Site-Directed Mutagenesis of Conserved Cysteine Residues in Porcine Membrane Dipeptidase. Cys 361 Alone Is Involved in Disulfide-Linked Dimerization[†]

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ABSTRACT: Membrane dipeptidase (EC 3.4.13.19) is a glycosylphosphatidylinositol-anchored glycoprotein of the renal brush border which exists as a disulfide-linked homodimer. Porcine membrane dipeptidase has a subunit M_r of 47 kDa, and the mature protein contains seven cysteine residues per subunit, six of which are conserved in the human enzyme. Chemical modification established that cysteine residues are not involved in enzyme activity. In order to determine which of the cysteine residues are involved in the interchain disulfide bond, we have used a site-directed mutagenesis approach. Each of the conserved cysteine residues was replaced by glycine or alanine. The single mutants (C71G, C93A, C154G, C226A, C258G, and C361G) were expressed in COS-1 cells and their enzymatic activity and oligomeric structure determined. Only the C361G mutant migrated as a polypeptide of 47 kDa when subjected to denaturing polyacrylamide gel electrophoresis under nonreducing conditions. Thus, cysteine 361 is the only residue involved in disulfide linkage between the subunits. This places the disulfide bond close to the site of GPI anchor addition (Ser 368 in the porcine enzyme) and to the membrane surface. Titration of the human and porcine proteins with 2-nitro-5-thiosulfabenzoate indicates that membrane dipeptidase additionally possesses two intrachain disulfide bonds. On native polyacrylamide gel electrophoresis, the C361G mutant migrates in a manner identical to that of the wild type, indicating that the protein remains associated as a noncovalent homodimer. The expressed C361G mutant, unlike the wild type, is released from COS-1 cell membranes by trypsin and by an endogenous serine protease.

Membrane dipeptidase (MDP,[1] microsomal dipeptidase, renal dipeptidase, dehydropeptidase-I, EC 3.4.13.19) is a mammalian cell-surface zinc metallopeptidase found predominantly in the lung and in the brush border membrane of the kidney. The enzyme displays a versatile substrate specificity, being able to hydrolyze dipeptides, including unsaturated dipeptides (dehydropeptides), and those with either an L- or a D-amino acid in the P_1' position [see Keynan *et al*. (1996) for review]. MDP is involved *in vivo* in the renal metabolism of glutathione (Kozak & Tate, 1982), and in the lungs, the enzyme appears to play a role in the conversion of leukotriene D_4 to leukotriene E_4 (Huber & Keppler, 1987; Campbell *et al*., 1990). MDP is also the only known example of a mammalian β-lactamase, hydrolyzing the carbapenem class of antibiotics (Kropp *et al*., 1982; Campbell *et al*., 1984). This activity has led to the development of specific inhibitors of the enzyme, notably cilastatin (Kahan *et al*., 1983). Some of the bacterial metallo-β-lactamases, e.g. the *cphA* gene product of *Aeromonas hydrophila*, are also inhibited by cilastatin, although less potently than is MDP (Keynan *et al*., 1995).

MDP is a glycosylphosphatidylinositol (GPI)-anchored cell-surface enzyme and was the first peptidase to be so recognized (Hooper *et al*., 1987). The enzyme is released efficiently from renal brush border membranes by bacterial phosphatidylinositol-specific phospholipase C (PI-PLC), and the solubilized enzyme can be purified in a single step by affinity chromatography on cilastatin−Sepharose (Littlewood *et al*., 1989). The GPI anchor on MDP has been extensively characterized in terms of its recognition by specific antibodies and its hydrolysis by phospholipases (Littlewood *et al*., 1989; Hooper & Turner, 1989; Hooper *et al*., 1990, 1991; Brewis *et al*., 1994). Recently, the complete structure of the GPI anchor on porcine MDP and the glycan core structure of the GPI anchor on human MDP have been determined, providing the first transspecies comparison of the GPI anchor structures of the same protein expressed in the same tissue (Brewis *et al*., 1995). Porcine MDP exists as a disulfide-linked homodimeric glycoprotein with a subunit M_r of 47 kDa. Assembly as a covalent oligomer is rare among the membrane peptidases, the only other known examples being meprin (Marchand *et al*., 1994), endothelin-converting enzyme (Takahashi *et al*., 1995), and aminopeptidase A (Wu *et al*., 1991).

MDP has now been cloned from a number of species (human, porcine, rabbit, rat, mouse, and sheep) (Adachi *et al*., 1990, 1992; Rached *et al*., 1990; Igarashi & Karniski, 1991; Satoh *et al*., 1993; Schmidt & Campbell, 1994). It shows no sequence homology with any other mammalian zinc peptidases but exhibits some similarity with a recently cloned bacterial peptidase (Adachi &Tsujimoto, 1995). Site-directed mutagenesis has shown His 219 (Keynan *et al*., 1994) and Glu 125 (Adachi *et al*., 1993) to be critical residues in MDP essential for functional protein, but the role of

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[1] Abbreviations: DipF, diisopropylfluorophosphate; DMEM, Dulbecco's Modified Eagles Medium; ECL, enhanced chemiluminescence; GPI, glycosylphosphatidylinositol; HPLC, high-performance liquid chromatography; MDP, membrane dipeptidase; NTB, nitrothiobenzoate; NTSB, 2-nitro-5-thiosulfabenzoate; PAGE, polyacrylamide gel electrophoresis; PI-PLC, phosphatidylinositol-specific phospholipase C; SDS, sodium dodecyl sulfate.

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cysteine residues has not been assessed. Comparison between all the cloned MDP sequences to date reveals six conserved cysteine residues between species [see Keynan *et al*. (1996)]. A seventh cysteine residue is also conserved in all species except the human. In the present study, we have used site-directed mutagenesis and chemical modification to explore the potential roles of the six fully conserved cysteine residues in porcine MDP. Each of the cysteine residues has been replaced by glycine or alanine, and the mutant cDNAs have been expressed in COS-1 cells and their enzymatic activity and oligomeric structure determined. We have identified a single cysteine residue (C361) responsible for the formation of the interchain disulfide bond and demonstrate that the expressed C361G mutant is fully functionally active.

EXPERIMENTAL PROCEDURES

Materials

T4 polynucleotide kinase and restriction enzymes were obtained from New England Biolabs (Hitchin, U.K.). Sequenase kit (version 2.0) and the enhanced chemiluminescence (ECL) kit were from Amersham International (Buckinghamshire, U.K.). [^{35}S]dαATP (1000 Ci/mmol) was from Du Pont (Stevenage, U.K.). Tissue culture media, serum, trypsin−EDTA, penicillin/streptomycin, L-glutamine, transfection reagent (lipofectAmine), and T4 DNA ligase were purchased from Gibco-BRL (Paisley, U.K.). Soybean trypsin inhibitor, Gly-D-Phe, bicinchoninic acid, and high-molecular mass protein standards were obtained from Sigma-Aldrich, Poole, (Dorset, U.K.). The mammalian expression vector pEF-BOS was a generous gift from Dr. Shigekazu Nagata (Osaka, Japan) (Mizushima & Nagata,1990). Cilastatin was a gift from Merck, Sharp & Dohme (Rahway, NJ). *Bacillus thuringiensis* phosphatidylinositol-specific phospholipase C was a gift from Dr. M. G. Low (New York). All reagents were of analytical grade.

Methods

Cell Growth and Expression. COS-1 cells were cultured in DMEM supplemented with 10% fetal calf serum (heat-inactivated), 100 units/mL penicillin, 100 μg/mL streptomycin, and 2 mM L-glutamine at 37 °C in 5% CO_2. The expression vector pEF-MDP (Keynan *et al*., 1994) was used to transfect COS-1 cells. For transient expression, COS-1 cells were plated in 24-well plates at 33% confluency or in 150 cm^2 flasks at approximately 2×10^6 cells. After 24 h of growth, the cells were washed twice with OptIMem and transfected (0.2 μg of DNA/well or 5 μg of DNA/flask) by using lipofectAmine as cationic lipid (1:10 DNA:lipid). The cells were incubated for 2 h, and DMEM containing 10% fetal calf serum was added. After 24 h, the medium was replaced with fresh and the cells were incubated for another 24 h. Transient expression was assayed in solubilized membrane preparations. Membranes were isolated and solubilized with 60 mM *n*-octyl β-D-glucopyranoside as described previously (Keynan *et al*., 1994).

Site-Directed Mutagenesis. Site-directed mutagenesis was performed according to the method of Kunkel *et al.* (1987). *Escherichia coli* CJ236 was transformed with the plasmid pEF-MDP. From an ampicillin resistant colony, single-strand 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 antisense. For construction of the different mutants, the following primers were used: C71G (TGC/GGC), 5′-TGT TCT GGG TGT CG**C** **C**GG GCA CGT ACG CG-3′; C93A (TGC/GCC), 5′-TCG GGA TAC GCC TGG **GC**C ATG CGC TGG ATG-3′; C154G (TGC/GGC), 5′-CCA GGG CGT GTT G**CC** GCT GTG GGT GA-3′; C226A (TGC/GCC), 5′-TTG CGC CGG TGC GGG **GC**C AGA CTG TAG GCC G-3′; C258G (TGC/GGC), 5′-TTG GCC TTG GCC GAG **CC**G GAA ACG TAG TCG TT-3′; and C361G (TGC/GGC), 5′-GTA ATT CGT CCG G**CC** GGA AGC CTC CAG-3′.

Mutations were verified by DNA sequencing. Each mutant was then subcloned back to the wild type plasmid pEF-MDP, and the region around the mutation was sequenced. Mutants C71G and C93A were restricted by *Sun*I-*Kpn*I and *Sph*I. C154G mutant cDNA was further restricted by *Eag*I, and the resultant fragments were subcloned back to the wild type cDNA. For subcloning the C361G mutant, *Eag*I and *Kpn*I were used. C226A and C258G were confirmed in four different mutants and retransformed, and the region around the mutation was sequenced again. The double mutant (C226A/C258G) was made in a single mutagenesis experiment, annealing the single-strand DNA in the presence of the two primers together. Cells were transfected with the mutated DNA, and enzyme activity was assayed 48 h after transfection.

Assay of Membrane Dipeptidase Activity. Solubilized membranes (10 μL; 3 μg of protein) were incubated in 0.1 M Tris/HCl buffer (pH 8.0) containing 3 mM Gly-D-Phe with or without the inhibitor cilastatin (0.25 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 12000*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 to 30% acetonitrile in 0.08% H_3PO_4 (Hooper *et al*., 1987).

SDS−PAGE and Immunoblotting. Polyacrylamide gel electrophoresis was performed as described by Laemmli (1970) using a 5% stacking and a 10% separating gel. Native gel electrophoresis in the absence of SDS was performed on a 7 to 17% gradient gel. The gels were blotted (Towbin *et al*., 1979) using a semidry blot and the detection of protein was performed by using chemical luminescence (ECL kit, Amersham). Molecular mass standards were run in parallel and visualized by Coomassie Blue staining. Levels of MDP protein expression were estimated by immunoblot analysis using purified pig kidney MDP as a standard. The linearity of exposure with protein concentration was routinely checked.

Immunocytochemistry. COS-1 cells were plated on six-well plates (containing cover slips) and transfected with wild type or C361G mutant cDNA. Cells were prepared for immunofluorescence 48 h post-transfection as described previously (Keynan *et al*., 1994). Affinity-purified anti-porcine membrane dipeptidase IgG (1:500 dilution) was used as the primary antibody (Littlewood *et al.*, 1989). The cover slips were mounted on microscope slides in Vectashield (Vector Laboratories Inc., Peterborough, U.K.) and were viewed by a Leitz confocal microscope.

Purification of Membrane Dipeptidase. MDP was purified to apparent homogeneity from porcine or human kidney cortex by solubilization with PI-PLC, followed by affinity

chromatography on cilastatin−Sepharose (Littlewood *et al*., 1989; Hooper *et al.*, 1990).

Triton X-114 Phase Separation. Membranes prepared from COS-1 cells that were transfected with wild type or C361G cDNA were subjected to phase separation in Triton X-114 essentially as described by Hooper and Turner (1988), either untreated or after pretreatment with PI-PLC or trypsin. Precondensed Triton X-114 was added to membranes at a 1% final concentration, and after 5 min of incubation at 4 °C, the mixture was layered on top of 0.3 mL of a 6% (w/v) sucrose cushion [in 10 mM Tris/HCl (pH 7.5) and 150 mM NaCl containing 0.06% Triton X-114]. After 3 min of 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 lower detergent-rich phase, and both phases were brought to the same volume. Aliquots (10 μl) from each phase were taken for the determination of MDP activity.

Determination of the Cystine Content of Membrane Dipeptidase. NTSB was prepared and assayed according to the procedure of Thannhauser *et al.* (1987). The NTSB assay solution containing 2 M guanidine thiocyanate, 50 mM glycine, 100 mM sodium sulfite, and 3 mM EDTA (pH 9.5) was mixed with 1 nmol of purified MDP (considering a dimer as 1 mol of enzyme) in a 1 cm path length glass cuvette. The reaction was started by adding 10 μL of NTSB (25 mM), and the change in the absorbance at 412 nm was recorded. An extinction coefficient of 13 600 M^{-1} cm^{-1} at 412 nm for NTB was used for quantitating the reaction.

Protein Determination. Protein concentrations were determined using the bicinchoninic acid method of Smith *et al*. (1985) modified for use in 96-well microtiter plates (Hooper, 1993) with bovine serum albumin as a standard.

RESULTS

Characterization of Cysteine Mutants of Membrane Dipeptidase Expressed in COS-1 Cells. Each of the six cysteine residues in MDP that are conserved between species was mutated to either alanine or glycine. After the mutations were verified by sequencing, the mutant cDNAs were expressed in COS-1 cells. The oligomeric structure of the various mutants was assessed by SDS−PAGE under nonreducing conditions followed by immunoblotting with a polyclonal antibody raised against porcine MDP (Figure 1). The wild type MDP was expressed in COS-1 cells and migrated as a single polypeptide of approximately 94 kDa (Figure 1, lane 9) which is not observed in nontransfected cells (Figure 1, lane 5). When cysteine 361 is replaced by glycine, the only polypeptide that is recognized under nonreducing conditions by the anti-MDP antibody is a band that runs at approximately 47 kDa which corresponds to the monomeric size of porcine membrane dipeptidase (Figure 1, lane 4). On nondenaturing PAGE, however, the C361G mutant migrates identically with the expressed wild type enzyme, consistent with the existence of the C361G mutant as a noncovalent homodimer in solution (Figure 2).

Mutants C226A and C258G are produced at levels comparable to that of the recombinant wild type enzyme, as is the double mutant C226A/C258G (Figure 1), and all show a major band at an M_r of 94 kDa. However, species with a higher M_r are also detected by immunoblot analysis of the C226A mutant (Figure 1, lane 7), presumably due to incomplete processing of hyperglycosylated intermediates.

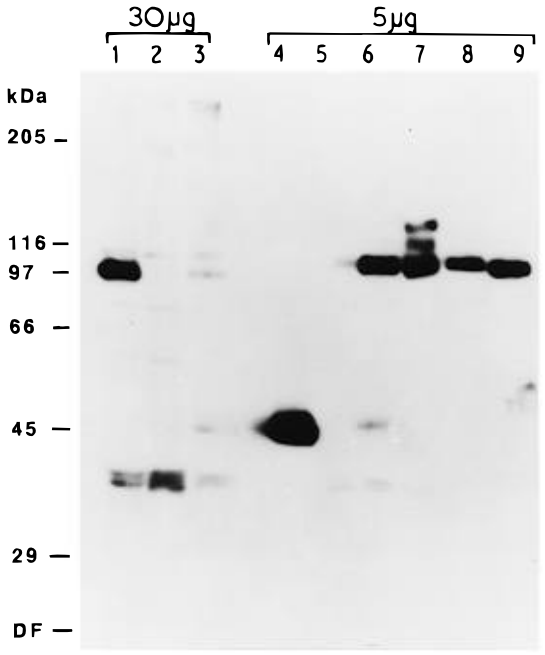

FIGURE 1: SDS−PAGE and immunoblotting of wild type and cysteine mutants of membrane dipeptidase expressed in COS-1 cells. COS-1 cells were transfected with the wild type or with the indicated mutants. Membranes were prepared from cells, and after solubilization with 60 mM β-octyl glucoside, 30 μg (lanes 1−3) or 5 μg (lanes 4−9) of protein was loaded onto the gel. For immunoblotting, the primary antibody, recognizing porcine MDP, was used at a 1:1000 dilution at 4 °C overnight. The lanes represent (1) C93A, (2) C154G, (3) C71G, (4) C361G, (5) nontransfected control cells, (6) C226A/C258G;, (7) C258G, (8) C226A, and (9) wild type. DF is the dye front.

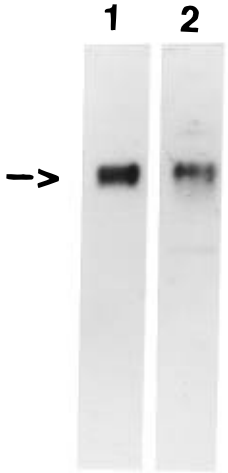

FIGURE 2: Native PAGE and immunoblotting of wild type and the C361G mutant of membrane dipeptidase expressed in COS-1 cells. Membrane fractions (5 μg of protein) were prepared from COS-1 cells transfected with either the wild type or the C361G mutant as described in Experimental Procedures and were then incubated with 5 units of PI-PLC for 1 h at 37 °C in a total volume of 100 μL. After centrifugation at 100000*g* for 1 h, the supernatant fractions (40 μL) were analyzed on a 7 to 17% polyacrylamide gradient gel followed by immunoblotting with anti-porcine MDP (1:500): lane 1, wild type; and lane 2, the C361G mutant. The arrow indicates the position of MDP (94 kDa).

Mutants C93A and C71G were detectable at M_r = 94 kDa on the immunoblot only upon loading the gel with 30 μg rather than 5 μg of protein. Under similar conditions, the C154G mutant shows no detectable band at 94 kDa. However, two immunoreactive polypeptides with substantially lower M_r values were observed, suggesting partial

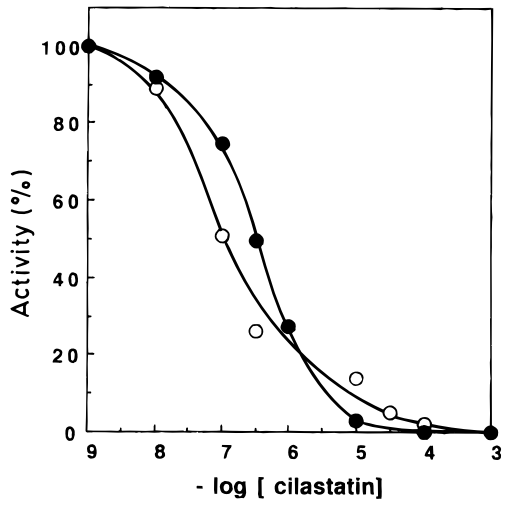

FIGURE 3: Cilastatin inhibition of expressed wild type and the C361G mutant of membrane dipeptidase. The wild type (●) and C361G mutant (○) of MDP were expressed in COS-1 cells, and their sensitivity to inhibition by cilastatin was examined. In both cases, solubilized membranes were incubated in a total 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 concentration indicated on the abscissa. The samples were incubated for 90 min at 37 °C, and the D-Phe product was determined by HPLC as described in Experimental Procedures.

degradation of the protein to smaller species that retain the relevant epitope recognized by the antibody (Figure 1, lane 2). Overexposure of the x-ray film does reveal a minor band at 94 kDa for the C154G mutant but no detectable band in the region of 47 kDa (data not shown).

The specific activity of each mutant was determined in solubilized membrane preparations and expressed relative to the amount of MDP protein quantified by immunoblot analysis (densitometric scanning of bands, Scanmaster 3, Howtek, Hudson, NH) (Figure 1). With the exception of the C154G mutant for which negligible protein and activity could be detected, all other mutants retained substantial enzymatic activity. In a representative experiment, 100% activity of the wild type corresponds to 35.1 ± 2.5 nmol min^{-1} (mg of protein)$^{-1}$. The other mutants reveal specific activities relative to the wild type of the following: C226A and C361G, 100%; C258G, 44%; C226A/C258G, 44%; C93A, 48%; and C71G, 60%. Transfection was carried out three times, and similar results were obtained in all cases.

Characterization of the C361G Mutant Expressed in COS-1 Cells. Some properties of the C361G mutant protein were examined in more detail in solubilized membrane preparations. The expressed protein appears to be fully catalytically active, and its sensitivity to inhibition by cilastatin is comparable to that of the wild type (Figure 3). The K_m of the C361G mutant for Gly-D-Phe was 9.1 ± 3.9 mM (n = 3) compared with 6.9 ± 2.3 mM (n = 3) for the wild type. The expressed protein could be detected immunocytochemically at the cell surface (Figure 4). As is evident from Figure 4a,b, C361G is expressed at the cell surface with the same fluorescent intensity as the wild type (Figure 4d). The staining had a punctate appearance and covered the entire surface of the cell. Since MDP is a GPI-anchored protein which can be released by PI-PLC, the sensitivity of the expressed wild type and Cys 361 mutant to release by PI-PLC was compared (Table 1). The appearance of hydrophilic forms of the enzyme was detected by phase separation in Triton X-114. Incubation of both the wild type and C361G mutant protein at 4 °C in the absence of PI-PLC prior to phase separation confirmed that the proteins were substantially amphipathic in character. After treatment with PI-PLC for 4 h at 37 °C, a marked shift occurred, with both enzymes now being distributed predominantly (>78%) into the detergent-depleted phase, indicating loss of the GPI anchor (Table 1). A distinct difference, however, was seen in the behavior of the wild type and C361G mutant proteins to phase separation when they were incubated at 37 °C in the absence of PI-PLC. The wild type enzyme was still predominantly (94%) amphipathic in character, but a substantial proportion (35%) of the C361G mutant had shifted into the detergent-depleted phase (Table 1). This suggested that the C361G mutant was significantly more susceptible to endogenous hydrolases (proteinases or phospholipases) present in the COS-1 cell membrane preparation which were able to generate a hydrophilic form of the protein. Consistent with this hypothesis was the marked susceptibility of the C361G protein to release from the membranes by mild trypsin treatment, whereas the wild type protein was essentially resistant to proteolysis (Table 2), and the observation that DipF could completely inhibit the endogenous release of the mutant protein from the membrane (Table 2).

In order to estimate the size of the truncated proteins released by trypsin and by the DipF sensitive protease in COS-1 cells, SDS−PAGE and immunoblot analysis were used to compare the different forms of the C361G mutant (Figure 5). The released form was separated from the membrane (amphipathic) form by centrifugation at 100000*g* for 1 h. The membrane form of MDP (Figure 5, lane 1) migrates slightly faster than the PI-PLC-cleaved form (Figure 5, lane 2) as observed previously (Littlewood *et al.*, 1989). The size of the C361G mutant released by trypsin or by the endogenous membrane enzyme is smaller than the PI-PLC-released form by approximately 5 kDa. Some further degradation of MDP protein occurs with trypsin producing two additional bands at around 35 kDa which are not seen in the case of the endogenous release.

Titration of Purified Porcine and Human Kidney Membrane Dipeptidase with NTSB and Other Thiol Reagents. To quantify the total disulfide bridge content of MDP, we have compared the reactivity of NTSB with purified porcine MDP or human MDP (Figure 6). One mole of NTB is released for each disulfide bond in the protein and for each free thiol (due to the ability of NTSB to react also with free thiols) (Thannhauser *et al.*, 1987). After completion of the reaction, a maximum of 8.5 mol of NTB was released per mole of purified porcine MDP (dimer) and 6.9 mol of NTB per mole of purified human MDP (dimer). The experiments with purified porcine MDP were carried out six times using several different MDP preparations with essentially the same results. The susceptibility of MDP to the action of other thiol reagents was also examined. Treatment of the protein for 1 h at 25 °C before the addition of substrate with DTNB (0.5 mM), 4,4-dithiopyridine (1 mM), and iodoacetic acid (5 mM) resulted in no loss in enzyme activity. However, dithiothreitol (10 mM) caused a loss of >95% in enzyme activity in accord with previous results (Hooper *et al.*, 1990).

DISCUSSION

MDP is a mammalian GPI-anchored protein that appears to be functionally active as a disulfide-linked dimer. Dis-

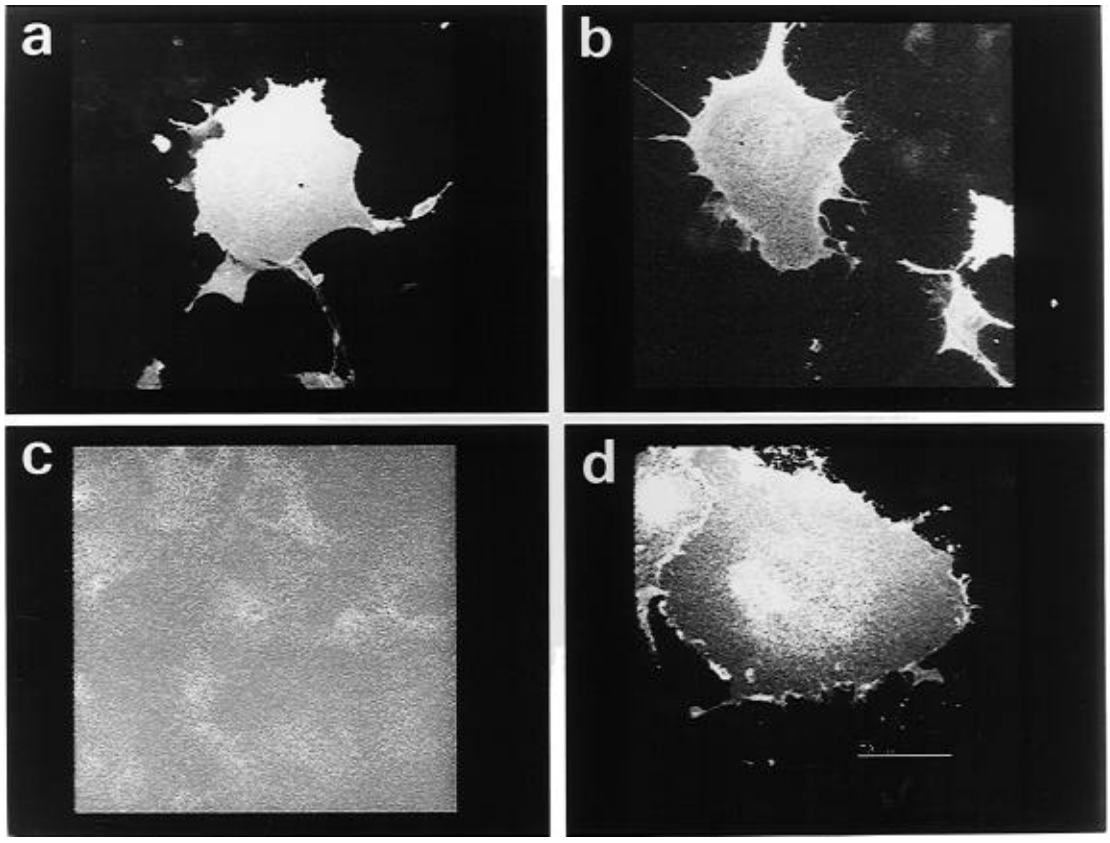

FIGURE 4: Immunostaining of transfected COS-1 cells for the wild type and C361G mutant of membrane dipeptidase using FITC fluorescence. Cells were prepared as described in Experimental Procedures. In panels a and b, the staining was performed in cells which were transfected with the mutant cDNA, C361G: (c) nontransfected control cells and (d) wild type transfected cells. Micrographs a, b, and d represent a composite image taken at several focal planes. Micrograph c was taken at a central plane. Bar = 50 μm.

Table 1: Phospholipase C Solubilization and Triton X-114 Phase Separation of the Wild Type and C361G Mutant of Membrane Dipeptidase[a]

	wild type[b]		wild type + PI-PLC	C361G		C361G + PI-PLC
Triton X-114 phase	4 °C	37 °C	37 °C	4 °C	37 °C	37 °C
detergent-rich phase	94.5	94.0	22.0	88.1	64.3	21.8
detergent-poor phase	5.5	6.0	78.0	11.9	35.7	78.2

[a] COS-1 cells were transfected with either of the following plasmids: pEF-MDP or pEF-C361G. Membrane fractions were prepared as described in Experimental Procedures. Membranes (50 μL) were diluted 1:2 in 50 mM Hepes buffer (pH 7.5) and were incubated for 4 h at 4 °C without PI-PLC or at 37 °C either with or without PI-PLC. The proportion of MDP present in a hydrophilic form was assessed by phase separation in Triton X-114. Aliquots (10 μL) from each phase were taken for determination of MDP activity. [b] The results are expressed as the percentage of the total activity recovered (detergent phase + aqueous phase) and are the mean of triplicate measurements, the SD being less than ±2% in all cases.

ulfide bonds are relatively common among the extracytoplasmic domains of cell-surface proteins, for example in dimeric growth factors and in the insulin receptor, where oligomerization appears to be a requirement for cell signalling to occur. Covalent association of subunits in the family of brush border peptidases is, however, a relatively rare event, MDP, meprin, aminopeptidase A, and endothelin-converting enzymes being the only established examples (Hooper & Turner, 1989; Marchand *et al*., 1994; Wu *et al.*, 1991; Takahashi *et al*., 1995). The number and location of the interchain disulfide bonds have only been reported to date for endothelin-converting enzyme (Shimada *et al.*, 1996). Some GPI-anchored enzymes are also disulfide-linked oligomers, for example, 5′-nucleotidase and mammalian erythrocyte acetylcholinesterase (Zimmermann, 1992; Inestrosa *et al*., 1987).

In the present study, we have used a site-directed mutagenesis approach to assign the cysteine(s) involved in dimer formation. Only mutagenesis of Cys 361 produced a monomeric form of MDP on SDS−PAGE under nonreducing conditions, indicating the involvement of a single disulfide bridge in dimerization. The C361G mutant was fully active and located at the cell surface and retained many of the characteristics of the wild type enzyme, especially K_m for substrate and sensitivity to inhibition by cilastatin. However, on native (nondenaturing) PAGE, the C361G mutant migrated identically with the wild type protein, indicating that the subunits are still able to associate noncovalently as a homodimer, and this may be a factor in allowing its efficient targeting to the plasma membrane. The majority of brush border hydrolases exist as noncovalently associated homodimers (Kenny & Maroux, 1982), and their dimeric assembly has been investigated by Danielsen (1994), who concluded that monomeric plasma membrane proteins show poor "transport competence".

Like the wild type, the C361G mutant is also GPI-anchored and released as a hydrophilic form by treatment with PI-PLC. Since the GPI anchor of MDP is attached to Ser 368 (Brewis *et al.*, 1995), the cysteine involved in disulfide bridge formation is close to the C-terminus of the mature protein and to the surface of the plasma membrane. We were able to show that this mutant is readily solubilized by mild trypsin treatment, whereas the wild type is resistant. It is noteworthy that the C361G mutant was also substantially more suscep-

Table 2: Endogenous and Trypsin Solubilization of the C361G Mutant Expressed in COS-1 Cells[a]

	C361G[b]		C361G +1 mM DipF	C361G + trypsin	wild type + trypsin
Triton X-114 phase	4 °C	37 °C	37 °C	37 °C	37 °C
detergent-rich phase	89.2 ± 11.7	70.9 ± 4.6	91.9 ± 20.5	31.8 ± 4.3	92.5 ± 1.4
detergent-poor phase	10.8 ± 3.0	29.1 ± 3.3	8.1 ± 4.7	68.2 ± 6.4	7.5 ± 0.2

[a] COS-1 cells were transfected with either of the following plasmids: pEF-MDP or pEF-C361G. Membrane fractions were prepared 48 h after transfection. One aliquot of membranes (175 μL; 10 μg of protein) was incubated with 1 mM DipF for 1 h at 4 °C to inhibit endogenous serine proteinases, followed by a further incubation for 1 h at 37 °C to assess susceptibility to solubilization. Aliquots of membranes were incubated either with trypsin (1:10 trypsin:protein) for 1 h at 37 °C or without trypsin for 1 h at 37 or 4 °C. Trypsin inhibitor was then added to the tubes containing trypsin and the hydrophilic form of MDP was separated by phase separation in Triton X-114. [b] The results are expressed as the percentage of the total activity (detergent phase + aqueous phase) recovered and are the mean ±SD of three determinations.

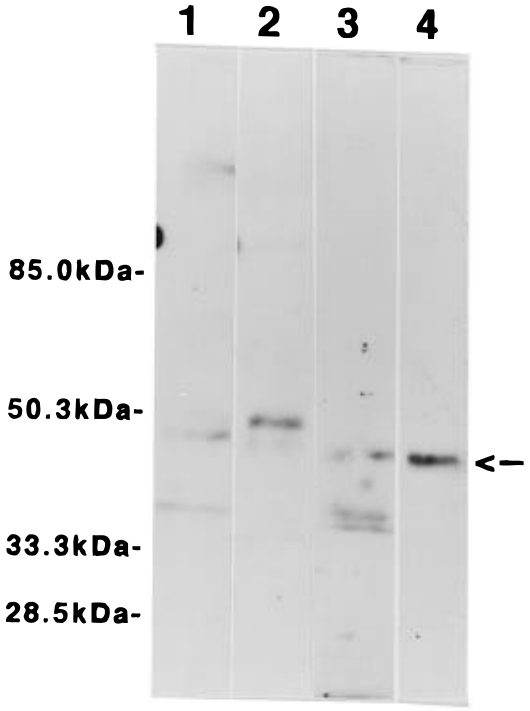

FIGURE 5: Immunoblot of the membrane form and the PI-PLC-solubilized and protease-solubilized forms of the C361G mutant of MDP. COS-1 cells were transfected with the C361G cDNA. Membrane fractions were prepared as in Experimental Procedures. The membranes were incubated for 1 h at 37 °C alone, or with either PI-PLC or trypsin. The hydrophilic forms of MDP generated in these procedures were obtained by centrifugation at 100000*g* for 1 h. Aliquots of the amphipathic (membrane) form and each of the solubilized forms were analyzed on 10% SDS−PAGE after immunoblotting with the anti-porcine MDP antibody (1:1000): lane 1, amphipathic membrane form solubilized by octyl glucoside; lane 2, hydrophilic form generated by PI-PLC treatment; lane 3, hydrophilic form generated by mild trypsin treatment; and lane 4, endogenously solubilized, hydrophilic form generated by incubation of membranes at 37 °C in the absence of PI-PLC or trypsin. The arrow indicates the position of the proteolytically solubilized form of the C361G mutant.

tible to hydrolysis by endogenous hydrolases present in the COS-1 cell membrane preparation. Furthermore, this endogenous release is blocked by DipF, implicating a membrane-associated serine protease in the event. Precise determination of the site of peptide bond hydrolysis is not possible using such electrophoretic approaches. Nevertheless, estimates of the difference in size of the PI-PLC-cleaved wild type and the trypsin-cleaved form of the C361G mutant, after allowing for the size of the sugar core, suggest that truncation by trypsin occurs some 30−50 amino acid residues from the mature C-terminus which would implicate one of several arginyl residues in the 314−332 region of the protein. The endogenous cleavage of the C361G mutant produces a

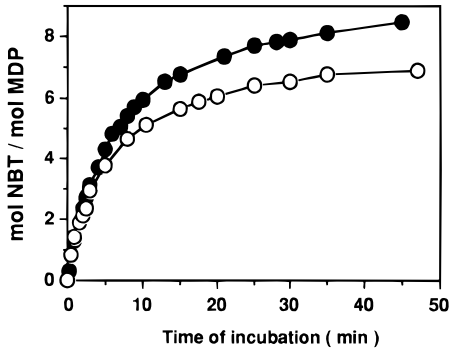

FIGURE 6: Spectrophotometric determination of the cystine content of porcine and human membrane dipeptidase. The cysteine content of purified porcine and human MDP was determined by reaction of each protein with NTSB as described in Experimental Procedures. The NTB released was quantified by measurement of A_{412}. The symbols represent (●) porcine MDP (90 μg) and (○) human MDP (118 μg).

similar size fragment as does trypsin cleavage, suggesting hydrolysis occurs in the same region of the protein. These data would suggest that the disulfide-linked dimerization seen in the wild type MDP confers resistance to proteolytic release from the membrane, presumably due to the steric hindrance introduced by the disulfide bridge preventing access of trypsin or related proteases to the critical residue(s). A number of cell-surface proteins are susceptible to proteolytic release from the plasma membrane by "secretases", including angiotensin-converting enzyme (Oppong & Hooper, 1993) and the amyloid precursor protein (Evin *et al.*, 1994). In several cases, these secretases have been characterized as serine proteases (Pandiella *et al.*, 1992; Bazil & Strominger, 1994), although one or more metalloproteases appear to predominate in the shedding of cell-surface proteins (Arribas *et al.*, 1996). Much interest has focused recently on membrane protein secretases as novel therapeutic targets. The serine protease(s) identified here might serve as one of the membrane protein secretases, and the expressed C361G mutant of MDP could provide a simple and convenient assay system for characterizing this activity.

Titration of porcine and human MDP with NTSB indicated the likely presence of two intrachain disulfide bonds, in addition to the interchain bridge formed by Cys 361. This would leave one free thiol in the human enzyme and two per subunit in the porcine enzyme. However, a range of thiol reagents (DTNB, dithiopyridine, and iodoacetamide) failed to affect MDP activity, suggesting that a free thiol is not essential for enzyme activity. Only dithiothreitol treatment resulted in inactivation of the enzyme, presumably as a result of reduction of disulfide bonds critical to the maintenance of structure. These chemical modification data are consistent with the observation that the cysteine mutants

exhibited a specific activity of the same order as the wild type except for the C154 mutant which failed to express MDP protein at any significant level.

The key finding of the present work is the identification of the single cysteine involved in the dimerization of MDP. In addition, expression of the C361G mutant in COS-1 cells has revealed the presence of an endogenous serine protease that can act as a membrane protein secretase. The C361G mutant of MDP should be a valuable tool for characterizing further the novel membrane protein secretase present in these cell membranes.

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