcine kidney MDP (lane 3) [11], and with anti-CRD antiserum (lane 4) [16]. Lane 5 shows the control reactivity of the anti-CRD antiserum with PI-PLC-treated ZGMs. The anti-CRD reactive band below the GP-2 band is a degradation product of GP-2 that is observed with porcine samples [35].

of MDP is restricted to dipeptides and related small molecules (e.g. leukotriene D_4 and β-lactam antibiotics), and the enzyme is unable to act on larger protein substrates. Thus MDP is unlikely to be involved in cleaving zymogens within the granule. Indeed, it is not clear if its catalytic activity has any role to play; instead, its GPI anchor may be conferring on MDP some packaging/structural role in the ZGM. The observation that the two major integral proteins of the ZGM remaining after high-pH bicarbonate washing are both GPI-anchored supports this hypothesis. Whether the cleavage of its GPI anchor by an endogenous ZGM PLA_1/PLA_2 has a role to play in releasing MDP from the membrane, thus altering the structure of the granule in some way, remains to be determined. In support of this hypothesis, however, is the observation that, like GP-2 [56], MDP is also secreted from the pancreas under atropine infusion (Figure 6, lane 3) in a CRD-negative form (Figure 6, lane 4). In addition, the lack of MDP enzymic and immunoreactivity in the zymogen granule contents (Table 2) suggests that the release of GPI-anchored proteins from the ZGM is a post-exocytotic event, taking place at the earliest just after fusion of the zymogen granule with the apical plasma membrane.

This work was supported by the Medical Research Council of Great Britain, the Canadian Cystic Fibrosis Foundation, NSERC (Canada) and by a NATO Collaborative Research Grant.

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Received 16 October 1996/7 January 1997; accepted 16 January 1997