Identification of Membrane Anchoring Site of Human Renal Dipeptidase and Construction and Expression of a cDNA for Its Secretory Form*

Hideki Adachi, Toyoko Katayama, Chikako Inuzuka, Shinzo Oikawa, Masafumi Tsujimoto, and Hiroshi Nakazato

From the Suntory Institute for Biomedical Research, Shimamoto-cho, Mishima-gun, Osaka 618, Japan

(Received for publication, April 2, 1990)

The chemical properties of human renal dipeptidase (hrDP) purified from the membrane fraction of kidney have been characterized. When treated with phosphatidylinositol-specific phospholipase C, hrDP was released from renal membrane fractions. After digestion with trypsin, carboxyl-terminal peptide was isolated employing anhydrotrypsin-agarose column chromatography and reversed-phase high performance liquid chromatography. The amino acid sequence of the peptide was identified at positions 363-369 in the primary sequence deduced from the cDNA sequence (Adachi, H., Tawaragi, Y., Inuzuka, C., Kubota, I., Tsujimoto, M., Nishihara, T., and Nakazato, H. (1990) J. Biol. Chem. 265, 3992-3995). Further examination of the chemical composition of the peptide showed that it contained, respectively, 2, 1, 5, 1, and 1 mol of ethanamine, glucosamine, mannose, inositol, and phosphate in addition to amino acids. These results suggest that the mature hrDP molecule lacks the carboxyl-terminal hydrophobic peptide extension predicted from the cDNA sequence and is anchored at Ser^369 via glycosyl-phosphatidylinositol to the membrane. To characterize further the action of the enzyme, we have established expression systems for both secretory and membrane anchored forms of hrDP using COS-1 cells and found that both recombinant forms were as active as natural enzyme. Our expression system made it possible to prepare large amounts of soluble enzyme, and will contribute toward elucidation of the physiological roles of the enzyme.

Human reninal peptidase (hrDP), its hydrolysis of some b-lactam antibiotics such as penem and carbenem (5-8).

Human reninal dipeptidase is a membrane-bound glycoprotein, which was recently shown to be released from kidney microvillar membranes by phosphatidylinositol-specific phospholipase C treatment, suggesting its anchorage to the membrane via glycosyl-phosphatidylinositol (GPI) (9, 10). Involvement of GPI is also suggested for a large number of membrane-bound proteins (11-15). Carboxyl-terminal domains attached to GPI were also isolated from several proteins such as the variant surface glycoprotein of Trypanosoma brucei (16), Thy-1 (17), acetylcholinesterase (18), decay-accelerating factor (19), placental alkaline phosphatase (20, 21), and carcinoembryonic antigen (22). Chemical analyses revealed that they contained a GPI, comprising ethanolamine, neutral sugars, hexosamine, and phosphatidylinositol, which is proposed to mediate anchoring of the protein to the membrane. Moreover, detailed structural analyses have been reported for the GPI anchors of the variant surface glycoprotein (23), Thy-1 (24), and acetylcholinesterase (25).

Recently, we have purified hrDP from renal membrane fractions, determined the amino-terminal sequence (4), and cloned the cDNA which has been expressed in COS-1 cells to produce enzymatically active hrDP (26). In the present study, we have isolated the carboxyl-terminal peptide from the trypsin digest of hrDP and analyzed its amino acid sequence and chemical composition in detail. Furthermore, we have constructed a plasmid containing a cDNA encoding hrDP lacking the carboxyl-terminal hydrophobic extension, amino acids 370-395, and have shown that the enzyme with normal activity is secreted into the medium when expressed in COS-1 cells.

EXPERIMENTAL PROCEDURES

Materials—PI-PLC from Bacillus thuringiensis was purchased from Funakoshi Pharmaceutical Co. Ltd. (Tokyo, Japan). Phospholipase C from Bacillus cereus (Grade I) was from Boehringer Mannheim (Federal Republic of Germany). Rat platelet secretory phospholipase A_2 was kindly supplied by Drs. K. Inoue and I. Kudo (University of Tokyo). One unit of phospholipase A_2 is defined as the amount of enzyme catalyzing the release of 1 nmol of fatty acid/min at 37 °C (27). One unit of phospholipase C and PI-PLC is defined as the amount of enzyme catalyzing the release of 1 nmol of phosphatidylcholine or phosphatidylinositol/min at 37 °C. Cilastatin-Sepharose was prepared as described previously (4). Anhydrotrypsin-agarose was obtained from Takara Shuzo Co., Ltd. (Kyoto, Japan).

Treatment of Membrane Fraction with Phospholipases—All procedures were carried out at 4 °C unless otherwise noted. Human kidney (7.5 g) was homogenized in 10 volumes of 150 mM NaCl, 10 mM Tris/HCl buffer, pH 7.4, and centrifuged at 8,000 × g for 15 min. The
supernatant was further centrifuged at 36,000 x g for 2 h. Then the pellet was suspended in 20 ml of 100 mM NaCl, 10 mM Tris/HCl buffer, pH 7.4 (buffer A), and centrifuged at 31,000 x g for 90 min. The final pellet was resuspended in buffer A to give a concentration of 1.2 mg/ml and used to examine the release of hrDP by treatment with various phospholipases. The membrane fractions were incubated with the same volume of phospholipase A2 (15,500 units/mg), phospholipase C (2,000 units/mg), and PL-PLC (356 units/mg) at 37 °C for 30 min. After centrifugation at 31,000 x g for 15 min, the supernatant was collected and subjected to hrDP assay. Activity recovered in the supernatant was expressed as percentage of activity which was determined on the total membrane fraction after solubilization with 120 mM n-octyl-D-glucopyranoside (4).

**Preparation of hrDP Tryptic Fragments**—Purified hrDP (400 μg) (4) was carboxymethylated with dithiothreitol and iodoacetate in the presence of 6 M guanidine hydrochloride at pH 8.0 (28), and then dialyzed against 0.1% (v/v) trifluoroacetic acid, purified by reversed-phase HPLC, and bophilized. Carboxymethylated hrDP was dissolved in 0.2 ml of 0.1 mM ammonium bicarbonate and digested with L-1-tyrosylamido-2-pentothylchloromethyl ketone-treated trypsin (Sigma) at a protein/enzyme ratio of 50:1 (w/w) for 16 h at 37 °C. For the purification of the carboxyl-terminal fragment, the tryptic digests were acidified to pH 5.0 by the addition of acetic acid and applied onto an anhydrotrypsin-agarose column (5 x 40 mm), which had been equilibrated with 50 mM ammonium acetate buffer, pH 5.0 (29). The pooled break-through fraction was separated by reversed-phase HPLC on a Vydac C8 column (4.6 x 150 mm) using a linear gradient of acetonitrile in 0.1% (v/v) trifluoroacetic acid.

**Peptide Sequence Analysis**—The amino acid sequence of the purified carboxy-terminal peptide (250 pmol) was obtained using an Applied Biosystems 477A gas-phase Sequencer (30).

**Chemical Analyses of the Carboxy-terminal Peptide of hrDP**—Amino acids, glucosamine, and ethanolamine were quantitated by a Hitachi amino acid analyzer (model L3500) by fluorogenic detection with o-phthalaldehyde (31). Samples were hydrolyzed in 6 N HCl at 110 °C in vacuo for 24 h.

Monosaccharides were quantitated by anion exchange chromatography with pulsed amperometric detection (32). In brief, the peptide (250 pmol) was hydrolyzed in 2 M trifluoroacetic acid at 100 °C for 8 h or 8 N HCl at 100 °C for 8 h under N2 and then evaporated. After dissolution in water, the sample was applied onto a column of Dionex Carbopac PA1 pellicular anion exchange resin (4.6 x 250 mm), equipped with Dionex Carbopac PAG guard column (5 x 25 mm), and then eluted isocratically with 20 mM NaOH. The detection system used consisted of a Dionex BioLC gradient pump module and model PAD2 detector equipped with a gold working electrode. In order to minimize base-line distortion around the amino sugar peaks, 400 mM NaOH was added to the postcolumn effluent.

Inositol was quantitated by measuring its benzylester as described (33). The peptide (1 nmol) was hydrolyzed in 6 N HCl at 100 °C for 8 h under N2, and then evaporated. Two ml of 2 N NaOH and subsequently 20 μl of benzyl chloride were added. After incubation for 20 min at ambient temperature, 2 ml of a saturated solution of NaCl in water and then 5 ml of diethyl ether was added. After vigorous shaking, 4 ml of the ether phase was removed, evaporated under N2 at 40 °C, and the residue was dissolved in 400 μl of methanol. The sample was loaded on a Vydac C8 (4.6 x 250 mm) column and eluted isocratically with 70% acetonitrile and detected at 230 nm. Glucose was used as an internal standard.

Glycerol was quantitated by measuring its dinitrobenzyl ester as described (34). The peptide (1 nmol) was hydrolyzed in 6 N HCl at 100 °C for 8 h under N2, and then evaporated. Dinitrobenzyl chloride (4 mg/ml in tetrahydrofuran) was added to the sample, and then the test tube was sealed and heated at 60 °C for 1 h. After cooling, the sample was subjected to reversed-phase HPLC. The sample was loaded on a Vydac C8 (4.6 x 250 mm) column and eluted isocratically with 15% acetonitrile and detected at 245 nm. Ethyleneglycol was used as an internal standard.

Phosphate was quantitated by ion chromatography (35) using a Shim-Pack IC-AL column (4.6 x 100 mm). As an eluent, a buffer containing 4.8 mM borate, 6.0 mM Tri, and 14.4 mM mannitol, pH 7.9, was used at a flow rate of 1.5 ml/min. Phosphate was measured by its electron conductivity monitored using a Shimadzu CDD-6A detector.

**Construction of cDNA for Secretory hrDP**—A cDNA for the expression of secretory hrDP was constructed as described in Fig. 1. In brief, the plasmid containing the cDNA for MDP4 (26) was partially digested with EcoRI and PstI. The 1.4-kilobase EcoRI-PstI fragment was isolated by agarose gel electrophoresis. For the introduction of a stop codon just after the codon for Ser99, we synthesized two oligodeoxyribonucleotides, HMDP2 and HMDP3, shown in Fig. 1. HMDP2 and HMDP3 (150 pmol each in 10 mM Tris/HCl, 1 mM EDTA, pH 8.0) were heated at 95 °C for 2 min and slowly chilled. The annealed oligodeoxyribonucleotides were ligated with the 1.4-kilobase EcoRI-PstI fragment using a Takara ligation kit. After digestion with EcoRI, the solution was heated at 55 °C for 10 min, and then the ligated fragment was subeluted into pUC19. After transfection into Escherichia coli JM109, the recombinant plasmids were prepared and sequenced using Sequenase (United States Biochemical Corp.). The cDNA was then ligated with the expression vector pPKCR-dhfr as described (26).

**Characterization of Recombinant hrDPs**—COS-1 cells were maintained in Dulbecco's modified Eagle's medium/10% fetal bovine serum (Gibco) and were transfected employing Cell-Phect transfection kit (Pharmacia). After transfection, media and cell extracts were assayed for hrDP activity using glycidylhydroxyphenylalanine as a substrate (36). Release of hrDP from intact cells by PI-PLC was monitored in the following manner. The cells, 72 h after transfection, were washed three times in phosphate-buffered saline, three times in release buffer (25 mM Tris/HCl, 0.25 mM succrose, 10 mM glucose, pH 5.0) and then were suspended in the same release buffer at a concentration of 1 x 106 cells/ml. One ml of cell suspension was then incubated either in the presence or absence of 0.1 unit of PI-PLC. After incubation, 700-μl aliquots of the cell suspensions were centrifuged at 15,000 x g for 2 min, and the resulting supernatants were assayed for hrDP activity (4). For the purification of hrDPs expressed in COS-1 cells, the culture media, the cell extracts, and the supernatants of the PI-PLC-treated cells were dialyzed against 50 mM Tris/HCl buffer, pH 7.6, containing 0.1% CHAPS (buffer B) and applied to a DEAE-Sepharose column (1 x 2 cm) and eluted with buffer B containing 0.5 M NaCl. Then the eluent was applied onto a refastatin-Sepharose column (1 x 2 cm) and washed with the same buffer. For the elution of hrDPs, chlorostatin...
solution was used as described (4). After extensive dialysis, the hrDP activity was assayed (4).

RESULTS

Susceptibility of hrDP to PI-PLC—Initially, we examined whether or not hrDP was released from renal membrane fraction by treatment with PI-PLC. As shown in Fig. 2, hrDP was released into the soluble fraction in a dose-dependent manner. In contrast, phospholipase A$_2$ and phospholipase C were unable to release hrDP. These results suggest that hrDP is anchored to the membrane via covalently attached GPI.

**Purification and Chemical Characterization of Carboxyl-terminal Peptide of hrDP—**The carboxyl-terminal tryptic peptide of hrDP was purified by employing anhydrotrypsin-agarose column chromatography. The tryptic map for hrDP is shown in Fig. 3A. Another aliquot containing the same amount of the tryptic digests was applied onto an anhydrotrypsin-agarose column, and the peptides in break-through fraction (where peptides with no lysine nor arginine residue at carboxyl-terminal end should appear) were separated by reversed-phase HPLC under the same conditions as for the tryptic map (Fig. 3B). By comparing the two HPLC chromatograms, the presence of a unique peak (designated as T11) that did not bind to the anhydrotrypsin agarose column was apparent. A single amino acid sequence was obtained by analysis with an automated gas phase sequencer as follows: H-Thr-His-Tyr-Gly-Tyr-Ser-Ser. By comparing with the primary structure of prepro-hrDP deduced from the cloned cDNA (26), the peptide is assigned at positions 363–369 (Fig. 4).

**FIG. 4. Amino acid sequence of purified carboxyl-terminal peptide and comparison with cDNA-predicted peptide sequence.** The purified peptide (260 pmol) was subjected to amino acid sequencing as described under “Experimental Procedures.” The determined sequence was compared with the carboxyl-terminal sequence (shown by single-letter code) predicted by the cDNA sequence (26). The determined sequence and a stretch of hydrophobic amino acids are indicated by double and single underlines, respectively.

<table>
<thead>
<tr>
<th>Component</th>
<th>Molar ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Threonine</td>
<td>0.9</td>
</tr>
<tr>
<td>Serine</td>
<td>1.6</td>
</tr>
<tr>
<td>Glucosamine</td>
<td>0.6</td>
</tr>
<tr>
<td>Glycine</td>
<td>1.0</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>2.4</td>
</tr>
<tr>
<td>Ethanolamine</td>
<td>1.6</td>
</tr>
<tr>
<td>Histidine</td>
<td>0.9</td>
</tr>
<tr>
<td>Mannose</td>
<td>4.5</td>
</tr>
<tr>
<td>Inositol</td>
<td>1.2</td>
</tr>
<tr>
<td>Phosphate</td>
<td>0.8</td>
</tr>
<tr>
<td>Glycerol</td>
<td>&lt;0.1</td>
</tr>
</tbody>
</table>

Minor peaks in Fig. 3B might be partial digests in addition to trypsin, which we did not analyze further for it was apparent that the T11 was the major peptide which contained the carboxyl-terminal end of hrDP (Fig. 3).

Next, we examined whether T11 peptide contained chemical components other than amino acids. Table I summarizes the results. All the components were normalized for their molar ratios by taking glycine as 1 mol. Our data indicated that the purified peptide contained 2 mol of ethanolamine and 1 mol of glucosamine. In addition, it contained 5 mol of mannose, 1 mol of inositol, and 1 mol of phosphate. These components are characteristic of the carboxyl-terminal peptide covalently attached to GPI (16–23). However, we could not detect the presence of glycerol.

Expression of Secretory Form of hrDP in COS-1 Cells—To express the secretory form of hrDP in order to characterize further the properties of the enzyme, we constructed a cDNA as described under “Experimental Procedures” and transfected COS-1 cells with it. When MDP4 (26) which coded the complete sequence for hrDP was expressed in COS-1 cells, hrDP activity was mainly localized on cell surface membrane, for most of it was released by PI-PLC treatment of the cells (Fig. 5). In contrast, the product of the cDNA, MDP4S, which lacked the sequence encoding hydrophobic carboxyl-terminal amino acids 370–395, was found predominantly in the culture medium (Table II). It would be noteworthy that the total activity of the MDP4S product expressed in COS-1 cells was about 2-fold more than that of the MDP4 product. These results further confirmed that hrDP is located at the cell surface and anchored to the membrane via covalently attached GPI.

When measured on the affinity purified enzymes, the specific activity of the secretory hrDP (MDP4S) was almost identical to both of the GPI-anchored natural and recombinant hrDPs. The membrane-bound hrDP retained its full activity after treatment with PI-PLC (Table III).

Fig. 6A shows SDS-PAGE analysis of hrDPs from various sources. It was revealed that both secretory and membrane-anchored forms of recombinant hrDPs migrated at around 62
Membrane Anchoring Site of Human Renal Dipeptidase

In agreement with the results reported by Hooper et al. (9, 10), we have shown that hrDP was anchored to the cellular membrane via GPI. We have further characterized the chemical composition of carboxyl-terminal peptide through which hrDP was anchored. The amino acid sequence of the carboxyl-terminal peptide indicated that the mature hrDP molecule lacks the hydrophobic amino acid extension (370-395 in the primary structure predicted from the cDNA sequence) and is

![Graph](image)

**FIG. 5.** Release of recombinant hrDP from pdKCR-dhfr-MDP4- and MDP4S-transfected COS-1 cells treated with (O) or without (●) PI-PLC. Total hrDP activity in samples were 6 milliunits.

**TABLE II**

hrDP activity from MDP4- and MDP4S-transfected COS-1 cells

<table>
<thead>
<tr>
<th>Source</th>
<th>Cell extract (milliunits)</th>
<th>Medium (milliunits)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDP4-transfected COS-1 cells</td>
<td>139.1 ± 15.0</td>
<td>61.8 ± 10.2</td>
</tr>
<tr>
<td>MDP4S-transfected COS-1 cells</td>
<td>20.7 ± 4.7</td>
<td>342.4 ± 31.6</td>
</tr>
</tbody>
</table>

*Mean ± S.D. of four individual preparations. The cell extract and medium from control COS-1 cells had little activity (<0.1 milliunit).

**TABLE III**

Specific activities of hrDPs after purification by DEAE-Sepharose column chromatographies

<table>
<thead>
<tr>
<th>Source</th>
<th>Specific activity* (units/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDP4 (cell extract)</td>
<td>156</td>
</tr>
<tr>
<td>MDP4 (released by PI-PLC treatm)</td>
<td>167</td>
</tr>
<tr>
<td>MDP4S (medium)</td>
<td>159</td>
</tr>
<tr>
<td>Natural hrDP (purified from human kidney)</td>
<td>134</td>
</tr>
</tbody>
</table>

*Mean value of duplicate determinations.

kDa as did natural hrDP (4). However, it should be noted that the secretory form of recombinant hrDP showed slightly broader bands than those of the membrane-anchored forms of natural and recombinant hrDPs, although the same amounts of the enzymes were loaded. After treatment with endoglycosidase F (4), recombinant hrDPs as well as natural hrDP were shown to migrate at around 42 kDa (Fig. 6B). These results indicated that hrDPs expressed in the COS-1 transfectants were also glycosylated. The possible differences in glycosylation among these hrDPs will be discussed below.

**DISCUSSION**

In agreement with the results reported by Hooper et al. (9, 10), we have shown that hrDP was anchored to the cellular membrane via GPI. We have further characterized the chemical composition of carboxyl-terminal peptide through which hrDP was anchored. The amino acid sequence of the carboxyl-terminal peptide indicated that the mature hrDP molecule lacks the hydrophobic amino acid extension (370-395 in the primary structure predicted from the cDNA sequence) and is attached at Ser\(^{260}\) to GPI. Furthermore, it was also shown that the carboxyl-terminal peptide contained 2 mol of ethanolamine, 1 mol of glucosamine, 5 mol of mannose, 1 mol of inositol, and 1 mol of phosphate per mol of peptide. These components are characteristic to GPI. In fact, we observed that purified hrDP partitioned almost completely (>95%) into the detergent-poor phase in the phase separation experiment using Triton X-114, further suggesting that the 1,2-diacylglycerol moiety is not present in the carboxyl-terminal end of purified hrDP.\(^3\) It was shown that alkaline phosphatase which contained the 1,2-diacylglycerol moiety partitioned into the detergent-rich phase (37). Possible explanation of our results might be that the GPI of the purified hrDP was hydrolyzed by a plasma phospholipase D (10) or endogeneous PI-PLC (38) during solubilization with n-octyl-\(\beta\)-D-glucopyranoside. We did not attempt to detect fatty acids because the amount of the hrDP required for the assay using the conventional gas-liquid chromatography was prohibitively large for the human enzyme.

Recently, mechanisms involved in the carboxyl-terminal processing of GPI-tailed protein were investigated using cDNA for placental alkaline phosphatase by Micanovic et al. (39). They classified amino acids found at the carboxyl-terminal end of recombinant hrDP into two categories, depending on their susceptibility to the putative "transamidating" enzyme. According to their criteria, the serine residue identified at the carboxyl-terminal end of hrDP should be classified into category I. The residues of category I (Asp, Gly, Ala, Cys, Asn, and Ser) are preferred because they serve as better substrates for the putative transamidating enzyme.

---

A secretory form of hrDP was successfully expressed in COS-1 cells transfected with a cDNA which encodes the proper hrDP lacking the carboxyl-terminal hydrophobic stretch. Like other GPI-anchored proteins, such as placental alkaline phosphatase (37, 40) and decay-accelerating factor (38), the secretory form of hrDP thus expressed was detected mainly in the culture medium. The specific activity of the secretory form of hrDP was identical to those of GPI anchored forms of hrDPs both from natural and recombinant sources.

Hooper et al. (10) reported that two bands were observed when the native hrDP treated with N-glycanase was analyzed on SDS-PAGE. Although the separation was not as clear, we also observed two bands for the endoglycosidase F-treated hrDP (Fig 6B, lane 1). It may indicate that there were two distinct populations of N-linked carbohydrate that differ in their susceptibility to cleavage by endoglycosidase F. Alternatively, it might reflect the presence of various amounts of O-linked carbohydrates, which is not cleavable by the endoglycosidase F.

The lower bands of the endoglycosidase F-treated secretory hrDPs migrated slightly faster than those of the endoglycosidase F-treated membrane-bound forms of hrDPs on SDS-PAGE, most likely because it lacks GPI moiety. What is unusual is that, even after extensive treatment with endoglycosidase F, the upper bands of secretory hrDPs (Fig. 6B, lanes 5 and 6) apparently have the same mobility as those of GPI-anchored hrDPs (Fig. 6B, lanes 1–4). It might reflect qualitative and/or quantitative differences in glycosylation between the secretory and GPI-anchored hrDPs. However, these are left for future studies.

Our system will enable us to prepare a large amount of secretory form of hrDP to study the mechanism of hydrolytic action of the enzyme. Moreover, production of the secretory form of hrDP in large scale will make it easy to crystallize the hrDP, which will be useful for the study of enzyme-substrate interactions.

REFERENCES

34. Jupille, T. H. (1976) Am. Lab. 8, 84