cDNA cloning and expression in *Xenopus laevis* oocytes of pig renal dipeptidase, a glycosyl-phosphatidylinositol-anchored ectoenzyme

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Clones expressing renal dipeptidase (EC 3.4.13.11) have been isolated from a pig kidney cortex cDNA library after employing the polymerase chain reaction technique to amplify a region of the dipeptidase cDNA. The complete primary sequence of the enzyme has been deduced from a full length cDNA clone. This predicts a protein of 409 amino acids, a cleavable *N*-terminal signal sequence of 16 residues and two *N*-linked glycosylation sites. At the *C*-terminus of the predicted sequence is a stretch of mainly hydrophobic amino acids which is presumed to direct the attachment of the glycosyl-phosphatidylinositol membrane anchor. Expression of the mRNA for pig renal dipeptidase in *Xenopus laevis* oocytes led to the production of a disulphide-linked dimeric protein of subunit M_r 48600 which was recognized by a polyclonal antiserum raised to renal dipeptidase purified from pig kidney cortex. Bacterial phosphatidylinositol-specific phospholipase C released renal dipeptidase from the surface of the oocytes and converted the amphipathic detergent-solubilized form of the dipeptidase to a hydrophilic form, indicating that *Xenopus laevis* oocytes can process expressed proteins to their glycosyl-phosphatidylinositol anchored form.

INTRODUCTION

A diversity of proteins are now known to be anchored in the plasma membrane by a covalently attached *C*-terminal glycosyl-phosphatidylinositol (G-PI) moiety (Low, 1989; Turner, 1990). The cDNA sequences encoding such proteins predict the presence of a cleavable *N*-terminal signal sequence and a hydrophobic region of approx. 10–15 amino acids at the *C*-terminus. This *C*-terminal sequence acts as a signal which is exchanged for a pre-formed G-PI structure early in biosynthesis (Cross, 1990; Doering *et al.*, 1990).

We have extensively characterized the brush border ecto-enzyme renal dipeptidase (dehydropeptidase-I; microsomal dipeptidase; EC 3.4.13.11) and shown it to possess a G-PI anchor. The enzyme is selectively released from pig and human kidney microvillar membranes by bacterial phosphatidylinositol-specific phospholipases C (PI-PLC) and by a G-PI-specific phospholipase D in plasma (Hooper *et al.*, 1987, 1990*a*; Hooper & Turner, 1989). Purified renal dipeptidase contains 1 molecule of inositol per subunit and also possesses the cross-reacting determinant, an epitope which is common to all G-PI anchored proteins (Little-wood *et al.*, 1989; Hooper *et al.*, 1990*a*). Renal dipeptidase is a zinc-metalloenzyme capable of hydrolysing a range of dipeptides (Campbell, 1970; Armstrong *et al.*, 1974). The enzyme has also been implicated in the renal metabolism of glutathione and its conjugates, e.g. leukotriene D_4 (Kozak & Tate, 1982) and is responsible for the hydrolysis of the β-lactam antibiotic imipenem (Kropp *et al.*, 1982). Pig and human renal dipeptidase are disulphide-linked dimeric glycoproteins of subunit M_r 47000 and 59000 respectively, and have highly similar *N*-terminal amino acid sequences (Hooper *et al.*, 1990*a*). We have suggested that the basis for the large difference in size between pig and human

renal dipeptidase is due to differences in the extent of *N*-linked glycosylation (Hooper *et al.*, 1990*a*).

In order to characterize further the structural and catalytic properties of renal dipeptidase, we have now isolated clones expressing the enzyme from a pig kidney cDNA library. The polymerase chain reaction (PCR) technique was used to amplify a region of the dipeptidase cDNA which was then used to screen the cDNA library. The complete primary sequence of the enzyme has been deduced from the cDNA sequence. This indicates the presence of a cleavable *N*-terminal signal sequence, a hydro-phobic stretch of amino acids at the *C*-terminus and two potential *N*-linked glycosylation sites. The mRNA for pig renal dipeptidase has been expressed in *Xenopus laevis* oocytes and the presence of the G-PI anchor demonstrated by digestion with PI-PLC and phase separation in Triton X-114.

EXPERIMENTAL

Materials

PI-PLC from *Bacillus thuringiensis* and *Staphylococcus aureus* were a gift 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. Units of PI-PLC activity are μmol/min. Ampli-Taq DNA polymerase was purchased from Perkin Elmer Cetus. pBluescript SK(−), λ-Zap bacteriophage DNA and packaging extract were purchased from Stratagene. DNAase 1 and T7 sequencing kits were purchased from Pharmacia. Collagenase type I and gentamycin were obtained from Sigma. All other materials were obtained from sources previously noted.

Purification and sequencing of renal dipeptidase

Renal dipeptidase was solubilized from pig kidney cortex by using bacterial PI-PLC and then purified by affinity chromatography on a column of cilastatin–Sepharose (Littlewood et al., 1989). The N-terminal sequence of the purified enzyme was determined by automated solid-phase Edman degradation (Hooper et al., 1990a) using the microsequence facility built by the Protein Sequence Unit, Department of Biochemistry, University of Leeds. Peptides were also derived from purified dipeptidase by cleavage with CNBr followed by further cleavage with Asp-N protease, and then analysed using an Applied Biosystems 470A gas-phase sequencer (Zurich).

Oligonucleotides

Based on the sequence of a 64-amino-acid N-terminal peptide, two oligonucleotides (48 and 128-fold degenerate, respectively, containing XhoI linkers at their 5' ends) were designed to amplify an internal 80 bp fragment of the dipeptidase cDNA by PCR. Oligonucleotides were synthesized with a Pharmacia Gene Assembler DNA Synthesizer.

PCR amplification

First strand cDNA was synthesized from 10 μg of poly(A)$^+$ RNA from pig kidney cortex (Gubler & Hoffman, 1983). The product was purified on a Sephacryl S-300 column and 50 ng was used in a 50 μl PCR containing 4.8 μM-degenerate primers/200 μM-deoxynucleoside triphosphates/2.5 mM-MgCl$_2$/10 mM-Tris/HCl (pH 8.3)/50 mM-KCl/0.01% (w/v) gelatin and 2.5 units of Ampli-Taq DNA polymerase. Forty cycles of PCR amplification were carried out (95 °C/45 s; 48 °C/2 min; 72 °C/30 s) followed by one final step of 10 min at 72 °C, using a DNA Thermal Cycler (Perkin Elmer Cetus). The products were separated on a 10% (w/v) polyacrylamide gel and a 96 bp fragment (80 bp dipeptidase DNA plus 16 bp from the XhoI linkers) was isolated. About 2 ng of this DNA was used as template in a second 100 μl PCR (20 cycles). The resulting 96 bp fragment was excised from a 7% (w/v) polyacrylamide gel, eluted, digested with XhoI and cloned into pBluescript SK(−).

cDNA synthesis and cloning

Double-stranded cDNA (Gubler & Hoffman, 1983) from pig kidney cortex poly(A)$^+$ RNA was treated with EcoRI methylase, ligated with EcoRI linkers, and digested with EcoRI. The fraction 1000–6000 bp in size was isolated by electrophoresis in low gelling temperature agarose, ligated to EcoRI-digested and dephosphorylated λ-ZAP bacteriophage DNA, and packaged to give about 80000 plaques. For use as a hybridization probe in screening the λ-ZAP cDNA bank, the PCR fragment was radioactively labelled according to Feinberg & Vogelstein (1983), except that the PCR oligonucleotides were used in place of random hexanucleotides. Hybridization was carried out as described in Maniatis et al. (1982), with final wash conditions 0.5 × SSPE (90 mM-NaCl/10 mM-sodium phosphate/0.5 mM-EDTA, pH 7.7) containing 0.2% SDS at 55 °C. Twenty positive clones (0.025%) were found. The EcoRI inserts of nine of them were purified (Maniatis et al., 1982) and subcloned into the EcoRI site of pBluescript SK(−).

Sequencing and analysis

DNA sequences were determined by the dideoxy chain termination method (Sanger et al., 1977; Sanger, 1981) adapted to accommodate [α-^{35}S]dATP using the T7 sequencing kit. One clone (pER11) was sequenced completely. Its EcoRI insert was digested separately with AluI, ApaI, AvaI, PalI, PvuII, RsaI, Sau3A and TaqI. The resulting fragments were separated by agarose gel electrophoresis, cloned into pBluescript SK(−), and sequenced. Various other fragments, suggested by the preliminary data, were sequenced to solve doubtful regions and to complete the total sequence on both strands. DNA and protein sequences were assembled and analysed with the Genetic Computer Group program package (Devereux et al., 1984) on a VAX8700. Signal peptidase cleavage sites were predicted with the weight matrix method of von Heijne (1986) with a program for the Macintosh computer.

In vitro transcription

The plasmid pER11 was linearized at the 3' end of the cDNA insert with XbaI. mRNA was synthesized in vitro with 500 mM-pm^{7}GpppG (cap analogue) in the reaction buffer according to Melton (1987). Before workup by phenol extraction and ethanol precipitation, the mixture was digested with 300 units of DNAase I/ml for 15 min at 37 °C.

Expression in Xenopus laevis oocytes

Adult Xenopus laevis females were anaesthetized by cooling in ice, and ovarian lobes were removed and treated overnight at 18 °C with collagenase type 1 (3 mg/ml) in modified Barth's solution (MBS), pH 7.5, containing gentamycin (10 μg/ml) (Gurdon & Wickens, 1983; Colman, 1984). Prior to injection the oocytes were washed, and then stored and handled in MBS containing 10 μg of gentamycin/ml. Oocytes were injected with 5–10 ng of in vitro synthesized dipeptidase mRNA in 50 nl of water for assay of activity in situ, after incubation at 18 °C (between 24 and 96 h). Batches of five oocytes were transferred to fresh MBS (0.1 ml) containing 10 μg of gentamycin/ml and 1 mM-Gly-D-Phe and incubated for 1 h at 37 °C before analysis by h.p.l.c. as described in Littlewood et al. (1989).

SDS/PAGE and immunoelectrophoretic blot analysis

SDS/PAGE was performed with a 7–17%-polyacrylamide gradient as described previously (Relton et al., 1983) in either the presence (reducing) or absence (non-reducing) of 0.08 M-dithiothreitol in the sample buffer. Immunoelectrophoretic ('Western') blot analysis was carried out with Immobilon P poly(vinylidene difluoride) membranes and a ^{125}I-labelled second antibody as detailed previously (Hooper & Turner, 1987; Hooper et al., 1990a).

Triton X-114 phase separation

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 detailed in Hooper & Turner (1989). Activity recovered in the detergent-poor phase is expressed as a percentage of the total activity.

RESULTS

Cloning, sequencing and cDNA characterization

A polynucleotide probe for renal dipeptidase was generated using the polymerase chain reaction (Mullis & Faloona, 1987; Saiki et al., 1988; Lee et al., 1988) to amplify an 80-nucleotide segment of dipeptidase cDNA coding for the known N-terminal amino acid sequence. This fragment was radioactively labelled and used as a hybridization probe to screen a pig kidney cortex cDNA bank in λ-ZAP. One of the positive clones (pER11) isolated appeared to be full length since it contained the known N-terminal amino acid sequence of pig renal dipeptidase and a poly(A) tail. The insert of pER11 was sequenced completely. It comprised 1389 bp, excluding the poly(A) tail. The sequence comprises 45 nucleotides of 5'-untranslated region, an open reading frame of 1227 nucleotides coding for 409 amino acids,

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   1                                        CAGACGTGAGGAGCGGCTGGGCAGCGGGTCCCCGGGAGTCGTGCC

  46                                        ATGTGGACCAGCTGGTGGCTCTGGCCCCTGGTGGCCGTCTGCGCTGCG
 -16                                        M  W  T  S  W  W  L  W  P  L  V  A  V  C  A  A

  94   GACCAATTCCGGGACCTGGCGGTGAGGATTATGCAGGACACGCCTGTCATTGACGGGCACAATGACCTGCCTTGGCAGCTGCTGAATCTG
   1    D  Q  F  R  D  L  A  V  R  I  M  Q  D  T  P  V  I  D  G  H  N  D  L  P  W  Q  L  L  N  L

 184   TTCAACAATCAGCTTCAGGACCCGGGGGCCAACCTCTCCAGCCTGGCCCACACGCACACCAACATCCCCAAGCTGAAGGCTGGCTTTGTG
  31    F  N  N  Q  L  Q  D  P  G  A  N  L  S  S  L  A  H  T  H  T  N  I  P  K  L  K  A  G  F  V
                                           *

 274   GGCGGCCCAGTTCTGGTCCGCGTACGTGCCCTGCGACACCCAGAACAGAGACGCCGTGAAGAGGACCCTGGAGCAGATCGACGTCATCCAG
  61    G  G  Q  F  W  S  A  Y  V  P  C  D  T  Q  N  R  D  A  V  K  R  T  L  E  Q  I  D  V  I  Q

 364   CGCATGTGCCAGGCGTATCCCGAGACCTTCGCCTGCGTCACCAGCAGCACAGGCATCCGGCAGGCCTTCCGGGAGGGCAAGGTGGCCAGC
  91    R  M  C  Q  A  Y  P  E  T  F  A  C  V  T  S  S  T  G  I  R  Q  A  F  R  E  G  K  V  A  S

 454   CTGGTGGGCGTGGAGGGTGGCCACTCCATCGACAGCAGCCTGGGCGTCCTGCGGGCCCTCTACCACCTGGGCATGCGGTACATGACCCTC
 121    L  V  G  V  E  G  G  H  S  I  D  S  S  L  G  V  L  R  A  L  Y  H  L  G  M  R  Y  M  T  L

 544   ACCCACAGCTGCAACACGCCCTGGGCCGACAACTGGCTGGTGGACACGGGCGATGACAAGGCCCAGAGCCAAGGCCTGTCGCACTTTGGG
 151    T  H  S  C  N  T  P  W  A  D  N  W  L  V  D  T  G  D  D  K  A  Q  S  Q  G  L  S  H  F  G

 634   CAGAGCGTCGTGAAGGAGATGAACCGGCTGGGCGTCATGATCGACTTGGCCCACGTGTCCGTGGCCACCATGAGGGCCGCTCTGAAGCTG
 181    Q  S  V  V  K  E  M  N  R  L  G  V  M  I  D  L  A  H  V  S  V  A  T  M  R  A  A  L  K  L

 724   TCCCAGGCCCCCGTCATCTTCAGCCACTCCTCGGCCTACAGTCTGTGCCCGCACCGGCGCAACGTGCCGGATGACGTGCTCCAGCTGGTG
 211    S  Q  A  P  V  I  F  S  H  S  S  A  Y  S  L  C  P  H  R  R  N  V  P  D  D  V  L  Q  L  V

 814   AAGGAGACGGGCAGCCTCGTGATGGTCAATTTCTACAACGACTACGTTTCCTGCTCGGCCAAGGCCAACTTGTCCCAAGTGGCAGATCAC
 241    K  E  T  G  S  L  V  M  V  N  F  Y  N  D  Y  V  S  C  S  A  K  A  N  L  S  Q  V  A  D  H
                                                                      *

 904   CTGGACCACATCAAGAAGGTGGCGGGGAGCTGCGGCCGTGGGCTTCGGTGGGGACTACGATGGTGTGTCCAGGGTCCCCTCAGGCCTGGAG
 271    L  D  H  I  K  K  V  A  G  A  A  A  V  G  F  G  G  D  Y  D  G  V  S  R  V  P  S  G  L  E

 994   GACGTGTCCAAGTACCCAGACCTGGTGGCCGAGCTGCTCAGGAGGCAGTGGACGGAGGCCGAGGTCAGGGGCGCCCTGGCCGACAACCTG
 301    D  V  S  K  Y  P  D  L  V  A  E  L  L  R  R  Q  W  T  E  A  E  V  R  G  A  L  A  D  N  L

1084   CTGAGGGTCTTCGAGGCAGTGGAGCAGGCCAGCAATCACGCTCAGGTTCCAGGGGAGGAGCCCATCCCGCTGGGCCAGCTGGAGGCTTCC
 331    L  R  V  F  E  A  V  E  Q  A  S  N  H  A  Q  V  P  G  E  E  P  I  P  L  G  Q  L  E  A  S

1174   TGCCGGACGAATTACGGCTACTCAGCCGCCCCCAGCCTCCACCTCCCGCCGGGCTCGCTGCTGGCCTCCCTCGTGCCCCTCCTCCTCCTC
 361    C  R  T  N  Y  G  Y  S  A  A  P  S  L  H  L  P  P  G  S  L  L  A  S  L  V  P  L  L  L  L

1264   AGTCTTCCGTGACTCCCCTGGGGACCAGCTGACACGCACTGTGCAGGCGTAGACTGGTCTGCCCTGAGCAGGGCCCCGGCTCCGGGAGGC
 391    S  L  P  End

1354   GCCCTGGGACCCTCAATAAAGGTACCGACACTTCGAAAAAAAAAAAAAAAA
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Fig. 1. cDNA sequence and deduced amino acid sequence of pig renal dipeptidase

The amino acid sequence is numbered from the *N*-terminal amino acid residue of the mature protein and after the predicted signal peptidase cleavage site. Amino acids −16 to −1 represent the cleaved signal sequence. The *N*-terminal amino acid sequence of mature dipeptidase as determined by Edman degradation is solid underlined. The partial sequences of the CNBr peptides are dashed underlined. The two *N*-linked glycosylation sites are indicated by asterisks. The underlined cDNA segments show the location of the (+ and −) degenerate PCR primers.

and 117 nucleotides of 3′-untranslated region (Fig. 1). A polyadenylation signal AATAAA is present beginning at bp 1368 [16 nucleotides away from the poly(A) tail] (Proudfoot & Brownlee, 1976). Residues 1–68 of the predicted amino acid sequence (underlined in Fig. 1) corresponded with the *N*-terminal amino acid sequence of mature dipeptidase determined by Edman degradation. Partial sequences of the CNBr peptides were also located in the predicted amino acid sequence (dashed underlined in Fig. 1).

Characteristics of the protein

The complete cDNA sequence encodes a protein of 409 amino acids with a calculated M_r of 44700. A hydropathy plot (Fig. 2)

reveals hydrophobic regions at both termini of the protein. At the *N*-terminus there is a typical cleavable signal sequence (amino acids −16 to −1 in Fig. 1). As determined by the weight matrix method of von Heijne (1986), maximal cleavage propensity ('score' of 7.5) was found before the *N*-terminal Asp determined by direct chemical sequencing of mature renal dipeptidase (Fig. 1). Also, at the *C*-terminus there is a stretch of 14 mainly hydrophobic amino acids. There are two potential *N*-linked glycosylation sites (Asn-41 and Asn-263; Fig. 1) present in the predicted protein sequence. During solid-phase sequencing, Asn-41 failed to sequence, consistent with the presence of attached carbohydrate.

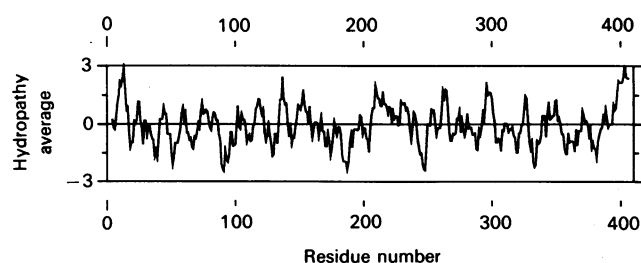The coding region of the pig renal dipeptidase cDNA sequence

Fig. 2. Hydropathy plot of pig renal dipeptidase

Hydropathy analysis of the deduced pig renal dipeptidase amino acid sequence was carried out as described by Kyte & Doolittle (1982) with a window size of seven residues. Positive values indicate increasing hydrophobicity. Amino acids are numbered from the initiating Met residue.

shows 82.7% identity to the analogous region of the recently sequenced human renal dipeptidase (Adachi et al., 1990). However, pig renal dipeptidase showed no significant similarity to any other protein (including peptidases and β-lactamases) in the Leeds/Birkbeck Owl, GenBank, Swissprot, NBRF or EMBL data bases.

Expression of pig renal dipeptidase in Xenopus laevis oocytes and identification of the glycosyl-phosphatidylinositol membrane anchor

Stage VI oocytes were microinjected with either renal dipeptidase mRNA or water, then incubated and assayed for dipeptidase activity as described in the Experimental section. The mRNA-injected oocytes were found to have expressed dipeptidase activity at their surface (27.0 pmol of D-Phe/min per oocyte), whereas no dipeptidase activity was detected in the water-injected oocytes. A time course of expression revealed maximal dipeptidase activity after 72 h of incubation at 18 °C (results not shown). To examine the mode of membrane anchorage of the expressed dipeptidase, protein was solubilized from the oocytes with either bacterial PI-PLC or octyl glucoside, a detergent which is effective at solubilizing G-PI anchored proteins (Hooper & Turner, 1988). When dipeptidase mRNA-injected oocytes were incubated with bacterial PI-PLC, dipeptidase activity was detected in the soluble fraction, whereas no activity was detected in the absence of PI-PLC (Table 1). Octyl glucoside also released dipeptidase activity into the soluble fraction from
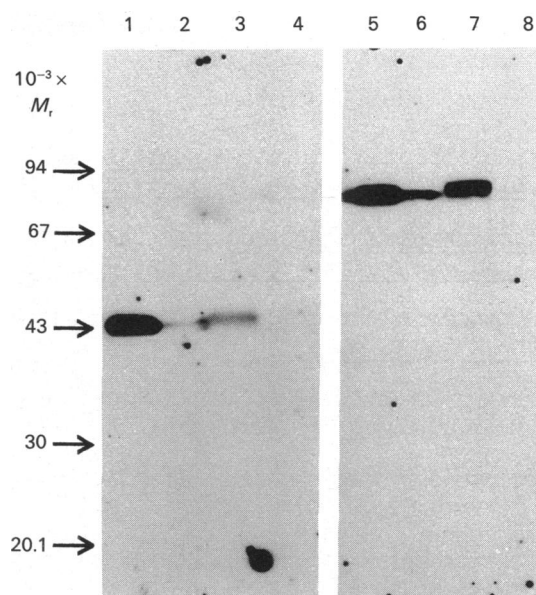

Fig 3. Western blot analysis of pig renal dipeptidase expressed in Xenopus laevis oocytes

Samples were prepared and analysed as described in the Experimental section. After electrophoretic transfer to poly(vinylidene difluoride) membranes, the membrane was blotted with antiserum to the phospholipase C-solubilized form of pig renal dipeptidase. Lanes 1 and 5, phospholipase-solubilized renal dipeptidase affinity purified from pig kidney cortex (0.5 μg of protein); lanes 2 and 6, phospholipase-solubilized renal dipeptidase affinity purified from pig kidney cortex (0.1 μg of protein); lanes 3 and 7, 31000 g, 1.5 h supernatant of oocytes injected with dipeptidase mRNA and then incubated with PI-PLC (1.95 units, 2 h at 37 °C; see legend to Table 1) (0.39 mg of protein); lanes 4 and 8, 31000 g, 1.5 h supernatant of oocytes injected with water and then incubated with PI-PLC (1.95 units, 2 h at 37 °C) (0.39 mg of protein). Lanes 1–4 were electrophoresed in reducing sample buffer, lanes 5–8 in non-reducing sample buffer.

mRNA-injected oocytes (Table 1). In both cases the enzyme activity was substantially inhibited by cilastatin (Table 1), a specific inhibitor of renal dipeptidase (Kahan et al., 1983). In contrast, no dipeptidase activity was detected in the PI-PLC or octyl glucoside solubilized fractions from oocytes injected with water.

Table 1. Solubilization of pig renal dipeptidase expressed in Xenopus laevis oocytes

Dipeptidase mRNA-injected oocytes (120) were incubated in MBS containing gentamycin (10 μg/ml) for 2 h at 37 °C in either the absence or presence of 1.95 units of B. thuringiensis PI-PLC. Alternatively, injected oocytes (10) were washed with 10 mM-Hepes, pH 7.4, and then incubated in 10 mM-Hepes/60 mM-octyl glucoside, pH 7.4, for 2 h at 4 °C. All samples were centrifuged at 31000 g for 1.5 h before assaying for enzyme activity. [a] The results are the mean (±S.E.M.) for four determinations. After solubilization, samples were subjected to phase separation in Triton X-114 as described in the Experimental section and the percentage of dipeptidase activity in the detergent poor (hydrophilic) phase determined. [b] The results are the mean (+S.E.M.) for three determinations. No dipeptidase activity was detected under identical solubilization conditions when oocytes were injected with water.

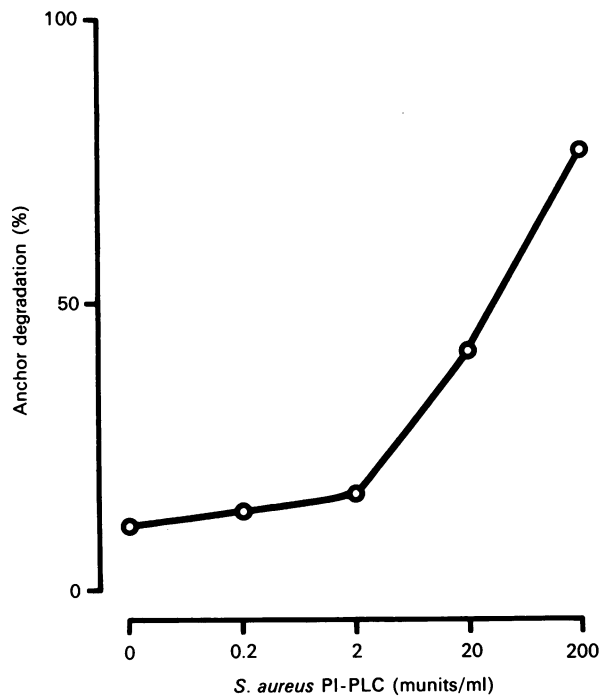| Solubilization conditions | Dipeptidase activity released into the soluble fraction[a] | | Inhibition by 0.1 mM-cilastatin (%) | Dipeptidase activity in detergent-poor phase[b] (% of total activity) |
	(nmol of D-Phe/min per mg)	(pmol of D-Phe/min per oocyte)		
None	Not detected	Not detected	–	–
PI-PLC	6.00±0.14	117.3±2.7	100.0	100.0±0.0
Octyl glucoside	3.23±0.19	400.2±23.5	97.8	11.2±0.1

Fig. 4. Hydrolysis by bacterial PI-PLC of the membrane anchor of renal dipeptidase expressed in *Xenopus laevis* oocytes

Oocytes injected with dipeptidase mRNA were treated with octyl glucoside to solubilize membrane proteins (see the legend to Table 1). Subsequently, the detergent-solubilized fraction (41 μg of protein) was incubated with PI-PLC from *S. aureus* for 2 h at 37 °C in 10 mM-Hepes, pH 7.4. After the incubation, samples were subjected to phase separation in Triton X-114 as described in the Experimental section. The results are the means of duplicate estimations. Anchor degradation of 100 % is equivalent to all of the dipeptidase activity being recovered in the detergent-poor phase after phase separation in Triton X-114.

Renal dipeptidase expressed in *Xenopus laevis* oocytes was analysed by Western blotting with an antiserum raised to pig renal dipeptidase (Littlewood *et al.*, 1989) (Fig. 3). When samples were electrophoresed under reducing conditions a single polypeptide of M_r 48600 was detected in the PI-PLC solubilized supernatant of mRNA-injected oocytes but not in the supernatant of water-injected oocytes (Fig. 3, lanes 3 and 4). In contrast, under non-reducing conditions a single polypeptide of M_r 80700 was detected in the supernatant from mRNA-injected oocytes but not in the supernatant from water-injected oocytes (Fig. 3, lanes 7 and 8). Under identical conditions renal dipeptidase purified from pig kidney cortex migrated with apparent M_r of 47000 and 79000, respectively (Fig. 3, lanes 1 and 5).

The dipeptidase activity solubilized from the oocytes with *B. thuringiensis* PI-PLC partitioned completely into the detergent-poor phase on phase separation in Triton X-114, whereas the octyl glucoside solubilized activity partitioned predominantly into the detergent-rich phase (Table 1). Also, the amphipathic detergent-solubilized dipeptidase activity was converted into a hydrophilic form upon incubation with *S. aureus* PI-PLC as measured by phase separation in Triton X-114 (Fig. 4).

DISCUSSION

Several attempts were made to isolate cDNA clones by screening with oligodeoxynucleotide probes corresponding to *N*-terminal sequences of pig renal dipeptidase. However, this approach was unsuccessful. The PCR technique was therefore

used to generate and amplify a segment of the dipeptidase cDNA coding for a region of the known *N*-terminal amino acid sequence. This polynucleotide probe was then employed to screen the pig kidney cortex cDNA library. A full length clone was identified, isolated and sequenced. The complete cDNA sequence predicts a protein of 409 amino acids. At the *N*-terminus there is a 16-residue cleavable signal sequence for translocation of this ecto-enzyme through the membrane of the endoplasmic reticulum (von Heijne, 1985, 1986). The calculated M_r for the predicted protein without the *N*-terminal signal sequence is 42800. This is slightly larger than the M_r of 40500 estimated by SDS/polyacrylamide-gel electrophoresis of the deglycosylated pig kidney enzyme (Littlewood *et al.*, 1989). This difference in size is probably accounted for by the removal of 20–25 amino acids from the *C*-terminus of the protein on processing to the G-PI anchored form. The *C*-terminal region of 14 mainly hydrophobic amino acids is characteristic of the *C*-terminal signal peptide present in all G-PI anchored proteins sequenced to date (Ferguson & Williams, 1988; Cross, 1990). The predicted amino acid sequence of pig renal dipeptidase contains two potential *N*-linked glycosylation sites. The results of the chemical sequencing indicate very strongly that Asn-41 is glycosylated. Earlier deglycosylation studies (Littlewood *et al.*, 1989) indicated the presence of two distinct populations of *N*-linked sugars that differed in their susceptibility to cleavage by *N*-glycanase. Thus, it is most probable that both potential *N*-linked glycosylation sites are glycosylated in the mature protein.

The mRNA corresponding to the cDNA sequence of pig renal dipeptidase was expressed in *Xenopus laevis* oocytes. The dipeptidase activity expressed on the surface of the oocytes was inhibited by the specific inhibitor cilastatin and was sensitive to release by bacterial PI-PLC. Phase separation in Triton X-114 revealed that the phospholipase-solubilized dipeptidase activity was hydrophilic, whereas the octyl glucoside-solubilized activity was amphipathic. Also, this detergent-solubilized amphipathic dipeptidase activity was converted by bacterial PI-PLC into a hydrophilic form. These observations are consistent with the removal by bacterial PI-PLC of the hydrophobic lipid anchor from renal dipeptidase expressed in *Xenopus laevis* oocytes, in agreement with previous results for the enzyme isolated from pig kidney (Hooper *et al.*, 1987; Hooper & Turner, 1989). Therefore, oocytes appear to possess the biochemical machinery required for synthesis of a G-PI anchor.

When renal dipeptidase mRNA was expressed in *Xenopus laevis* oocytes a single polypeptide of M_r 48600 was recognized on Western blotting of the PI-PLC solubilized fraction with an antiserum to the pig kidney enzyme. Under non-reducing conditions, a single polypeptide of approx. M_r 80700 was detected. These results indicate that renal dipeptidase expressed in *Xenopus laevis* oocytes exists as a disulphide-linked dimer of identical subunits, as observed for the enzyme purified from pig kidney cortex (Littlewood *et al.*, 1989; Hooper & Turner, 1989). The slight difference in size observed between the dipeptidase expressed by *Xenopus laevis* oocytes and that purified from pig kidney cortex probably reflects species-specific differences in glycosylation. The antiserum to renal dipeptidase appears to be less sensitive in the detection of protein samples electrophoresed under reducing conditions compared with those electrophoresed under non-reducing conditions (compare Fig. 3, lanes 2 and 6). This is probably because the antiserum was raised against the native disulphide-linked form of renal dipeptidase, some antigenicity being lost on reduction of the enzyme.

Pig renal dipeptidase shows extensive similarity at the amino acid level with the recently sequenced human renal dipeptidase (Adachi *et al.*, 1990). The human enzyme consists of 411 residues, beginning with a 16-residue *N*-terminal signal peptide and a

highly hydrophobic sequence at the C-terminus. The two additional amino acid residues in the human sequence, as compared to the pig, occur in the C-terminal 25 amino acids which would probably be cleaved from the mature protein on processing to the G-PI anchored form. As well as the two N-linked glycosylation sites at Asn-41 and Asn-263, the human dipeptidase contains two additional sites at residues 316 and 342 consistent with our previous observation that the difference in size of renal dipeptidase between pig and human is due to differences in the extent of N-linked glycosylation (Hooper et al., 1990a).

Although renal dipeptidase is a Zn^{2+} metalloenzyme (Armstrong et al., 1974), the characteristic signature identified in other Zn^{2+} metalloendopeptidases (His-Glu-Xaa-Xaa-His) (Jongeneel et al., 1989) is not found within the protein sequence of renal dipeptidase. However, comparison of the dipeptidase sequence with other Zn^{2+} enzymes (Vallee & Auld, 1990) reveals close similarity of the amino acids potentially comprising the active site Zn^{2+} ligands with those in D-alanyl-D-alanine-cleaving carboxypeptidase of Streptomyces albus (Dideberg et al., 1982). It is of interest that both these enzymes can cleave peptides with a C-terminal D-amino acid and both are sensitive to inhibition by β-lactam antibiotics. In particular the Zn^{2+} binding motif of the bacterial enzyme, Asp^{192}-His^{193}-Xaa-Xaa-His^{196}, is present in both pig and human renal dipeptidase: Asp^{269}-His^{270}-Xaa-Xaa-His^{273}. In D-alanyl-D-alanine-cleaving carboxypeptidase the third Zn^{2+} ligand is located 40 residues on the N-terminal side of this motif (His^{152}). In renal dipeptidase there is a candidate His at position 219, 50 residues on the N-terminal side of the motif, which may be the third ligand. However, the precise identity and location of the Zn^{2+} ligands in renal dipeptidase will require chemical modification, site-directed mutagenesis and X-ray crystallographic data.

The majority of the dozen or so mammalian brush border peptidases are anchored in the plasma membrane by a hydrophobic transmembrane polypeptide located at either the N- or C-terminus (Turner & Hooper, 1990). Renal dipeptidase is one of only two of these ectoenzymes which is known to possess a G-PI membrane anchor, the other being aminopeptidase P (Hooper et al., 1990b). Thus, the isolation of the cDNA encoding renal dipeptidase and its expression in a G-PI anchored form in Xenopus laevis oocytes should allow further characterization of the functional role of a G-PI anchor in this group of proteins.

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