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Spontaneous Release of Glycosylphosphatidylinositol (GPI)-anchored Renal Dipeptidase from Porcine Renal Proximal Tubules

Sung Wook Park[1], Bok Yun Kang[1], Hyun Joong Yoon[2], Eun Mi Park[1], Kyong Choi[2], Hwanghee Blaise Lee[2], Nigel M. Hooper[3], and Haeng Soon Park[1]

[1]Department of Pharmacy, College of Pharmacy, [2]Department of Biological Sciences, College of Natural Science, Chonnam National University, Kwangju 500-757, Korea, and [3]Proteolysis Research Group, School of Biochemistry and Molecular Biology, University of Leeds, Leeds, LS2 9JT, U.K.

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The incubation of porcine renal proximal tubules (PTs) resulted in the release of the glycosylphosphatidylinositol (GPI)-anchored renal dipeptidase (RDPase, EC 3. 4. 13. 19) from the membrane after a lag period of approximately 6 hours. This spontaneous release of RDPase from the membrane was inhibited by antibiotics. When the incubation supernatant was added back to fresh PTs, both the antibiotic inhibition of RDPase release and the lag period disappeared. The released RDPase reacted with an anti-cross reacting determinant antibody indicating the presence of the Ins (1,2-cyc)*P* moiety. These results suggest that bacteria in the PTs, when incubated, grow and secrete a phosphatidylinositol-specific phospholipase C (PI-PLC). This enzyme then hydrolyses the GPI-anchored RDPase and is transferable. RDPase was purified following its release from the membrane by this simple and inexpensive method which may also be applied to other GPI-anchored proteins.

Key words: Spontaneous release, glycosylphosphatidylinositol (GPI)-anchor, cross-reacting determinant (CRD), phospholipase C, antibiotics

INTRODUCTION

Renal dipeptidase (RDPase, EC 3.4.13.19) is a well known glycosylphosphatidylinositol (GPI)-anchored ectoenzyme of renal proximal tubules (PTs) microvilli. The same enzyme activity detected in urine, called urinary dipeptidase, was identified as the released form of RDPase on the basis of amino acid sequence (We *et al.*, 1997), and it has been proposed as a diagnostic enzyme of renal disease (Ito *et al.*, 1984; Lee *et al.*, 1999; Fukumura *et al.*, 1999).

Traditionally, the solubilization of RDPase from the membrane was achieved with organic solvents such as n-butanol (Campbell, *et al.*, 1966; Park *et al.*, 1993) or with detergents such as Triton X-100 (Hitchcock *et al.*, 1987) and octyl-β-D-glucopyranoside (Adachi *et al.*, 1989; Hooper *et al.*, 1989), followed by an extensive dialysis or other measures to remove the solubilizing agents. The solubilization achieved by these methods produces an amphipathic form of RDPase, containing the intact GPI anchor. Recently, however, GPI-anchored proteins including RDPase were also released from the membrane with phosphatidylinositol-specific phospholipase C (PI-PLC) which is purified of various bacterial species such as *Bacillus, Clostridium, Staphylococcus, Listeria* (Hooper *et al.*, 1987; Ferguson, 1992; Lehto and Sharom, 1998; Iwasaki *et al.*, 1998; Griffith and Ryan, 1999) and with trypanosomal GPI-specific PLC (Stieger and Brodbeck, 1991). The use of these enzymes for solubilization of RDPase results in the hydrophilic form containing the Ins (1,2-cyc) *P* moiety, also called the cross reacting determinant (CRD), which is formed by PI-PLC cleavage of the GPI anchor.

Kang *et al.* (1999) examined an *in vitro* assay system of rabbit PTs to use as a model to study nephrotoxicity of various drugs. The system was found unsuitable for screening purpose in general, but it behaved in a very peculiar way. RDPase spontaneously released itself from the rabbit PTs without the addition of any commercial hydrolase,

Correspondence to: Haeng Soon Park, Department of Pharmacy, College of Pharmacy, Chonnam National University, Kwangju 500-757, Korea
E-mail: haspark@chonnam.ac.kr

even though the rabbit PTs had been prepared in the normal laboratory condition with necessary precautions to prevent contamination.

In this study, we examined the nature of this spontaneous release of RDPase from its membrane anchorage using rabbit and porcine PTs, and purified RDPase following this simple and inexpensive solubilization method.

MATERIALS AND METHODS

Materials

Porcine kidneys were kindly provided by Samho Slaughter House, Kwangju, Korea. New Zealand white rabbits (approximately 1.5 kg) were purchased from Duksan animal farm, Kwangju, Korea. The polyclonal antibody against porcine RDPase was raised in rabbits. The anti-CRD antiserum was prepared as described in Broomfield and Hooper (1993). The 83 and 253 μm nylon meshes from Tetko, Inc. (Kansas City, MO, USA) were soaked in 70% ethanol before use. Furosemide, ampicillin, kanamycin, gentamicin, leukotriene D_4 (LTD_4), Dulbecco's modified Eagle's medium (DMEM) and CNBr-activated Sepharose 4B were purchased from Sigma (St. Louis, MO, USA). DMEM was filter-sterilized. The ECL® kit for immunoblot analysis was purchased from Amersham Pharmacia Biotech (Buckinghamshire, UK). Glycyldehydrophenylalanine (Gdp), the substrate of RDPase, was synthesized according to the method of Campbell *et al.* (1963). Cilastatin was a kind gift from Dr. H. Kropp (Merck, Sharp and Dohme Research Laboratories, Rahway, NJ, USA). Solutions, surgical tools, glassware and other necessary equipment were autoclaved before use. The PTs were incubated at 37°C, and the enzyme was purified at 4°C.

Preparation of PTs

Rabbits were sacrificed by cervical dislocation and the abdominal area was shaved and wiped with 70% ethanol. The PTs were prepared as described in Kang *et al.*(1999). Rabbits' fresh kidneys were perfused briefly with ice-cold phosphate-buffered saline (pH 7.4) followed by iron oxide (0.5% w/v) prepared by the method of Cook and Pickering (1958). The iron oxide-entrapped glomeruli were removed from the 83 μm mesh with a magnetic bar. The remaining portion was incubated for 2 min with soybean trypsin inhibitor (0.025% w/v) and centrifuged (600 *g*, 10 min). The pellet was resuspended in DMEM, and is referred to as 'PTs' in this paper. Porcine PTs were prepared in the same manner but using a catheter instead of a needle for perfusion.

Release of RDPase from PTs as a function of time

Rabbit or porcine PTs were incubated at 37°C with gentle shaking and aliquots were removed with 1 h intervals followed by centrifugation (18,000 *g*, 5 min). The supernatants were assayed for the released RDPase according to the method of Campbell *et al.* (1963) with Gdp (5.2×10^{-5} M in 2 mM Tris-HCl, pH 7.4, $E^{275\ nm} = 1.56 \times 10^4\ M^{-1}cm^{-1}$) hydrolysis monitored at 37°C. The enzyme activity, unit (U), was defined as μmol Gdp hydrolyzed per min. The 'long term' incubation supernatant refers to those supernatants obtained from the PTs with the maximum release which took place in 8-15 h incubations.

Effects of various compounds on the release of RDPase from rabbit PTs

The effect of various compounds on the release of RDPase was examined on rabbit PTs. In a typical assay, a total reaction mixture of 300 μl contained 250 μl of rabbit PTs (approximately 2 mg/ml, RDPase of 170 mU/ml) and various compounds such as furosemide, LTD_4, antibiotics, or DMEM for the control. They were incubated for short term (2 h) or long term (6 h) with gentle shaking followed by centrifugation (18,000 *g*, 5 min). The supernatants were assayed for the released RDPase with the activity in the control tube (no additional compound) taken as 100%.

Effects of long term incubation supernatant and gentamicin on the release of RDPase

The long term incubation supernatant (6 h) was added to another batch of rabbit PTs and incubated both in the presence of and in the absence of gentamicin for 2 h, respectively. After centrifugation, the released RDPase was assayed and calibrated by subtracting the carry-over activity in the long term incubation supernatant.

Purification of porcine RDPase released by long term incubation

A large volume of porcine PTs (300 ml) was incubated for a long term (9 h) and the supernatant was subjected to ammonium sulfate (AS) fractionations, 0-50 and 50-75% saturation. The 50% saturation was obtained by adding AS powder (314 g/l) to the supernatant with stirring in the ice-bath followed by overnight standing at 4°C. It was centrifuged (23,000 *g*, 20 min) and additional AS was added to the supernatant (172 g/l) to precipitate the 50-75% saturation fraction. After the centrifugation and dialysis of the resuspended pellet, it was further purified by affinity chromatographic technique (Park *et al.*, 1993). Briefly, cilastatin, the competitive inhibitor of RDPase, was coupled to one gram of CNBr-activated sepharose 4B (1 mg/ml, 5 ml) according to the method of Kropp *et al.* (1982). The resuspended AS fraction (50-75%) was loaded onto the column and was thoroughly washed. The cilastatin-bound enzyme

was eluted with cilastatin (1 mg/ml, 10 ml). The protein concentration was determined by the Bradford method (Bradford, 1976) with BSA as the standard protein.

Effect of porcine AS fractions on RDPase release from rabbit PTs

The porcine AS fractions obtained from the long term incubation supernatant (9 h, 37°C) were incubated with rabbit PTs in time- and dose-dependent manners to examine if the RDPase-releasing activity is applicable beyond its own species.

Western blot analyses

A Partial characterization of purified RDPase was carried out with electrophoresis and the Western blot analyses. The purified RDPase was incubated with 0.25 M HNO_2 by reacting with 0.25 M sodium acetate and 0.25 M $NaNO_2$ (pH 4.0) for 3 h or with 1 M HCl for 30 min at 23°C and was neutralized prior to SDS-PAGE.

The Western blot analysis was carried out as described previously (Park *et al.,* 2001). Briefly, the protein bands were transferred to a nitrocellulose membrane and were incubated with polyclonal antiserum. The bound primary antibodies were detected with horseradish peroxidase-conjugated secondary antibody, and the immune complex identified with the ECL® kit. Reprobing was carried out to detect the CRD epitope with the anti-CRD serum after removing the polyclonal antibodies from the membrane by incubation with the stripping buffer [62.5 mM Tris-HCl, pH 6.8, 3.0% (w/v) SDS and 50mM dithiothreitol] for 1 h at 55 °C.

RESULTS AND DISCUSSION

Antibiotics inhibit the release of RDPase from rabbit PTs

The effects of several compounds on the spontaneous release of RDPase from rabbit PTs were examined and the results are summarized in Table I. The enzyme release in the control tube, PTs incubated in DMEM only, was taken as 100% (191.3 mU released RDPase/mg protein). Furosemide, a potent diuretic agent, and LTD_4, a substrate of RDPase, as well as a biogenic sulfidipeptide (Farrell *et al.,* 1987), did not affect the release of RDPase significantly. However, RDPase release was inhibited in a concentration-dependent manner by ampicillin, kanamycin and gentamicin after a long term (6 h) incubation. There was no detectable RDPase release after a short term incubation (2 h). Previously, Kang *et al.* (1999) reported that a nephrotoxic compound cisplatin, an inflammation factor lipopolysaccharide, and an anti-inflammatory factor indomethacin

Table I. Effect of various chemicals on the release of RDPase from the rabbit PTs with 6 h incubation

Treatment	Released RDPase activity (%)[a]
Control	100
Diuretic agent	
Furosemide (1.2×10^{-4} M)	100.5 ± 11.0
Furosemide (1.2×10^{-3} M)	94.5 ± 2.3
Leukotriene	
LTD_4 (1×10^{-8} M)	94.5 ± 1.9
LTD_4 (1×10^{-6} M)	103.5 ± 1.0
Antibiotics	
Ampicillin (1×10^{-5} M)	104.6 ± 0.6
(1×10^{-4} M)	6.8 ± 0.1
Kanamycin (1.7×10^{-6} M)	63.0 ± 0.5
(1.7×10^{-5} M)	3.6 ± 0.0
Gentamicin (8×10^{-7} M)	88.7 ± 5.5
(8×10^{-4} M)	0

[a]Released RDPase of the control experiment was taken as 100% (191.3 mU/mg protein).
Each value represents mean ± S.D. of 3 or more determinations.

did not affect the RDPase release, but gentamicin exhibited a concentration-dependent inhibition of RDPase release from the rabbit PTs with a long term incubation. Of the chemicals examined so far, only the antibiotics significantly inhibited the release of the GPI-anchored RDP ase.

The RDPase-releasing entity was transferable as the 6 h incubation supernatant (1.3 mg/ml, 50 μl) of the control rabbit PTs caused the release of RDPase from the new rabbit PTs with only a 2 h incubation. The 600 mU RDPase activity/mg protein of the supernatant in the control tube was taken as 100%. At this condition, the system was gentamicin-insensitive as the presence of gentamicin (8.4×10^{-4} M) made no significant difference in the release of RDPase from the PTs; 95.2 ± 13.5% of the control release.

Spontaneous RDPase release also observed with porcine PTs

The incubation of porcine PTs also produced a significant amount of RDPase which was released upon long term incubation (Fig. 1). RDPase releases took place with a large variation of the lag period from 4 to 9 h. The activity of the released RDPase increased almost twice as much as the unit activity in the PTs. Such an increase of enzyme activity with the release from the membrane has been observed repeatedly and was understood to have resulted from the altered accessibility to the active site as a result of a conformational change in the protein (Hooper and Turner, 1989; Kang *et al.,* 1999; Campbell *et al.,* 1990; Brewis *et al.,* 1994).

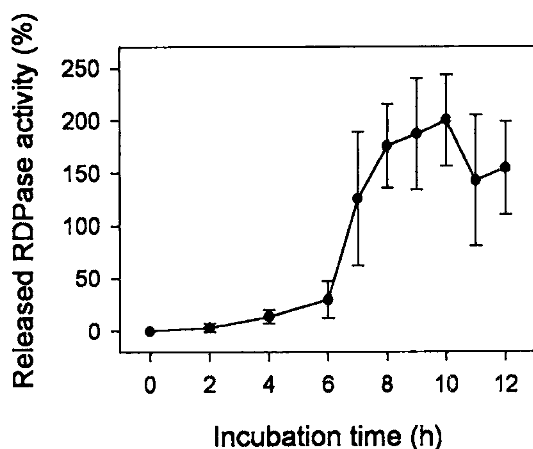

Fig. 1. Release of RDPase from porcine PTs as a function of time. Aliquots of porcine PTs (500 μl) were incubated at 37°C for the indicated time followed by centrifugation (18,000 *g*, 5min). The RDPase released into the incubation supernatants was measured spectrophotometrically using Gdp as substrate according to the method of Campbell *et al.* (1963). The released RDPase activity was expressed as the percentile of the total activity of PTs prior to the incubation (mean ± S.D., n = 3).

Fifty percent AS fraction caused RDPase release

The long term incubation supernatant from the porcine PTs was subjected to AS fractionation as described previously. When the 50% AS fraction (1.7 mg/ml) of the long term incubation supernatant was incubated with the rabbit PTs, RDPase release took place as a function of time without a lag period (Fig. 2A). This immediate release was confirmed as a function of protein concentration with a 2 h incubation (Fig. 2B). RDPase release by 50-75% and by 75-100% saturation fractions was not significant (data not shown). These results demonstrated that the RDPase GPI anchor hydrolyzing entity was exclusively enriched in the 50% AS fraction. These results also demonstrate that the immediate solubilization of RDPase by the long term incubation supernatant was not limited to its own species.

Purification of porcine RDPase following long term incubation of PTs

The purification of porcine RDPase was done by a long term incubation supernatant as shown in Table II. The activity yield was 51.9% with a 2,156 fold enrichment which is greater than the 34.7% yield and 2,119 fold enrichment of porcine kidney RDPase following its release from the membrane with PI-PLC purified from *B. cereus* (Littlewood *et al.,* 1989).

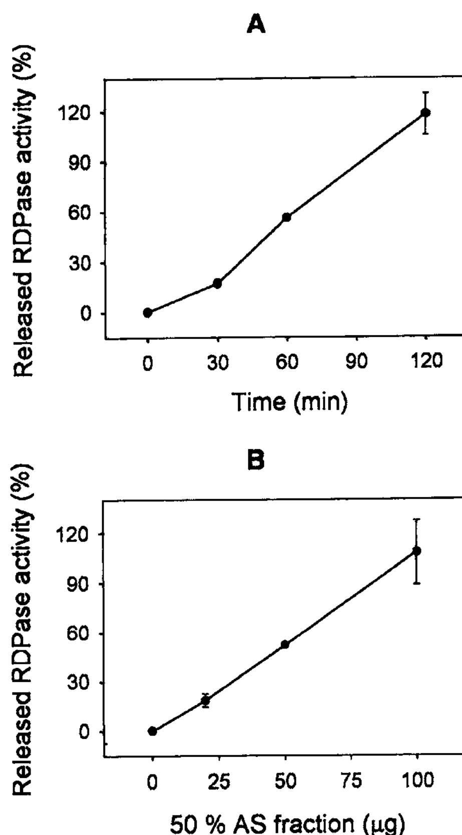

Fig. 2. Release of RDPase from the rabbit PTs in the presence of long term incubation supernatant of porcine PTs. A large volume of porcine incubation supernatant (1.7 mg/ml, 275 ml) obtained by a long term incubation (9 h) (see Fig. 1) was subjected to AS fraction (0-50% saturation). (A) Release of rabbit RDPase as a function of time. The porcine PTs 50% AS fraction (1 μg) was added to the rabbit PTs. (B) Release of rabbit RDPase as a function of protein concentration. The released RDPase activity was expressed as the percentile of the total activity of rabbit PTs prior to incubation (mean ± S.D., n=3).

On SDS-PAGE the purified RDPase exhibited a single band of 47kDa (Fig. 3A, lane 2), the same mobility as the porcine RDPase purified after solubilization with *B. cereus* PI-PLC (Fig 3A, lane 1). The subunit MW is in good

Table II. Purification of porcine RDPase from long term incubation of PTs

Fraction	Total activity (U)	Activity yield (%)	Total protein (mg)	Protein yield (%)	S.A. (U/mg)	Fold purification
PTs	61.2	100	19380	100	0.0032	1
Incubation supernatant	38.5	62.9	467.5	2.41	0.0824	25.75
AS fraction (50-75%)	33.3	54.5	183.3	0.95	0.182	56.88
Affinity chromatography	31.7	51.9	4.6	0.02	6.9	2156

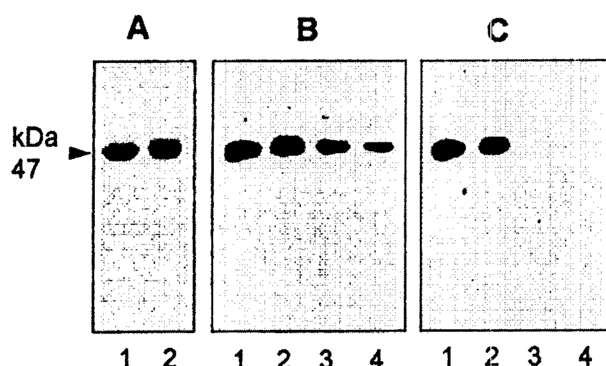

Fig. 3. Protein staining and Western blot. The purified RDPase was subjected to electrophoresis on a 12% polyacrylamide SDS gel with MW markers. (A) Protein staining with Coomassie Brilliant Blue R-250. (B) Western blot with polyclonal anti-RDPase serum. (C) Western blot with anti-CRD serum. Lane 1, Porcine RDPase (2 μg) solubilized with *B. cereus* PI-PLC; lane 2, porcine RDPase purified from a long term incubation supernatant (2μg); lane 3, the same sample (2 μg) of lane 2 was treated with 1 M HCl; lane 4, the same sample (2 μg) of lane 2 was treated with 0.25 M HNO_2 as described in "Materials and methods" section.

agreement with a previous report (Park *et al.,* 2001). The RDPase band was confirmed by Western blot with a polyclonal anti-RDPase serum (Fig. 3B, lane 2).

The presence of the Ins(1,2-cyc)*P* moiety on the released RDPase was identified with the polyclonal anti-RDPase and anti-CRD sera (Fig. 3B and C, respectively). The intact protein band of the acid-treated RDPase (Fig. 3B, lanes 3,4) as well as the positive control (lane 1) and the untreated RDPase (lane 2) were detected by the polyclonal anti-RDPase serum. The acid-treated RDPase samples were not recognized by the anti-CRD serum due to the breakdown of its epitope (Fig. 3C, lanes 3,4) though the positive control (Fig. 3C, lane 1) and the untreated purified RDPase (Fig. 3C, lane 2) were observed with the similar band intensity. The disappearance of the bands by HNO_2 and HCl, which are known to hydrolyse the glucosamine-inositol bond and to selectively decyclize the Ins(1,2-cyc)P ring (Ferguson *et al.*, 1985), respectively, confirms the presence of the CRD epitope (Broomfield and Hooper, 1993). Thus the immune complexes of porcine RDPase with the polyclonal anti-RDPase and anti-CRD sera demonstrate the presence of the exposed Ins(1,2-cyc)*P* moiety indicating that the release of RDPase from the PTs was carried out by a PI-PLC.

The inhibition of this spontaneous release of RDPase from PTs by antibiotics indicates that certain bacteria grow and secrete a hydrolase in the case of a long-term PTs incubation. This hydrolase which cleaves the GPI anchor on RDPase is almost certainly a PI-PLC as demonstrated by the Western blot results with the anti-CRD antibody, which selectively detects the Ins(1,2-cyc)*P* formed on the phospholipase C cleavage of the GPI anchor. The antibiotics prevent this release by blocking the bacteria growth, thus no PI-PLC is secreted. However, the long-term incubation supernatant can be added to new PTs and cause the release of RDPase even in the presence of antibiotics, because the PI-PLC has already been secreted into this supernatant and is active. The species of bacteria and its source are under investigation.

This is a simple, easy and inexpensive method of solubilizing RDPase from its GPI-anchor, which may be applicable to the solubilization of other GPI-anchored proteins without the use of commercial bacterial PI-PLC. The novelty and significance of this finding are that this *in vitro* system can be allowed to do its own work (i.e. to release GPI anchored proteins) without any external interference.

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REFERENCES

Adachi, H., Kubota, I., Okamura, N., Iwata, H., Tsujimoto, M., Nakazato, H., Nishihara, T., and Noguchi, T., Purification and characterization of human microsomal dipeptidase, *J. Biochem.*, 105, 957-961 (1989).

Bradford, M. M., A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, *Anal. Biochem.*, 72, 248-254 (1976).

Brewis, I. A., Turner, A. J., and Hooper, N. M., Activation of the glycosylphosphatidyl-inositol-anchored membrane dipeptidase upon release from pig kidney membranes by phospholipase C, *Biochem. J.*, 303, 633-638 (1994).

Broomfield, S. J., and Hooper, N. M., Characterization of an antibody to the cross- reacting determinant of the glycosyl-phosphatidylinositol anchor of human membrane dipeptidase. *Biochim. Biophys. Acta*, 1145, 212-218 (1993).

Campbell, B. J., Baker, S. F., Shukla, S. D., Forrester, L. J., and Zahler, W. L., Bioconversion of leukotriene D_4 by lung dipeptidase, *Biochim. Biophys. Acta*,1042, 107-112 (1990).

Campbell, B. J., Lin, Y. C., and Bird, M. E., Renal aminopeptidase- and copper-activated peptide hydrolysis, *J. Biol. Chem.*, 238, 3632-3640 (1963).

Campbell, B. J., Lin, Y. C., Davis, R. V, and Ballew, E., The purification and properties of a particulate renal dipeptidase, *Biochim. Biophys. Acta*, 118, 371-386 (1966).

Cook, W. F., and Pickering, G.W., A rapid method for separating glomeruli from rabbit kidney, *Nature*, 182, 1103-1104 (1958).

Farrell, C. A., Allegretto, N. J., and Hitchcock, M. J., Cilastatin-

sensitive dehydropeptidase I enzymes from three sources all catalyze carbapenem hydrolysis and conversion of leukotriene D_4 to leukotriene E_4, *Arch. Biochem. Biophys.*, 256, 253-259 (1987).

Ferguson, M. A., Glycosyl-phosphatidylinositol membrane anchors: the tale of a tail, *Biochem. Soc. Trans.*, 20, 243-256 (1992).

Ferguson, M. A., Low, M. G., and Cross, G. A., Glycosyl-sn-1,2-dimyristyl-phosphatidylinositol is covalently linked to Trypanosoma brucei variant surface glycoprotein, *J. Biol. Chem.*, 260, 14547-14555 (1985).

Fukumura, Y., Kera, Y., Oshitani, S., Ushijima, Y., Kobayashi, I., Liu, Z., Watanabe, T., Yamada, R., Kikuchi, H., Kawazu, S., and Yabuuchi, M., Behaviour of urinary dipeptidase in patients with chronic renal failure, *Ann. Clin. Biochem.*, 36, 221-225 (1999).

Griffith, O. H., and Ryan, M., Bacterial phosphatidylinositol-specific phospholipase C: structure, function, and interaction with lipids, *Biochim. Biophys. Acta*, 1441, 237-254 (1999).

Hitchcock, M. J., Farrell, C. A., Huybensz, S., Luh, B.Y., and Phelps, D. J., Affinity purification of renal dipeptidase solubilized with detergent, *Anal. Biochem.*, 163, 219-223 (1987).

Hooper, N. M., Low, M. G., and Turner, A. J., Renal dipeptidase is one of the membrane proteins released by phosphatidylinositol-specific phospholipase C, *Biochem. J.*, 244, 465-469 (1987).

Hooper, N. M., and Turner, A. J., Ectoenzymes of the kidney microvillar membrane. Isolation and characterization of the amphipathic form of renal dipeptidase and hydrolysis of its glycosyl-phosphatidylinositol anchor by an activity in plasma, *Biochem. J.*, 261, 811-818 (1989).

Ito, Y., Watanabe, Y., Hirano, K., Sugiura, M., Sawaki, S., and Ogiso, T., A fluorometric method for dipeptidase activity measurement in urine, using L-alanyl-L-alanine as substrate, *J. Biochem., (Tokyo)* 96, 1-8 (1984).

Iwasaki, Y., Tsubouchi, Y., Ichihashi, A., Nakano, H., Kobayashi, T., Ikezawa, H., and Yamane, T., Two distinct phosphatidylinositol-specific phospholipase Cs from Streptomyces antibioticus, *Biochim. Biophys. Acta*, 1391, 52-66 (1998).

Kang, B. Y., We, J. S., Choi, K., Lee, H. B., Han, H. J., and Park, H. S., Release of renal dipeptidase from rabbit renal proximal tubules and its inhibition by gentamicin, *Arch. Pharm. Res.*, 22, 367-371 (1999).

Kropp, H., Sundelof, J. G., Hajdu, R., and Kahan, F.M., Metabolism of thienamycin and related carbapenem antibiotics by the renal dipeptidase: dehydropeptidase-I, *Antimicrob. Agent. Chemother.* 22, 62-70 (1982).

Lee, S. H., Kang, B. Y., We, J. S., Park, S. K., and Park, H. S., Differentiation of acute renal failure and chronic renal failure by 2-dimensional analysis of urinary dipeptidase versus serum creatinine, *Renal Fail.*, 21, 169-176 (1999).

Lehto, M. T., and Sharom, F. J., Release of the glycosylphosphatidylinositol-anchored enzyme ecto-5'-nucleotidase by phospholipase C: catalytic activation and modulation by the lipid bilayer, *Biochem. J.*, 332, 101-109 (1998).

Littlewood, G. M., Hooper, N. M., and Turner, A. J., Ectoenzymes of the kidney microvillar membrane. Affinity purification, characterization and localization of the phospholipase C-solubilized form of renal dipeptidase, *Biochem. J.*, 257, 361-367 (1989).

Park, S. W., Choi, K., Kim, I. C., Lee, H. B., Hooper, N. M., and Park, H. S., Endogenous glycosylphosphatidylinositol-specific phospholipase C releases renal dipeptidase from kidney proximal tubules in vitro, *Biochem. J.*, 353, 339-344 (2001).

Park, H. S., Kim, D. H., Kwark, H. S. E., Park, S. K., Kang, S. K., Chung, B. H., and Yoo, G. S., Human renal dipeptidase from kidneys of renal stone patients: Partial purification, *Arch. Pharm. Res.*, 16, 295-299 (1993).

Stieger, S., and Brodbeck, U., Glycosyl-phosphatidylinositol anchored acetylcholinesterase as substrate for phosphatidylinositol-specific phospholipase C from Bacillus cereus, *Biochimie.*, 73, 1179-1186 (1991).

We, J. S., Kang, B. Y., Lee, J. C., Lee, H. B., and Park, H. S., Identification of urinary dipeptidase as the released form of renal dipeptidase, *Kidney Blood Press. Res.*, 20, 411-415 (1997).