Characterization of the glycosyl-phosphatidylinositol-anchored human renal dipeptidase reveals that it is more extensively glycosylated than the pig enzyme

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Renal dipeptidase (EC 3.4.13.11) has been purified from human kidney cortex by affinity chromatography on cilastatin–Sepharose following solubilization with either *n*-octyl-β-D-glucopyranoside or bacterial phosphatidylinositol-specific phospholipase C (PI-PLC). Phase separation in Triton X-114 revealed that the detergent-solubilized form was amphipathic and retained the glycosyl-phosphatidylinositol membrane anchor whereas the phospholipase solubilized form was hydrophilic. Both forms of the enzyme existed as a disulphide-linked dimer of two identical subunits of M_r 59000 each. The glycosyl-phosphatidylinositol anchor of purified human renal dipeptidase was hydrolysed by a range of bacterial PI-PLCs and by a plasma phospholipase D. Mild acid treatment and nitrous acid deamination of the hydrophilic form revealed that the cross-reacting determinant, characteristic of the glycosyl-phosphatidylinositol anchor, was due exclusively to the inositol 1,2-cyclic phosphate ring epitope. The *N*-terminal amino acid sequences of the amphipathic and hydrophilic forms were identical, locating the membrane anchor at the *C*-terminus. The *N*-terminal sequence of human renal dipeptidase showed a high degree of similarity with that of the pig enzyme, and enzymic deglycosylation revealed that the difference in size of renal dipeptidase between these two species is due almost entirely to differences in the extent of *N*-linked glycosylation.

INTRODUCTION

Renal dipeptidase (dehydropeptidase-I; microsomal dipeptidase; EC 3.4.13.11) is one of several ectoenzymes anchored in the plasma membrane by a covalently attached glycosyl-phosphatidylinositol (G-PI) moiety (reviewed in Low, 1987, 1989*a*; Ferguson & Williams, 1988; Low & Saltiel, 1988; Turner, 1989). Pig renal dipeptidase can be selectively released from kidney microvillar membranes by bacterial phosphatidylinositol-specific phospholipase C (PI-PLC) or by a G-PI-specific phospholipase D present in plasma (Hooper *et al.*, 1987; Hooper & Turner, 1989). The enzyme also gives a pattern of solubilization by a range of detergents that is characteristic of a G-PI-anchored protein (Hooper & Turner, 1988*a*). Pig renal dipeptidase contains inositol and possesses the cross-reacting determinant (CRD) which is common to G-PI anchored proteins (Littlewood *et al.* 1989).

Human renal dipeptidase is also selectively released from kidney microvillar membranes by bacterial PI-PLC (Hooper & Turner, 1988*b*). However, the human enzyme is reported to be substantially larger than the pig enzyme, subunit M_r 59000 (Campbell *et al.*, 1984) compared with M_r 47000 (Littlewood *et al.*, 1989), even though both enzymes are very similar in terms of their substrate specificity and inhibitor profile (see for example Kozak & Tate, 1982; Campbell *et al.*, 1988). Although the pig enzyme is a glycoprotein (Kim & Campbell, 1983; Littlewood *et al.*, 1989) there are conflicting reports as to whether the human enzyme contains carbohydrate on the basis of the periodic acid/Schiff's reaction (Sugiura *et al.*, 1978; Campbell *et al.*, 1984). Thus, the molecular basis for the difference in size between human and pig renal dipeptidase is unclear. Although more than 50 G-PI-anchored proteins have been identified (Low, 1989*b*), studies on the anchor structure of only a few of these proteins have been carried out in more than one species.

In the present study we have purified renal dipeptidase from human kidney cortex following solubilization from the membrane with either n-octyl-β-D-glucopyranoside or bacterial PI-PLC. These resulting amphipathic and hydrophilic forms of the enzyme, respectively, have been used to characterize the G-PI anchor and compare its structure with that of the pig enzyme. We have also obtained *N*-terminal amino acid sequence data for human renal dipeptidase and show that it displays a high degree of similarity with that of the pig enzyme. Enzymic deglycosylation of the human and pig dipeptidase reveals that the difference in size of the enzyme between these two species is almost entirely due to differences in the extent of *N*-linked glycosylation.

EXPERIMENTAL

Materials

PI-PLC from *Bacillus thuringiensis* and from *Staphylococcus aureus* were gifts from Dr. M. G. Low, Department of Physiology and Cellular Biophysics, College of Physicians and Surgeons of Columbia University, New York, NY, U.S.A. *B. cereus* phospholipase C type

Abbreviations used: CRD, cross-reacting determinant; G-PI, glycosyl-phosphatidylinositol; I_{50}, concentration of inhibitor causing 50% inhibition; PI-PLC, phosphatidylinositol-specific phospholipase C; PVDF, polyvinylidene difluoride.

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III was purchased from Sigma. Units of PI-PLC activity are μmol/min. Anti-(cross-reacting determinant) (anti-CRD) rabbit serum was a gift from Dr. M. A. J. Ferguson, Department of Biochemistry, University of Dundee, U.K. 2D-Silver Stain Kit for detection of proteins on polyacrylamide gels was purchased from Koch–Light (Haverhill, Suffolk, U.K.) Immobilin P polyvinylidene difluoride (PVDF) membranes were purchased from Millipore. All other materials were obtained from sources previously noted.

Purification of renal dipeptidase

Renal dipeptidase was solubilized from human kidney cortex either with PI-PLC or with n-octyl-β-D-glucopyranoside and then purified by affinity chromatography on cilastatin–Sepharose as described previously for the pig enzyme (Littlewood *et al.*, 1989; Hooper & Turner, 1989).

Enzyme and protein assays

Renal dipeptidase was assayed by an h.p.l.c. method using glycyl-D-phenylalanine as described in Littlewood *et al.* (1989). Protein was determined as described previously.

SDS/polyacrylamide-gel electrophoresis and immunoelectrophoretic blot analysis

SDS/polyacrylamide-gel electrophoresis was performed with a 7–17% polyacrylamide gradient as described previously (Relton *et al.*, 1983). Protein was detected using a 2D-Silver Stain Kit as directed by the manufacturers. Immunoelectrophoretic ('Western') blot analysis was carried out as detailed previously (Hooper & Turner, 1987), except that Immobilin P PVDF membranes were used and a ^{125}I-labelled goat anti-(rabbit IgG) was employed as second antibody. After extensive washing of the membranes as for the first antibody, labelled protein was detected by autoradiography at −70 °C with Konica A2 film.

Triton X-114 phase separation

Triton X-114 was pre-condensed before use (Bordier, 1981). Enzyme samples were made up to 0.2 ml with 10 mM-Tris/HCl/0.15 M-NaCl/1.0% (w/v) Triton X-114, pH 7.4, and subjected to phase separation at 30 °C for 3 min as in Bordier (1981). The detergent-rich and detergent-poor phases were separated through 0.3 ml of 6% (w/v) sucrose by centrifugation at 3000 *g* for 3 min and assayed for enzyme activity.

Mild acid and nitrous acid treatment

The inositol 1,2-cyclic phosphate ring, formed by PI-PLC cleavage, was selectively decyclized by incubation of renal dipeptidase with 1 M-HCl for 30 min at 23 °C (Zamze *et al.*, 1988). After neutralizing with NaOH, samples were subjected to Western blot analysis. Renal dipeptidase was deaminated by treatment with 0.25 M-sodium acetate/0.25 M-$NaNO_2$, pH 4.0, for 3 h at 23 °C (Zamze *et al.*, 1988). Control samples were treated with 0.25 M-acetate/0.25 M-NaCl, pH 4.0. After neutralizing, samples were acetone precipitated and then subjected to Western blot analysis.

Enzymic deglycosylation

Renal dipeptidase was deglycosylated with *N*-glycanase (glycopeptide *N*-glycosidase, EC 3.2.2.18) as described in Hooper & Turner (1987) and then analysed by SDS/polyacrylamide-gel electrophoresis.

Solid-phase sequencing

Lyophilized protein was dissolved in 0.1 M-$NaHCO_3$/0.1 M-Na_2CO_3/0.25% SDS (pH 10) and coupled to D-phenylenedi-isothiocyanate glass (17 nm pore size, 200–400 mesh) for 60 min at 56 °C under N_2. The glass-coupled peptide was then sequenced by automated solid-phase Edman degradation using the microsequence facility built by the Protein Sequence Unit, Department of Biochemistry, University of Leeds (Findlay *et al.*, 1989). Anilinothiazolinone-amino acids were converted in 30% (v/v) aqueous trifluoroacetic acid for 20 min at 70 °C (under N_2) and the corresponding phenylthiohydantoin-amino acids identified by reverse-phase (C_{10}) microbore h.p.l.c. (Brownlee Spheri-5 RP-18 column, 220 mm × 2.1 mm) on a Hewlett Packard 1090M HPLC system, using a gradient of acetonitrile in 35 mM-acetic acid (pH 4.9 with NaOH) at a flow rate of 0.21 ml/min. Data were collected at 269 nm and 313 nm using a diode array detector, serine and threonine residues being confirmed by the detection of their dehydro derivatives at the latter wavelength.

RESULTS AND DISCUSSION

Purification of the amphipathic and hydrophilic forms of human renal dipeptidase

Renal dipeptidase was purified from human kidney cortex by affinity chromatography on cilastatin–Sepharose after solubilization from the membrane by

Table 1. Purification of dipeptidase from human kidney cortex

Dipeptidase was solubilized with bacterial PI–PLC from 130 g of human kidney cortex and then purified as described in the Experimental section. The purified, n-octyl-β-D-glucopyranoside-solubilized form of human renal dipeptidase had a specific activity of 8.6 μmol of D-Phe produced/min per mg of protein.

	Protein (mg)	Total activity (μmol of D-Phe/min)	Specific activity (nmol of D-Phe/min per mg)	Recovery (%)	Enrichment (-fold)
Homogenate	22140	90.8	4.1	100	1
Microsomal membrane fraction	1150	28.3	24.6	31	6
PI-PLC solubilized supernatant	520	7.7	14.9	8	4
Affinity chromatography	0.13	1.1	8800	1	2146

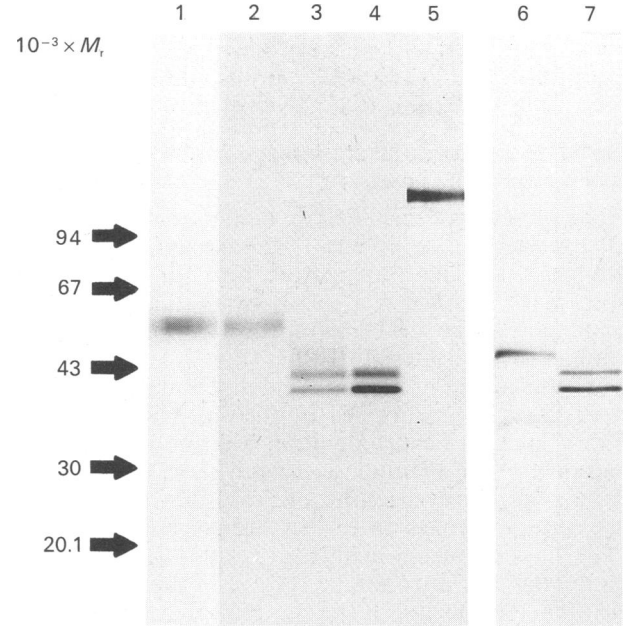

Fig. 1. SDS/polyacrylamide-gel electrophoresis of human and pig renal dipeptidase before and after deglycosylation

Samples were prepared and analysed as described in the Experimental section. Lane 1, detergent-solubilized, affinity-purified human renal dipeptidase (5 μg of protein); lanes 2–4, phospholipase-solubilized, affinity-purified human renal dipeptidase (5 μg of protein); lane 2, untreated; lane 3, incubated with *N*-glycanase (0.75 units) for 24 h at 37 °C; lane 4, incubated with *N*-glycanase (1.5 units) for 24 h at 37 °C; lane 5, phospholipase-solubilized, affinity-purified human renal dipeptidase (5 μg of protein) in non-reducing sample buffer; lanes 6 and 7, phospholipase-solubilized, affinity-purified pig renal dipeptidase (2 μg of protein); lane 6, untreated; lane 7, incubated with *N*-glycanase (0.3 units) for 24 h at 37 °C. The lanes were silver stained.

either PI-PLC or n-octyl-β-D-glucopyranoside. Details of the purification of the PI-PLC-solubilized enzyme are provided in Table 1. Both the phospholipase- and detergent-solubilized forms of human renal dipeptidase were purified to apparent homogeneity as assessed by SDS/polyacrylamide-gel electrophoresis. In contrast with the sharp polypeptide band of apparent M_r 47000 observed for the pig enzyme (Littlewood *et al.*, 1989; Fig. 1, lane 6), the human enzyme appeared as single, broad, diffuse band of apparent M_r 59000 (Fig. 1, lanes 1 and 2) consistent with earlier observations (Campbell *et al.*, 1984; Mitsuhashi *et al.*, 1988). Under non-reducing conditions the enzyme migrated with an apparent M_r of 105000 (Fig. 1, lane 5) revealing that the enzyme is a disulphide-linked dimer, consistent with an earlier report for an apparent M_r of 135000 by gel filtration analysis (Mitsuhashi *et al.*, 1988). The purified phospholipase-solubilized form of human renal dipeptidase was potently inhibited by the specific inhibitor cilastatin (Kahan *et al.*, 1983) with an I_{50} value of 0.97 ± 0.17 μM ($n = 3$), comparable with that for the pig enzyme (Littlewood *et al.*, 1989). On phase-separation in Triton X-114 the phospholipase-solubilized form of the enzyme partitioned predominantly into the detergent-poor phase (97.1 ± 2.5%, $n = 3$) demonstrating its hydrophilic nature. In contrast, the detergent-solubilized form partitioned predominantly into the detergent-rich phase (98.1 ± 0.1%, $n = 3$), implying retention of the hydrophobic membrane anchor.

Hydrolysis of the amphipathic form of human renal dipeptidase by bacterial PI-PLC and plasma phospholipase D

The action of PI-PLC from *B. thuringiensis*, *S. aureus* and *B. cereus* on the hydrophobic membrane anchor of purified renal dipeptidase was examined by phase separation in Triton X-114 (Table 2). All three bacterial PI-PLCs converted the amphipathic form of human renal dipeptidase into a hydrophilic form consistent with the

Table 2. Hydrolysis of the membrane anchor of human renal dipeptidase by PI–PLC and plasma

Detergent-solubilized, affinity-purified human renal dipeptidase (1 μg of protein) was incubated with either bacterial PI–PLC in 10 mM-Hepes, pH 7.4, or plasma in 0.2 M-Hepes/0.1% (v/v) Nonidet P-40, pH 7.0 (50 μl total volume) for 2 h at 37 °C. Incubations with *B. cereus* phospholipase C contained 0.1 M-NaCl. The plasma and inhibitors were preincubated at 4 °C for 15 min. After the incubation, samples were subjected to phase separation in Triton X-114 as described in the Experimental section. The results are the mean of duplicate incubations. Anchor degradation of 0% is equivalent to all of the dipeptidase activity being recovered in the detergent-rich phase after phase separation in Triton X-114.

Phospholipase or plasma	Inhibitor	Degradation of dipeptidase anchor (%)
None	–	0
B. thuringiensis PI–PLC (100 units/ml)	–	72.5
S. aureus PI–PLC (0.2 units/ml)	–	78.6
B. cereus PLC (10 units/ml)	–	76.2
Plasma (2 μl)	–	93.2
Plasma (2 μl)	*p*-Hydroxymercuriphenylsulphonate (2.5 mM)	70.6
Plasma (2 μl)	EDTA (0.25 mM)	0
Plasma (2 μl)	EGTA (2.5 mM)	0
Plasma (2 μl)	Phenylmethanesulphonyl fluoride (1.0 mM)	89.3

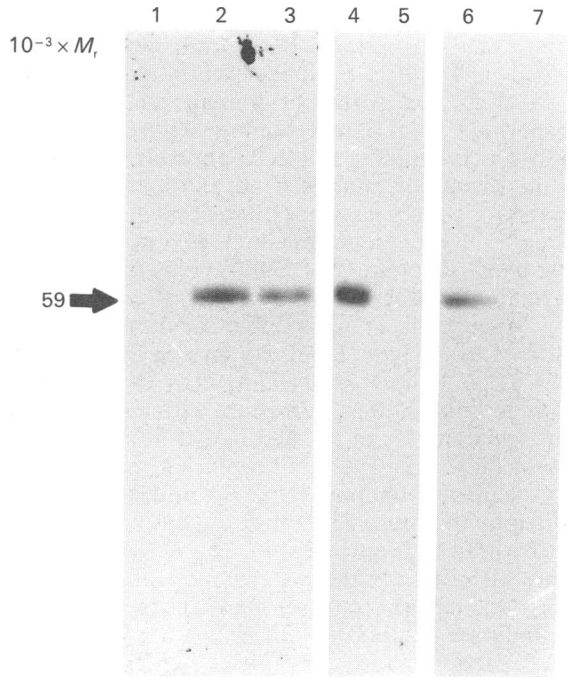

Fig. 2. Western blot of human renal dipeptidase with anti-CRD antiserum

Samples were prepared and analysed as described in the Experimental section. After electrophoretic transfer to PVDF membranes, the tracks were blotted with antiserum to the CRD of the variant surface glycoprotein 221. Lanes 1–3, detergent-solubilized, affinity-purified renal dipeptidase (5 μg of protein) incubated in 0.1 M-Hepes, pH 7.0, for 6 h at 37 °C; lane 1, untreated; lane 2, incubated with 0.2 units of *S. aureus* PI-PLC/ml; lane 3, incubated with 10 units of *B. cereus* phospholipase C/ml and 0.1 M-NaCl. Lanes 4–7, phospholipase-solubilized, affinity-purified renal dipeptidase (5 μg of protein); lane 4, untreated; lane 5, incubated in the presence of 1 M-HCl for 30 min at 23 °C; lane 6, incubated with 0.25 M-sodium acetate/0.25 M-NaCl, pH 4.0, for 3 h at 23 °C; lane 7, incubated with 0.25 M-sodium acetate/0.25 M-$NaNO_2$, pH 4.0, for 3 h at 23 °C.

loss of the membrane anchor. The membrane anchor of human renal dipeptidase was also hydrolysed by an activity in pig plasma (Table 2). This anchor-degrading activity in plasma was inhibited by EDTA and EGTA and to some extent by *p*-hydroxymercuriphenylsulphonic acid, but not by phenylmethanesulphonyl fluoride and hence resembles a previously characterized phospholipase D (Davitz *et al.*, 1987; Cardoso de Almeida *et al.*, 1988; Low & Prasad, 1988; Hooper & Turner, 1989).

Characterization of the cross-reacting determinant on human renal dipeptidase

The presence of the CRD on human renal dipeptidase was examined using an anti-CRD antiserum in Western blot analysis. The amphipathic form of human renal dipeptidase was not recognized by the anti-CRD antiserum unless the CRD epitope was exposed by prior treatment with bacterial PI-PLC (Fig. 2, lanes 1–3). The hydrophilic form of human renal dipeptidase solubilized by PI-PLC was recognized by the anti-CRD antiserum (Fig. 2, lane 4). This recognition was abolished by prior treatment of the dipeptidase either with mild acid (Fig. 2, lane 5), which decyclizes the inositol 1,2-cyclic phosphate ring epitope formed on PI-PLC cleavage (Zamze *et al.*, 1988), or with nitrous acid (Fig. 2, lanes 6 and 7), which deaminates the glucosamine residue in the G-PI anchor releasing the inositol 1,2-cyclic phosphate group (Ferguson *et al.*, 1985). These results indicate that the only site of recognition on human renal dipeptidase by the anti-CRD antiserum is the inositol 1,2-cyclic phosphate ring epitope, and that human renal dipeptidase, like pig renal dipeptidase (Hooper & Turner, 1989), does not contain a galactose branch in its G-PI anchor as present on the variant surface glycoprotein (Zamze *et al.*, 1988).

N-Terminal sequence analysis of human renal dipeptidase and comparison with that of pig renal dipeptidase

Affinity-purified samples of both forms of human renal dipeptidase were subjected to *N*-terminal sequence analysis as described in the Experimental section (Fig. 3). The hydrophilic form of the enzyme gave a single amino acid sequence. The amphipathic form was identical up to residue 15, the extent to which this form was sequenced. Thus the presence of the same *N*-terminal amino acid sequence in both the amphipathic and hydrophilic forms of human renal dipeptidase confirms that the G-PI membrane anchor is located at the *C*-terminus of the protein, in common with all other G-PI-anchored proteins (Ferguson & Williams, 1988). A comparison of the sequence of human renal dipeptidase with that obtained for the hydrophilic form of pig renal dipeptidase (Fig. 3) revealed a high degree of similarity with 17 of the first 23 residues (74 %) being identical.

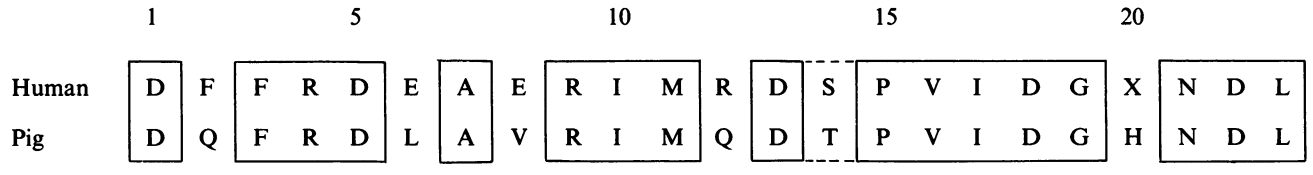

Fig. 3. *N*-Terminal sequences of human and pig renal dipeptidases

Samples (0.1–0.5 nmol) of affinity-purified human or pig renal dipeptidase were coupled to D-phenylenedi-isothiocyanate glass and then sequenced by automated solid-phase Edman degradation as described in the Experimental section. The sequences are those of the phospholipase-solubilized forms of human and pig renal dipeptidase. Identical residues are boxed. Conservative substitutions are shown in dotted lines. The detergent-solubilized form of human renal dipeptidase was identical with the phospholipase-solubilized form up to residue 15.

Deglycosylation of human renal dipeptidase

Human renal dipeptidase was enzymically deglycosylated with *N*-glycanase and the products analysed by SDS/polyacrylamide-gel electrophoresis (Fig. 1). Two polypeptide bands were produced by *N*-glycanase treatment with apparent M_r values of 46000 and 42500 (Fig. 1, lanes 3 and 4), confirming an earlier report that the enzyme is a glycoprotein as shown by a positive periodic acid/Schiff's reaction (Sugiura *et al.*, 1978). As *N*-glycanase only removes *N*-linked oligosaccharides, up to 28% by weight of the M_r 59000 subunit is due to the presence of *N*-linked sugars. The presence of two polypeptide bands upon deglycosylation with *N*-glycanase would suggest that there are two distinct populations of *N*-linked sugars that differ in their susceptibility to cleavage by *N*-glycanase. Similarly, pig renal dipeptidase is also deglycosylated by *N*-glycanase to two polypeptides but of apparent M_r values 44000 and 40500 (Fig. 1, lanes 6 and 7; Littlewood *et al.*, 1989). The difference in the extent of glycosylation between the two species may account for the observation that the detergent-solubilized form of human renal dipeptidase was not recognized on Western blot analysis by a polyclonal antiserum raised against the pig enzyme (result not shown).

General conclusions

The *C*-terminal G-PI anchor of purified human renal dipeptidase is identical to that of pig renal dipeptidase with respect to its hydrolysis by bacterial PI-PLC and plasma phospholipase D. Renal dipeptidase from both species cross-reacts with an anti-CRD antiserum, the only site of recognition being the inositol 1,2-cyclic phosphate ring epitope. Thus the G-PI anchors of human and pig renal dipeptidase are very similar. Although the human enzyme is substantially larger in M_r (12000) than the pig enzyme they both exist as disulphide-linked dimers and have a highly homologous *N*-terminal amino acid sequence. The deglycosylated polypeptide chains of human and pig renal dipeptidase are of similar M_r, indicating that the difference in size of renal dipeptidase between these two species is almost entirely due to differences in the extent of *N*-linked glycosylation.

We thank the Wellcome Trust, the Medical Research Council and the Royal Society for financial support, and Mr. J. Hryszko for expert technical assistance. N.M.H. is a Mr. and Mrs. John Jaffé Donation Research Fellow of the Royal Society.

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Received 7 August 1989/18 September 1989; accepted 20 September 1989