Endogenous glycosylphosphatidylinositol-specific phospholipase C releases renal dipeptidase from kidney proximal tubules *in vitro*

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Spontaneous enzymic release of renal dipeptidase (RDPase; EC 3.4.13.19), a glycosylphosphatidylinositol (GPI)-linked ectoenzyme, was observed *in vitro* during incubation of porcine proximal tubules at 37 °C. Triton X-114 phase separation of the released RDPase showed that the majority of the enzyme activity partitioned into the aqueous phase, indicating its hydrophilic nature. Immunoblot analyses using an antibody against the cross-reacting determinant (CRD) inositol 1,2-cyclic monophosphate, the epitope formed by phospholipase C (PLC) cleavage of the GPI anchor on a protein, detected the released RDPase. Reprobing the immunoblot with an anti-RDPase serum showed the RDPase band co-migrating with the CRD band. The release of RDPase from the proximal tubules was a Ca^{2+}-dependent process and had a pH optimum of 9.0. These results indicate that RDPase is released from the proximal tubules by the action of a distinct endogenous GPI-specific PLC.

Key words: cross-reacting determinant, inositol 1,2-cyclic monophosphate, GPI-anchor, GPI-specific phospholipase C, membrane dipeptidase.

INTRODUCTION

An increasing number of membrane proteins are linked to the outer leaflet of the lipid bilayer via a glycosylphosphatidylinositol (GPI) anchor [1,2]. Such GPI-anchored proteins may be localized in microdomains [3] and have been implicated in processes such as sorting in polarized cells [4], T-cell activation [5] and cell signalling [5–7], in which the release of GPI-molecules by specific phospholipases may trigger signals *in vivo*. Renal dipeptidase (RDPase; also known as membrane dipeptidase; EC 3.4.13.19), a GPI-anchored ectoenzyme of the kidney, is predominantly present in the brush-border membrane of the renal proximal tubules, but is also present in the lungs, in pancreatic zymogen granules and in adipocytes [8–10]. In a previous report from this laboratory, a soluble form of dipeptidase purified from human urine was identified as RDPase based upon substrate specificity, immunoblot analyses and N-terminal amino-acid sequencing [11]. Thus the existence of a soluble form of dipeptidase is direct evidence of certain enzymic processes resulting in the release of RDPase from its GPI anchor *in vivo*.

Based upon their structural specificity, GPI anchors are susceptible to phospholipases A, C and D (PLA, PLC and PLD respectively) [12–15]. PLC and PLD with specificities for GPI anchors have been purified from *Trypanosoma brucei* [16,17] and rat liver membranes [14], respectively. The mammalian GPI-specific PLD that hydrolyses the glycolipid anchors to *myo*-inositol has been shown to exist as a soluble protein in serum, although a cell-associated form has also been identified in keratinocytes and neuronal cells [18,19]. Bacterial and protozoan PLCs also readily cleave GPI-anchored proteins, exposing inositol 1,2-cyclic monophosphate [Ins(1,2cyc)*P*], called the cross-reacting determinant (CRD), which is specifically recognized by anti-CRD sera [20]. Based upon generation of the CRD epitope, the insulin-stimulated release of RDPase from 3T3-L1 adipocytes was due to the action of a PLC [10]. In the pig exocrine pancreas, however, it was suggested that the membrane-bound RDPase might be released into pancreatic secretions via the action of a PLA [9]. Recently, Kang et al. [21] introduced an *in vitro* system using rabbit proximal tubules from which the release of RDPase was measured during a long-term incubation (6–10 h) in a gentamicin-sensitive manner.

The present study was undertaken to examine and characterize the release mechanism of RDPase from porcine proximal tubules *in vitro*, in order to understand the *in vivo* process more thoroughly.

MATERIALS AND METHODS

Materials

Porcine kidneys were supplied by the Samho Slaughter House (Kwangju, Korea) and were transferred to an ice bath immediately after death. Ala-Ala, alanine dehydrogenase, β-NAD^+, diaphorase, resazurin and dithiothreitol (DTT) were purchased from the Sigma–Aldrich Chemical Company (St. Louis, MO, U.S.A.) and the bicinchoninic acid (BCA) protein assay kit was obtained from Pierce (Rockford, IL, U.S.A.). CNBr-activated Sepharose CL 6B and the ECL® kit for immunoblot analysis were purchased from Amersham Pharmacia Biotech (Little Chalfont, Bucks., U.K.). The anti-CRD serum was described previously [20]. Cilastatin was a gift from Dr Kropp (Merck, Sharp and Dohme, Research Laboratories, Rahway, NJ, U.S.A.). All procedures were carried out at 4 °C, unless otherwise stated.

Preparation of porcine proximal tubules

Proximal tubules were freshly prepared for each experiment, with a slight modification of the protocol described by Taub et al. [22]. Briefly, the kidneys were perfused with cold PBS, to

Abbreviations used: BCA, bicinchoninic acid; CRD, cross-reacting determinant; DTT, dithiothreitol; GPI, glycosylphosphatidylinositol; Ins(1,2-cyc)*P*, inositol 1,2-cyclic monophosphate; KRH, Krebs–Ringer-Hepes; PLA, phospholipase A; PLC, phospholipase C; PLD, phospholipase D; RDPase, renal dipeptidase.

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completely remove the blood, via a polyethylene catheter, which was inserted above the bifurcation of the renal artery. The renal outer sack was removed, and the cortex was minced into small pieces and homogenized by one stroke with a Polytron tissue homogenizer and one stroke with a Teflon/glass homogenizer (GlasCol, Terre Haute, IN, U.S.A.) set on low speed. The crude homogenate was passed through a 253 μm nylon mesh and then an 83 μm mesh (Tetko, Kansas City, MO, U.S.A.). The proximal tubules retained on the 83 μm mesh were washed with 3 vols. of cold PBS and then with an equal volume of Krebs–Ringer-Hepes (KRH) buffer consisting of 25 mM Hepes (pH 8.0), 125 mM NaCl, 5 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 2 mM $CaCl_2$ and 6 mM glucose. The scraped proximal tubules were then resuspended in 2 vols. of KRH buffer and centrifuged at 600 ***g*** for 5 min. The pellet was resuspended in 3 vols. of KRH buffer and was referred to as the 'proximal tubules' and was used for the subsequent study as a source of GPI-specific PLC, as well as an RDPase-enriched compartment, i.e. the substrate of GPI-specific PLC.

Time course of RDPase release

Aliquots of proximal tubules (500 μl) were incubated at 37 °C for different time intervals (0, 2, 5, 10, 15, 30 and 60 min) with gentle agitation, after which the reaction tubes were transferred to a solid CO_2/acetone bath to stop the reaction. The frozen tubes were slowly thawed at room temperature, with brief swirling, and were then centrifuged at 18000 ***g*** for 3 min. In subsequent experiments, the samples were incubated for 20 min and were centrifuged immediately without freezing and thawing. The supernatant is referred to as the 'incubation supernatant' and is used as the source of released RDPase.

Spectrofluorometric assay for RDPase

The enzymic activity of the released RDPase was assayed according to the fluorometric method of Ito et al. [23]. A typical 2.5 ml reaction mixture consisted of 1.5 μM L-Ala-L-Ala, 0.4 unit of L-alanine dehydrogenase, 1.5 μM β-NAD^+, 0.06 unit of diaphorase and 0.1 μM resazurin sodium in 12.5 mM Na_2CO_3 buffer (pH 9.0). The incubation supernatant (20 μl), containing the released RDPase, was the final addition to the preincubated reaction mixture, and the reaction was carried out for 2 min at 25 °C. The concentration of resorufin, the fluorescent product of the coupled reactions, was measured photometrically for 2 min using its excitation and emission maximums of 568 nm and 589 nm respectively using a JASCO spectrofluorimeter (JASCO Corporation, Hachioji City, Tokyo, Japan). GPI-specific PLC activity is expressed as the arbitrary fluorescent intensity resulting from the amount of RDPase released.

Phase separation in Triton X-114

Samples were prepared in 200 μl of TS buffer [10 mM Tris/HCl (pH 7.6) and 150 mM NaCl] containing 1 % (v/v) Triton X-114 at 0 °C, and were subjected to phase separation into an aqueous phase and a Triton X-114 phase [24]. Protein samples were overlaid on to 300 μl of a chilled sucrose cushion [TS buffer containing 6 % (w/v) sucrose and 0.06 % Triton X-114]. The tube was incubated at 37 °C for 5 min until the solution became turbid, and was subsequently centrifuged (300 ***g*** for 3 min) at room temperature. The upper aqueous phase was removed to another tube and supplemented with Triton X-114 to 0.5 %. The aqueous phase was overlaid on to the same sucrose cushion as before and phase separation was carried out again. The volumes of the two phases were made equal through the addition of TS buffer to the detergent phase and aliquots from both phases were used to assay the released RDPase.

Purification of released RDPase

A large quantity of pooled 30-min incubation supernatant (approx. 300 ml) was used to purify the released RDPase by affinity chromatography, using cilastatin as the ligand [11]. The hydrophilic form of RDPase was obtained by treating the proximal tubules with *Bacillus cereus* PI-specific PLC (1 unit/50 mg protein) for 2 h at 37 °C and then centrifugation (18000 ***g*** for 3 min) followed by affinity chromatography. The eluent was concentrated using an Amicon concentrator with a 30000 molecular-mass cut-off.

Immunoblot analyses

Samples were resolved by SDS/PAGE (12 % polyacrylamide gels) and were electrotransferred on to a sheet of nitrocellulose, followed by treatment with blocking buffer [1 % (w/v) non-fat dry milk and 0.5 % Tween-20 in PBS] for 1 h at room temperature. The sheet was then rinsed twice with wash buffer (milk-free blocking buffer). The sheet was subsequently incubated for 1 h with the anti-CRD serum, as the primary antibody, in the wash buffer. After three washes, it was reacted with the horseradish-peroxidase-conjugated goat anti-rabbit IgG, the secondary antibody, in the wash buffer and incubated overnight at 4 °C. The immune complex was detected on an X-ray film after a 5 min exposure using the chemiluminescence method of the manufacturer (ECL® kit). Reprobing was carried out by first stripping the antibodies off the nitrocellulose sheet with stripping buffer [62.5 mM Tris/HCl (pH 6.8), 3 % (w/v) SDS and 50 mM DTT] for 30 min at 55 °C. This was repeated a second time. The sheet was then treated with a blocking buffer containing 3 % (w/v) non-fat dry milk, and then probed with an anti-RDPase serum having the secondary antibody in the same buffer. Protein concentrations were determined using a BCA protein assay kit, with BSA as the protein standard.

Mild acid treatments

Aliquots of the incubation supernatant were deaminated by treatment with 0.25 M HNO_2 [0.25 M sodium acetate/0.25 M $NaNO_2$ (pH 5.0)] for 3 h at 23 °C [20]. The Ins(1,2-cyc)*P* ring, formed on PI-specific PLC cleavage, was selectively decyclized by incubation of the affinity-purified released RDPase in 1 M HCl for 30 min at 23 °C. The samples were neutralized with NaOH and immunoblotted as described above.

Extracellular [Ca^{2+}] influence on RDPase release

Proximal tubules were resuspended in 3 vols. of Ca^{2+}-free KRH buffer and the suspensions were incubated with 2 mM EGTA and various concentrations of Ca^{2+} (0, 1, 2, 4, 6 and 8 mM). Aliquots of the incubation supernatant were then assayed for RDPase release.

Proximal tubules were also treated with 1,10-phenanthroline in a concentration-dependent manner.

pH profiles of the enzyme activities

Buffers with different pH ranges were made as follows: 50 mM sodium acetate/acetic acid buffer (pH 4.5–5.5); 50 mM Tris/maleic acid buffer (pH 6.0–8.0); and 50 mM glycine/NaOH buffer (pH 8.5–10.5). All contained 50 mM NaCl and 2 mM $CaCl_2$. Aliquots of proximal tubules were prewashed twice with

each buffer and then resuspended in the corresponding buffer. They were incubated at 37 °C for 20 min followed by centrifugation at 18000 ***g*** for 3 min. All the supernatants were then adjusted to a pH of 7.0 and to the same volume, and assayed for RDPase activity.

RESULTS

RDPase is released from proximal tubules by an endogenous hydrolase

To determine whether RDPase was released from its GPI anchor through enzymic hydrolysis, the release of RDPase from porcine proximal tubules was examined as a function of time. Antibiotics were included to examine whether an enzyme originating from bacterial contamination caused the RDPase release. As shown in Figure 1, the RDPase release was almost proportional to the incubation time for the first 15 min and then reached a plateau. The same time curve was obtained in the presence of 10 mM lincomycin, and with other antibiotics, such as gentamicin and neomycin (results not shown), which were present throughout the experiment. Similar results were also obtained when protease inhibitors (trypsin inhibitor, EDTA and PMSF) were used during the tubule preparation and incubation to prevent non-specific hydrolysis of membrane-bound RDPase (results not shown). These results suggest that the release of RDPase from the proximal tubules is accomplished by an endogenous hydrolase that does not originate from bacterial contamination. Therefore all subsequent experiments were carried out in the absence of antibiotics.

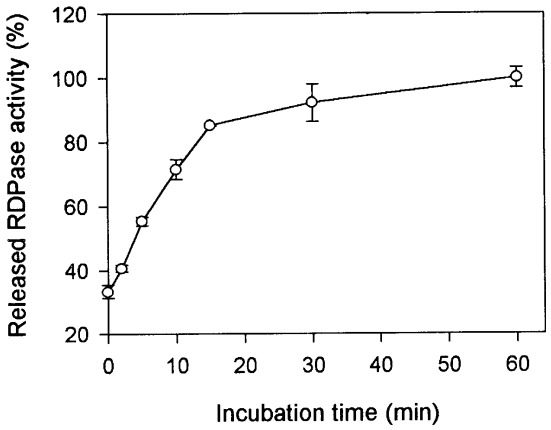

Figure 1 Endogenous release of RDPase from proximal tubules

Porcine proximal tubules were prepared in KRH buffer and aliquots of 0.5 ml (approx. 4 mg/ml) were incubated for the indicated times at 37 °C followed by centrifugation (18000 ***g*** for 3 min). The RDPase released into the incubation supernatant was measured as described in the Materials and methods section. Results are presented as means ± S.D. ($n = 3$).

Triton X-114 phase separation of released RDPase

The kidney cortex homogenate was solubilized with 20 % (v/v) n-butanol for 48 h at 4 °C, and the butanol-solubilized RDPase was further purified by affinity chromatography, using cilastatin as the ligand [11]. When the RDPase affinity-purified after butanol-solubilization was subjected to Triton X-114 phase separation, its activity was recovered predominantly in the detergent phase (65.2 ± 1.5 %; mean ± S.D.). Treatment of this butanol-solubilized RDPase with bacterial PI-specific PLC resulted in a shift of most of the RDPase activity into the aqueous phase (90.4 ± 2.0 %). The RDPase affinity-purified after incubation of the proximal tubules with bacterial PI-specific PLC partitioned predominantly into the aqueous phase (94.7 ± 2.7 %). When the RDPase released from the proximal tubules by the endogenous hydrolase was subjected to phase separation, 90.2 ± 1.0 % was recovered in the aqueous phase. These results are consistent with the complete removal of the acyl chains from the GPI anchor of RDPase upon its release from the proximal tubules by the endogenous hydrolase.

Identification of the CRD epitope on the released RDPase

RDPase purified following release from the membrane with either bacterial PI-specific PLC (Figure 2A, lane 1) or the endogenous hydrolase (Figure 2A, lane 2), gave a single band having the same mobility after Coomassie Blue staining of the native polyacrylamide gel. Immunoblot analysis with the anti-CRD serum, as the primary antibody, showed multiple bands

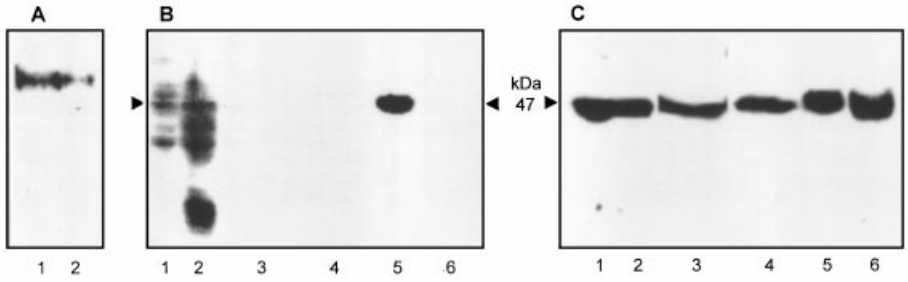

Figure 2 Electrophoretic analyses of RDPase

Samples were prepared as described in the Materials and methods section. (**A**) Protein staining. Samples were subjected to native PAGE (10% polyacrylamide gel), followed by staining with Coomassie Brilliant Blue R-250. Lane 1, affinity-purified bacterial PI-specific PLC-digested RDPase (5 μg); lane 2, affinity-purified endogenously released RDPase (5 μg). (**B**) Immunoblot with anti-CRD serum. Samples were resolved by SDS/PAGE (12% polyacrylamide gel). After electrophoretic transfer on to a nitrocellulose sheet, the samples were reacted with the anti-CRD serum. Lane 1, supernatant after digestion of the proximal tubules with bacterial PI-specific PLC followed by ammonium sulphate precipitation (50–75% saturation; 200 μg); lane 2, supernatant after 30-min incubation of the proximal tubules, i.e. endogenously released supernatant (200 μg); lane 3, 30-min incubation supernatant treated with 0.25 M HNO_2 (200 μg); lane 4, butanol-solubilized purified RDPase (5 μg); lane 5, affinity-purified endogenously released RDPase (5 μg); lane 6, affinity-purified endogenously released RDPase treated with 1 M HCl (5 μg). (**C**) Immunoblot with polyclonal anti-RDPase serum. The nitrocellulose sheet was stripped to remove the anti-CRD serum and was reprobed with anti-RDPase serum as described in the Materials and methods section. Lanes as in (**B**).

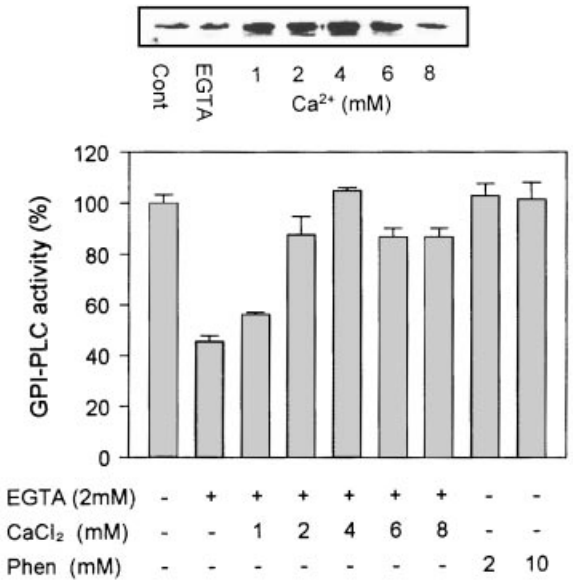

Figure 3 Effect of Ca^{2+} on the release of RDPase

Bottom panel: the proximal tubules were resuspended in Ca^{2+}-free KRH buffer and aliquots were treated with either Ca^{2+} (0, 1, 2, 4, 6 and 8 mM) in the presence of 2 mM EGTA or with 1,10-phenanthroline (phen; 2 and 10 mM), for 20 min at 37 °C. The RDPase released into the incubation supernatant was assayed as described in the Materials and methods section, and the GPI-specific PLC (GPI-PLC) activity was expressed as a percentage compared with the control (cont), i.e. the activity present in proximal tubules in Ca^{2+}-free KRH buffer. Results are presented as means ± S.D. ($n \geqslant 3$). Top panel: the corresponding protein samples (40 μg) were resolved by SDS/PAGE (12% polyacrylamide gel) and immunoblotted with anti-RDPase serum.

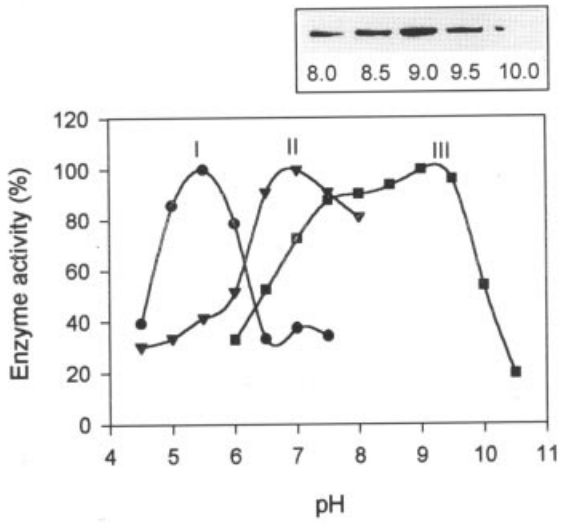

Figure 4 pH profiles of bacterial PI-specific PLC, RDPase and the endogenous GPI-specific PLC

Bottom panel: proximal tubules were prepared in buffers with pH values, ranging from 4.5–10.5, as described in the Materials and methods section. Curve I, aliquots of proximal tubules at pH 4.5–7.5 were treated with *B. cereus* PI-specific PLC (1 unit/50 mg protein) for 2 h at 37 °C, followed by centrifugation (18000 ***g*** for 3 min). The activity of bacterial PI-specific PLC was determined by measuring the released RDPase, which was assayed by a UV-spectrophotometric method using glycyldehydrophenylalanine as a substrate [40]. Curve II, the RDPase (0.1 mg/ml) that was partially purified after bacterial PI-specific PLC-digestion was preincubated in each buffer (pH 4.5–8.0) for 2 min and assayed using the above UV-spectrophotometric method. Curve III, aliquots of proximal tubules were incubated for 20 min at 37 °C followed by centrifugation (18000 ***g*** for 3 min). The released RDPase in supernatants was assayed by the fluorometric method described in the Materials and methods section. The maximum activities were expressed as 100%. Top panel: protein samples (30 μl) from the incubation supernatants corresponding to each point of the endogenous GPI-specific PLC activity (curve III) between pH 8.5 and 10 were resolved by SDS/PAGE (12% polyacrylamide gel) and were immunoblotted with anti-RDPase serum.

with the bacterial PI-specific PLC-digested supernatant of the proximal tubules (Figure 2B, lane 1) and with the 30-min incubation supernatant in which the proteins had been released from the tubules by the endogenous hydrolase (Figure 2B, lane 2). Treatment of the 30-min incubation supernatant with 0.25 M HNO_2 and of the affinity-purified released RDPase with 1 M HCl prior to electrophoresis removed the CRD epitopes (Figure 2B, lanes 3 and 6 respectively). Butanol-solubilized RDPase was not recognized by the anti-CRD serum due to the cryptic nature of the epitope (Figure 2B, lane 4). The purified RDPase released from the tubules by the endogenous hydrolase gave a single band at 47 kDa with the anti-CRD serum (Figure 2B, lane 5).

When the anti-CRD antibody was removed from the nitrocellulose sheet with the stripping buffer and the protein bands were reprobed with the polyclonal anti-RDPase serum, a single 47 kDa band was seen in all of the samples (Figure 2C). Disappearance of the CRD epitope (Figure 2B, lanes 3 and 6) following nitrous acid or HCl treatment was not due to the degradation of the protein, as shown by the immune complex with the anti-RDPase serum (Figure 2C, lanes 3 and 6 respectively). These results demonstrate that the RDPase released from the proximal tubules by the endogenous hydrolase contains the Ins(1,2-cyc)*P* moiety, which is evidence of cleavage by a PLC.

GPI-specific PLC that releases RDPase is Ca^{2+}-dependent

The activities of PLC isoenzymes and peanut GPI-specific PLC are known to depend on the presence of Ca^{2+} [25,26], whereas trypanosomal GPI-specific PLC does not require Ca^{2+} for activity [17,27]. We therefore investigated if the activity of the endogenous GPI-specific PLC that releases RDPase is dependent on the extracellular Ca^{2+} concentration in the proximal tubules. As shown in Figure 3 (bottom panel), the release of RDPase diminished to approx. 46% in the presence of 2 mM EGTA, but release was restored with Ca^{2+} concentrations greater than 2 mM, whereas the release was not affected with 2–10 mM 1,10-phenanthroline, which chelates mainly iron but not Ca^{2+}. The release of RDPase by the GPI-specific PLC, as monitored by measuring the enzyme activity of the released RDPase, matched closely with the amount of released RDPase that was detected by the polyclonal antibody, with the highest level of RDPase protein being detected at a concentration of 4 mM Ca^{2+} (Figure 3, top panel).

GPI-specific PLC that releases RDPase has a basic pH optimum

The pH optimum for the release of RDPase by the endogenous GPI-specific PLC was determined using pH values ranging from 6.5 to 10.5. The activity of the released RDPase was measured at pH 7.0, since this is its optimal pH (Figure 4, peak II). The bacterial PI-specific PLC (*B. cereus*) was most active at pH 5.5 (Figure 4, peak I). The optimal pH for the endogenous GPI-specific PLC was found to be pH 9.0 (Figure 4, peak III). The asymmetrical shape of the curve in the lower pH range (below 8.5) is probably due to the activity of RDPase itself. The trend of peak III was quantitively confirmed with the results of the

immunoblot analysis using polyclonal anti-RDPase serum (Figure 4, upper panel).

DISCUSSION

Many proteins anchored to the cellular membrane via a GPI moiety are found in soluble forms in extracellular fluids. We have previously identified a soluble form of dipeptidase in human urine as the released form of RDPase [11]. Soluble dipeptidase activity was also observed in the urine of various animals, such as cow, rat, rabbit, dog and pig (S. W. Park, K. Choi, H. H. B. Lee, S. K. Park, A. J. Turner, N. M. Hooper and H. S. Park, unpublished work). These findings allow for the putative suggestion that there are certain endogenous release mechanism(s) for GPI-anchored proteins during homoeostasis through either constitutive release or in response to extrinsic stimuli.

The butanol-solubilized RDPase was amphipathic in nature, indicating the presence of the acyl chains on the GPI anchor, whereas the released RDPase was hydrophilic, as it was recovered in the aqueous phase after Triton X-114 phase separation. Similar results were obtained with the soluble dipeptidase in human and porcine urine (S. W. Park, K. Choi, H. H. B. Lee, S. K. Park, A. J. Turner, N. M. Hooper and H. S. Park, unpublished work). The hydrophilic nature of the released RDPase indicates the involvement of a PLC or PLD, ruling out PLA or non-specific carboxylesterases, because PLA produces an amphipathic form that partitions into the detergent phase [9].

GPI-specific PLD has been studied in depth in mammalian plasma and serum, as well as in the placenta, brain and liver plasma membrane fragments and in HeLa cells [19,28–30]. GPI-specific PLD generates *myo*-inositol from the variant surface glycoprotein of *Trypanosoma brucei* [14,18,31], but is not able to hydrolyse GPI anchors from intact cells in the absence of detergents [30,32]. In contrast, GPI-specific PLCs have been purified from or identified in rat liver membranes and mouse brain membranes [12,33], and have been shown to be responsible for the cleavage of the GPI anchor on the Trypanosome variant surface glycoprotein, leading to the production of Ins(1,2-cyc)*P* and Ins(1)*P*. The anti-CRD serum exclusively recognizes the Ins(1,2-cyc)*P* determinant and does not react with other protein epitopes, including *myo*-inositol [20]. Based upon the CRD epitope, Paul et al. [34] reported the involvement of an endogenous PLC in the solubilization of GPI-anchored glycoprotein-2 ('GP-2'), an exocrine pancreatic secretory protein.

The immunoblot analysis of bacterial PI-specific PLC-digested or endogenously released supernatants of the proximal tubules identified many protein bands with Ins(1,2-cyc)*P* moieties. Abolition of the CRD epitope by nitrous acid and HCl, which are known to hydrolyse the glucosamine-inositol bond and to selectively decyclize the Ins(1,2-cyc)*P* ring [20], respectively, confirmed the involvement of a PLC in the release of several proteins from their GPI anchors. The identification of the Ins(1,2-cyc)*P* epitope in the released RDPase provides unequivocal evidence that a GPI-specific PLC, rather than a PLD, is involved in the release of RDPase from the proximal tubules, and not only RDPase but also other GPI-anchored proteins, as indicated by the multiple bands detected with the anti-CRD antibody. The concentration-dependent effect of Ca^{2+} on the release of RDPase and lack of effect of 1,10-phenanthroline strongly suggest that this endogenous GPI-specific PLC requires extracellular calcium for its activity. No effect of 1,10-phenanthroline on RDPase release from the proximal tubules is further evidence for excluding the involvement of GPI-specific PLD, because 1,10-phenanthroline is known to inhibit GPI-specific PLD [35]. The Ca^{2+} dependency of this GPI-specific PLC is similar to already known peanut GPI-specific PLC and PI-specific PLCs [25,26], but different from trypanosomal GPI-specific PLC and the bacterial PI-specific PLC (*B. cereus*), which are Ca^{2+}-independent [27,36]. The pH optimum of this endogenous GPI-specific PLC is pH 9.0, clearly different from the PI-specific PLC of *B. cereus* (pH 5.5). The intracellular PLC isoenzymes, the GPI-specific PLC in the mouse brain membrane, and the ecto-PLC of the Swiss 3T3 cell surface showed acidic pH optima of approx. pH 5.5 [12,25,37], while trypanosomal GPI-specific PLC had a maximum at approx. pH 8.5 [27]. The Ca^{2+} dependency and the basic pH optimum (pH 9.5) suggest that the GPI-specific PLC in the porcine proximal tubules that releases RDPase and other GPI-anchored proteins may be a novel enzyme distinct from the previously reported mammalian GPI-specific PLCs.

Soluble dipeptidase has been proposed as a diagnostic marker enzyme, because its activity in urine samples of acute and chronic renal failure patients decreases [38,39]. This allows us to postulate that the GPI-specific PLC may be regulated by various physiological conditions, possibly dependent on [Ca^{2+}]. GPI-specific PLC, through its release of GPI-anchored proteins, may play important role(s) in cellular events, which occur under normal physiological conditions to maintain the proximal tubular homoeostasis and during pathological conditions. This endogenous GPI-specific PLC may also be implicated in extracellular signal transduction, which is non-receptor mediated, and in cell proliferation through the release of GPI-anchored proteins *in vivo* and *in vitro* from normal epithelial cells, as was proposed for immune cells [6].

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