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Directed mutagenesis of pig renal membrane dipeptidase

His^{219} is critical but the DHXXH motif is not essential for zinc binding or catalytic activity

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Abstract

Pig renal membrane dipeptidase cDNA has been expressed in COS-1 cells. Directed mutagenesis was used to investigate the roles of some conserved histidyl and aspartyl residues. Mutation of His^{219} to Arg, Lys or Leu results in complete abolition of enzyme activity, although the mutants are expressed at the cell-surface. Residues in a proposed motif (DHXDH; residues 269–273) for zinc binding have been mutated individually. Each retained activity comparable to that of the wild-type, excluding an essential role for components of this motif. The zinc-binding ligands in membrane dipeptidase therefore represent a novel domain for a metallopeptidase with His^{219} being one candidate.

Key words: Site-directed mutagenesis; Membrane dipeptidase; Zinc metallopeptidase; Renal brush border

1. Introduction

Membrane dipeptidase (MDP; microsomal dipeptidase; dehydropeptidase-I; EC 3.4.13.19) is a glycosylphosphatidylinositol (GPI)-anchored protein of the kidney brush border membrane. It was originally described as a renal enzyme that could hydrolyse a variety of dipeptides; glycyl-D-phenylalanine (Gly-D-Phe) commonly being used as an assay substrate. The enzyme exhibits a number of other catalytic activities including the conversion of leukotriene D_4 to leukotriene E_4 and hydrolysis of the β-lactam antibiotic, imipenem (see [1] for review). All of these activities are blocked by the selective MDP inhibitor, cilastatin. Immobilized cilastatin has proved highly effective in affinity purification of MDP after solubilization of the enzyme by treatment with phosphatidylinositol-specific phospholipase C (PI-PLC) [2].

MDP exists as disulphide-linked homo-dimeric glycoprotein of subunit M_r 47,000 in pig and subunit M_r 59,000 in human. The differences in size between the two species being entirely attributable to differences in *N*-linked glycosylation [2–4]. cDNAs encoding the protein were originally isolated and sequenced from human placental and renal cDNA libraries [5] and from a pig kidney library [6]. The pig and human enzymes show extensive similarity, consisting of 409 and 411 residues, respectively. Both possess a cleavable N-terminal signal peptide and a highly hydrophobic sequence at the C-terminus which provides the signal for GPI-anchor attachment. Pig MDP has two *N*-linked glycosylation sites, also found in the human enzyme, but the latter contains two additional sites consistent with the greater extent of glycosylation in this species.

MDP is a zinc metallopeptidase [7] containing 1 mol of Zn^{2+} per subunit [8]. However, it does not contain within its sequence the characteristic signature identified in many other Zn^{2+} metallopeptidases (HEXXH), including a peptidyldipeptidase (angiotensin converting enzyme; EC 3.4.15.1) [9–11]. The inverse motif (HXXEH) seen in some other Zn^{2+}-metallopeptidases such as insulin-degrading enzyme [12] is also absent. Comparison of the human and pig MDP sequences with other Zn^{2+} enzymes [6,13] revealed a sequence (DHLDH; residues 269–273) highly similar to a sequence (DHTH) containing the active site Zn^{2+}-ligands in *Streptomyces albus* D-alanyl-D-alanyl-cleaving carboxypeptidase [14]. The DHLDH motif in MDP is also conserved in the more recently cloned rat and mouse enzymes [15,16]. In the carboxypeptidase from *S. albus*, a further Zn^{2+} ligand is located 40 residues on the N-terminal side of the DHXH motif. We previously speculated that in MDP, His^{219}, 50 residues N-terminal to the putative motif, may fulfill this role [6]. In the present work we have employed directed mutagenesis to explore the roles of these resi-

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Abbreviations: DMEM, Dulbecco's Modified Eagle Medium; FITC, fluorescein isothiocyanate; GPI, glycosylphosphatidylinositol; MDP, membrane dipeptidase; PBS, phosphate-buffered saline; PI-PLC, phosphatidylinositol-specific phospholipase C; TBS, Tris-buffered saline.

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dues in the catalytic activity of MDP. Surprisingly, the dipeptidase DHLDH sequence does not appear to be required for catalytic activity, although His^{219} is essential. Since the predicted amino acid sequence contains no other recognised zinc motif, this metallopeptidase must contain a novel zinc-binding domain in which His^{219} may be one of the important residues involved in zinc binding or catalysis.

2. Materials and methods

2.1. Materials

T4 polynucleotide kinase was obtained from New England Biolabs. Sequenase kit (version 2.0) was from Amersham. [^{35}S]dαATP (1000 Ci/mmol) was from New England Nuclear. Tissue culture media, serum, trypsin-EDTA, penicillin, streptomycin, L-glutamine, transfection reagent (lipofectAmine) and T4 DNA ligase were purchased from Gibco-BRL. The mammalian expression vector pEF-BOS was a generous gift from Dr. S. Nagata (Osaka, Japan) [17]. Gly-D-Phe and bicinchoninic acid were obtained from Sigma. Cilastatin was a gift from Merck, Sharp and Dohme, Rahway, NJ, USA. *Bacillus thuringiensis* PI-PLC was a gift from Dr. M.G. Low (New York, USA). All other reagents were of analytical grade.

2.2. Methods

2.2.1. Cell culture. COS-1 cells were cultured in DMEM supplemented with 10% foetal calf serum (heat inactivated), 100 units/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine at 37°C in 5% CO_2.

2.2.2. Construction of the expression vector (pEF-MDP) and transient expression in COS-1 cells. The Bluescript SKII$^-$ plasmid containing an insert of 1.4 kb encoding the cloned pig MDP (pER11) [6] was digested with *Eco*RI and a fragment of 1.4 kb was purified by agarose gel electrophoresis. The eukaryotic expression vector pEF-BOS [17] was digested at *Xba*I sites and the two fragments were ligated using standard cloning procedures [18]. The orientation of the insert relative to the pEF promoter was verified by *Sma*I and *Sac*I digestions. The resulting plasmid (pEF-MDP) was used for transfection of COS-1 cells. For transient expression of pEF-MDP in COS-1 cells, the cells were plated in 24-well plates at 33% confluency or in 150 cm^2 flasks at approx. 2×10^6 cells per flask. After 24 h of growth, the cells were washed twice with OptI-Mem and transfected (0.2 μg DNA/well) by using lipofectAmine as cationic lipid (DNA : lipid, 1 : 10). The cells were incubated for 2 h and DMEM containing 10% foetal calf serum was added. After 24 h, the medium was replaced with fresh, and transient expression was assayed on the cell surface or in a solubilized membrane preparation 48 h after transfection.

2.2.3. Preparation of membrane fraction. Adherent cells were washed three times with phosphate-buffered saline (PBS), scraped from the plates and spun down ($1000 \times g$, 10 min). Subsequently, the pellet was resuspended in 50 mM HEPES/NaOH, pH 7.5, containing 20 mM $CaCl_2$ and cells disrupted by using N_2 cavitation. After a brief centrifugation at $1000 \times g$, the supernatant was centrifuged at $100{,}000 \times g$ for 1 h and the membrane pellet was resuspended in 50 mM HEPES/NaOH, pH 7.5. Membranes were solubilized by incubation with 60 mM *n*-octyl-β-D-glucopyranoside for 1 h at 4°C and centrifuged at $12{,}000 \times g$ for 10 min. The solubilized membranes were stored in aliquots at −20°C.

2.2.4. Assay of membrane dipeptidase activity. Solubilized membranes (10 μl; 1.2 μg protein) were incubated in 0.1 M Tris/HCl, pH 8.0, containing 3 mM Gly-D-Phe with or without the inhibitor cilastatin (0.3 mM) in a total volume of 100 μl. After incubation for 3 h at 37°C, samples were boiled for 4 min and centrifuged at $12{,}000 \times g$ for 10 min at 4°C. The product, D-Phe, was resolved from the substrate and quantified by HPLC on a μBondapak C_{18} column by using a linear gradient of 4.5–30% acetonitrile in 0.08% H_3PO_4 [19].

2.2.5. Site-directed mutagenesis. Site-directed mutagenesis was performed according to the method of Kunkel [20]. *E. coli* CJ236 was transformed with the plasmid pEF-MDP. From an ampicillin-resistant colony single-stranded DNA (containing uracil) was isolated using helper phage R408 (according to the standard protocol of Boehringer-Mannheim). All the mutagenic primers were designed to be anti-sense. For construction of the different mutants the following primers were used with the changes indicated in bold in parentheses:

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(H270LR) : 5'-ATGTGGTCCAGG(AC)GATCTGCCACTT-3'
(H273LR) : 5'-ACCTTCTTGATG(AC)GGTCCAGG-3'
(D272A) :  5'-ACCTTCTTGATGTG(TG)CCAGGTGATCTGCCAC-
           TTGGGACAA-3'
(D269A) :  5'-GTCCAGGTG(TG)TCTGCCACTTG
(H270Q) :  5'-CTTGATGTGGTCCAGCT(G)ATCTGCCACT-
           TGGGA-3'
(H270K) :  5'-GATGTGGTCCAG(C)T(T)ATCTGCC
           ACTT-3'
(H219K) :  5'-TGTAGGCCGAGGA(C)T(T)GCTGAAGA-
           TGAC-3'
(H219L) :  5'-GTAGGCCGAGGAG(A)GGCTGAAGAT-3'
(H219R) :  5'-GTAGGCCGAGGAG(C)GGCTGAAGAT-3'.
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Mutations were verified by DNA sequencing (at least three clones for each mutation). COS-1 cells were transfected with the mutated DNA (at least two samples for each point mutation) and enzyme activity was assayed 48 h after transfection.

2.2.6. Immunocytochemistry. COS-1 cells were plated in 24-well plates (containing coverslips) and transfected with wild type, H219K or H219R mutant cDNAs. 48 h post-transfection, cells were prepared for immunofluorescence as described previously [21]. Cells were washed gently 3 times with warm PBS (37°C) and fixed at room temperature in 4% paraformaldehyde for 20 min. Subsequently the cells were washed 3 times with Tris-buffered saline (TBS) and blocked for 30 min in TBS containing 0.2% gelatin at room temperature. The cells were incubated with affinity purified anti-dipeptidase IgG (1:500 dilution) [2]. After an overnight incubation at 4°C, cells were washed and incubated for 30 min with goat anti-rabbit IgG conjugated with fluorescein isothiocyanate (FITC) (1 : 50 dilution). After washing three times with TBS, the coverslips were mounted in vectashield (Vector Laboratories, Peterborough, UK) and viewed with a Leitz confocal microscope.

2.2.7. Protein determination. Protein concentrations were determined by the bicinchoninic acid method [22] using bovine serum albumin as standard.

3. Results and discussion

MDP is a metalloenzyme which exhibits a variety of catalytic activities, including the ability to hydrolyse dipeptides as well as the β-lactam antibiotic imipenem. In addition it is able to convert leukotriene D_4 to leukotriene E_4. We had previously isolated a cDNA clone encoding MDP from a pig kidney cortex cDNA library [6]. The predicted amino acid sequence of the protein showed no significant similarity to any other protein in the database; not even with other metallopeptidases or β-lactamases. The absence of the HEXXH motif typical of zinc peptidases has prompted us to examine whether the DHXXH motif (residues 269–273), similar to an identified zinc motif in a bacterial carboxypeptidase [14], was important for activity. Like MDP, this carboxypeptidase is able to hydrolyse peptides in which the C-terminal amino acid is a D-isomer. We have therefore initiated a programme of directed mutagenesis of histidines-270 and -273 and aspartates-269 and -272 to assess their importance for catalytic activity. The possible importance of His-219 has also been evaluated.

Fig. 1. Immunostaining of transfected COS-1 cells for membrane dipeptidase using FITC-fluorescence. Cells were prepared as described in section 2. (A) Control cells; (B) wild-type MDP expressed in COS-1 cells. In (C) and (D) the staining was performed in cells which had been transfected with either mutant H219K (C) or H219R (D). All the four micrographs represent a composite image taken at all focal planes. Bar = 10 mm.

3.1. *Expression of wild type membrane dipeptidase in COS-1 cells*

When wild-type MDP was transiently expressed in COS-1 cells using the mammalian expression vector pEF-BOS, high level expression of enzyme activity was achieved which exhibited the characteristics appropriate of a GPI-anchored protein. Expressed protein could be detected at the cell-surface immunocytochemically by using a polyclonal antibody to pig MDP [2] (Fig. 1B). Phase-separation in Triton X-114 of membrane preparations from the transfected cells showed that the protein partitioned into the detergent-rich phase, indicating amphipathic character (Table 1). After PI-PLC treatment followed by phase-separation, MDP appeared predominantly in the detergent-poor phase (Table 1) confirming cleavage of the GPI anchor by the phospholipase and release of a hydrophilic form of the enzyme. Expressed activity was fully sensitive to the MDP specific inhibitor, cilastatin, and cilastatin-sensitive activity was undetectable in cells transfected with control plasmid (Table 2).

3.2. *Mutation of the DHXXH motif*

Each histidine in the proposed motif was changed to leucine; additionally His^{270} was mutated to Gln and Lys and His^{273} to Arg. Aspartates-269 and -272 were changed to Ala. After verification by sequencing of at least two mutants for each mutation, the insert containing the open reading frame was subcloned into the wild-type vector and the mutant cDNAs were expressed in COS-1 cells as described for wild-type MDP. All the mutants express MDP activity at levels approaching that of the wild-type (Table 2). The activity was cilastatin-sensitive (Table 2) with an identical I_{50} value to the wild-type enzyme (Fig. 2). A detergent-solubilized membrane fraction revealed similar activity to intact cells (data not shown) and all the mutants were releasable in a soluble form by PI-PLC treatment (Table 1). Immunoblotting of membrane fractions from cells expressing wild-type or mutant MDP all revealed a polypeptide of M_r approx 90,000 after SDS-PAGE under non-reducing conditions (data not shown).

Table 1
Phase separation in Triton X-114 of wild-type and His^{270} mutants of membrane dipeptidase

Triton X-114 phase	pEF-MDP (−PI-PLC)	pEF-MDP (+PI-PLC)	H270R (−PI-PLC)	H270R (+PI-PLC)	H270L (−PI-PLC)	H270L (+PI-PLC)
Detergent-rich phase	92.2 ± 0.0	6.5 ± 0.5	96.2 ± 5.0	8.6 ± 0.7	86.2 ± 3.5	10.5 ± 0.1
Detergent-poor phase	7.8 ± 0.5	93.5 ± 0.0	3.8 ± 1.9	91.4 ± 3.1	13.8 ± 0.3	89.5 ± 8.2

MDP activity was assayed in the membrane fraction (12.6 μg) before and after PI-PLC treatment. In each case, the proportion of activity partitioning into the detergent-rich and detergent-poor fractions was assessed after Triton X-114 phase-separation [3]. COS-1 cells were transfected with each of the following plasmids: pEF-MDP, H270R or H270L. Membrane fractions from the cells (40 μl) were diluted 1:3.5 into 50 mM HEPES/NaOH, pH 7.5. To half of the samples was added 1 unit of PI-PLC followed by incubation for 2 h at 37°C. Pre-condensed Triton X-114 was added to a final concentration of 1%. After 10 min of incubation at 4°C followed by 10 min incubation at 30°C, the samples were centrifuged at 3000 × *g* for 3 min at 25°C. The upper (detergent-poor) phase was separated from the detergent-rich phase and both phases were brought to the same volume. Aliquots (10 μl) from each phase were taken for determination of MDP activity as described in section 2. The results are expressed as the percentage of total activity recovered and are the mean ± S.E.M. of four determinations.

Table 2
Cell-surface activity of wild-type membrane dipeptidase and of histidyl and aspartyl mutants expressed in COS-1 cells

Transfected cells	Mutated to	Specific activity (nmol D-Phe/min/mg)	Specific activity + cilastatin (nmol D-Phe/min/mg)
pEF-MDP	(wild-type)	19.9 ± 0.6	0.5
Control cells		0.5 ± 0.0	0.5
H270L	(CAC → CTC)	16.4 ± 2.6	0.5
H270Q	(CAC → CAG)	24.5 ± 0.6	0.5
H270K	(CAC → AAG)	10.0 ± 1.0	0.5
H273L	(CAC → CTC)	24.5 ± 1.5	0.5
H273R	(CAC → CGC)	23.1 ± 1.1	0.5
D269A	(GAT → GCA)	9.2 ± 1.4	0.4
D272A	(GAC → GCA)	9.2 ± 0.8	0.5
H219R	(CAC → CGC)	0.4 ± 0.0	0.4
H219K	(CAC → AAG)	0.4 ± 0.0	0.4
H219L	(CAC → CTC)	0.5 ± 0.0	0.5

Transfection and expression were performed as described in section 2. Cell-surface expression was determined on 24-well plates 48 h after transfection. The cells were washed twice with PBS and assayed by addition of 200 μl 0.1 M Tris-HCl, pH 8.0, containing Gly-D-Phe (3 mM) as substrate. After 2.5 h at 37°C the buffer was collected and the product (D-Phe) quantified by HPLC as described in section 2. Results are the mean ± S.E.M. of triplicate determinations where indicated.

3.3. Mutation of His^{219}

His^{219} was mutated to Leu, Lys or Arg. In all cases there was complete abolition of cilastatin-sensitive enzyme activity. His^{219} may therefore be a critical residue involved in zinc-binding. As can be seen in Fig. 1 for the H219K and H219R mutants, the mutated protein is expressed on the cell-surface (Fig. 1C, D) with the same intensity as the wild-type (Fig. 1B). The staining had a punctate appearance and covered the entire surface of the membrane.

3.4. General conclusions

There are seven histidines and nine glutamates conserved in MDP between all species whose cDNA derived amino acid sequences have so far been isolated. We have ruled out the histidines and aspartates in the DHLDH motif, which is conserved in the human, pig, rat and mouse enzymes, as being critical to enzyme activity but the conserved His^{219} is essential for functional protein. A rabbit kidney 43 kDa protein which is 81% homologous with human MDP has been suggested as the rabbit homologue of MDP [23], although the cDNA clone has not been expressed so its functional activity is unknown. This protein has a tyrosine residue in place of the second histidine (His^{273}) of the motif. Our data would suggest that this change should not affect activity and would be consistent with the rabbit 43 kDa protein representing MDP, particularly since His^{219} is conserved. The only other residue in MDP reported to date whose mutation abolishes activity is Glu^{125} [24]. It is clear from the present data that the zinc-binding ligands in MDP must represent a novel motif for a metallopeptidase.

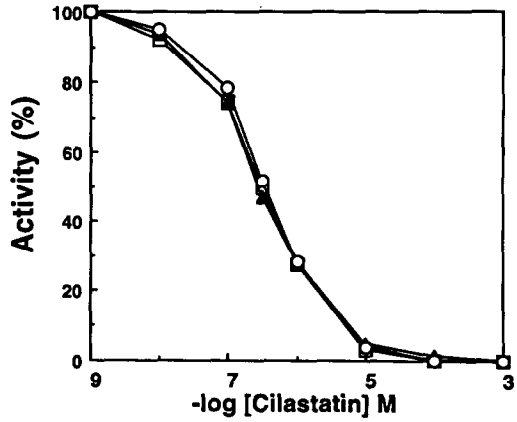

Fig. 2. Cilastatin inhibition of expressed membrane dipeptidase activity. Solubilized membranes (10 μl; 1.6 μg) were incubated in a total volume of 100 μl in 0.1 M Tris-HCl, pH 8.0, containing 3 mM Gly-D-Phe. Cilastatin was present at the final concentrations indicated on the abscissa. The samples were incubated for 2 h at 37°C and D-Phe product was then determined by HPLC as described in section 2. The forms of MDP expressed were (□), pEF-MDP; (▲), H270Q and (○), H273R. Activity of 100% represents 56.6 nmol D-Phe/min/mg protein.

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