Regulated Expression, Processing, and Secretion of Dog Mast Cell Dipeptidyl Peptidase I*

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Paul J. Wolters, Wilfred W. Raymond, John L. Blount, and George H. Caughey‡
From the Department of Medicine and the Cardiovascular Research Institute, University of California, San Francisco, California 94143-0911

Dipeptidyl peptidase I (DPPI) is a cysteine protease found predominantly in myelomonocytic cells, cytotoxic T-cells, and mast cells. Recent studies identify an intracellular role for mast cell-DPPI (MC-DPPI) by activating prochymase and protryptase to their mature forms. To better define MC-DPPI and to explore the possibility of extracellular roles, we purified MC-DPPI from mastocytoma cells. We found the dog C2 mastocytoma cell line to be the richest source yet described for DPPI, purifying up to 200 μg of enzyme per g of cells. Dog MC-DPPI has an M₉ of ~175,000 and consists of four subunits, each composed of a propeptide, light chain, and heavy chain. The heavy chain is N-glycosylated and is heterogeneous processed to three different forms. NH₂-terminal sequences of the heavy chain and propeptide are identical to those predicted from a cDNA clone we sequenced from a mastocytoma cDNA library. The dog cDNA-derived sequence is 86% identical to that of human DPPI. Dog mastocytoma cells incubated with 12-O-tetradecanoylphorbol-13-acetate increase expression of MC-DPPI mRNA. MC-DPPI maintains its activity for tetradecanoylphorbol-13-acetate increase expression of MC-DPPI in parallel with the granule-associated mediators tryptase and histamine. Thus, dog mastocytoma cells secrete DPPI that is active at the pH of extracellular fluids, suggesting that MC-DPPI may act outside the cell.

The C1 family of cysteine proteases includes the lysosomal proteases cathepsins B, C, H, S, and L. Initially, it was believed that these proteases function primarily or exclusively in intracellular protein degradation and turnover (1). This perspective has expanded as members of this group were found to play a role in antigen presentation (2, 3) and protease activation (4–8). In addition, cathepsins B, L, and S can be secreted into the extracellular compartment and may play a part in extracellular matrix degradation and tumor metastasis (9–11). These observations suggest more diverse roles for this group of proteases.

Dipeptidyl peptidase I (DPPI), also known as cathepsin C, is a unique member of the lysosomal group of cysteine proteases. DPPI differs from the other members in structure and substrate preferences. By unknown mechanisms, human DPPI is processed into a mature, proteolytically active enzyme consisting of the M, 23,000 heavy chain (amino acids 231–394), the M, 7000 light chain (amino acids 395–483), and an M, 16,000 propeptide that remains associated with the active enzyme. In contrast to the endopeptolytic activity of cathepsins B, S, and L, DPPI has relatively promiscuous exopeptidase activity, hydrolyzing most NH₂-terminal dipeptides except those with P1 proline or P2 basic residues (12–15). By removing NH₂-terminal dipeptides intracellularly, DPPI activates a number of granule-associated serine proteases, including lymphocyte granymes, neutrophil elastase, and cathepsin G (5). In addition, DPPI may play a role in cell growth (16, 17) and in the activation of platelet factor XIII (18) and neuraminidase (19).

Mast cells have granules that contain histamine, heparin, and serine proteases, such as tryptase and chymase (20). Tryptase and chymase are synthesized as inactive zymogens and then are activated by removal of NH₂-terminal dipeptides by DPPI (7, 8). These activated proteases are released by mast cells when stimulated to degranulate by various physiological stimuli (21). Mast cell granules also contain and secrete lysosomal hydrolases, such as β-hexosaminidase (22). Therefore, mast cell granules contain a diverse group of molecules, some of which reside in lysosomes in other types of cells, which can be secreted by exocytosis.

We have initiated studies designed to improve our understanding of the intracellular and possible extracellular roles of mast cell DPPI (MC-DPPI). This work reports the purification and biochemical properties of MC-DPPI. We show that dog mast cells produce large amounts of MC-DPPI, which is active over a broad pH range. Unlike other reported DPPIs, MC-DPPI exhibits heterogeneous processing of its heavy chain, and a large fraction of the enzyme can be released from intracellular storage sites. We also found that the levels of MC-DPPI mRNA are subject to modulation. These studies of MC-DPPI show possible novel processing, regulated expression, and extracellular activity.

EXPERIMENTAL PROCEDURES

Materials—All chemicals are from Sigma unless otherwise specified. Cell Culture—Dog BR and C2 mastocytoma cells were a gift of S. Lazarus and were cultured in Dulbecco’s modified Eagle’s medium-H16 medium containing 2% supplemented calf serum as described previously (23). Cells were maintained at 37 °C in 5% CO₂ and 95% air and were cultivated to a density of 1 × 10⁶ cells/mL. In experiments requiring stimulation with phorbol, cells were incubated with 25 ng/mL of 12-O-tetradecanoylphorbol-13-acetate for 18 h and then harvested by centrifugation.

Purification of DPPI—C2 cell pellets were resuspended in 100 mM NaCl, 300 mM NaCl, 1 mM EDTA (pH 6.0) (Buffer A), sonicated, and then centrifuged at 10,000 × g for 15 min. The supernatant was adjusted to pH 4.2, heated at 37 °C for 2 h, and then centrifuged at 14,000 × g for 15 min. The resulting supernatant was dialyzed overnight in 20 mM bis[2-hydroxyethyl]iminotris(hydroxymethyl)methane, 1 mM EDTA (pH 6.0) (Buffer B). The dialysate was loaded onto a lenti
P-40) and 0.05M Na2HPO4 (pH 7.5) at 37 °C for 2 h. Proteins were the mixture incubated in 1% nonylphenoxypolyethoxyethanol (Nonidet captoethanol at 100 °C for 10 min. 2 units of PNGase F were added, and proteins in active fractions were subjected to SDS-PAGE and then stained with Coomassie Blue.

**Deglycosylation**—Purified MC-DPPI was deglycosylated with N-glycosidase F (PNGase F; New England Biolabs, Beverly, MA). 5 μg of DPPI were denatured in a 100-μl solution of 0.5% SDS and 1% 2-mercaptoethanol at 100 °C for 10 min. 2 units of PNGase F were added, and the mixture incubated in 1% nonylphenoxypolyethoxyethanol (Nonidotin P-40) and 0.05M Na2HPO4 (pH 7.5) at 37 °C for 2 h. Proteins were precipitated with quinine sulfate prior to electrophoresis.

**NH2-terminal Sequence Analysis**—5 μg of purified and deglycosylated MC-DPPI were subjected to SDS-PAGE, transferred to polyvinylidine difluoride membrane (Bio-Rad) in 10 mM CAPS buffer containing 0.1M NaCl. Active fractions were diluted 1:2 with Buffer B and then applied to a Mono Q HR 5/5 anion exchange column (Amersham Pharmacia Biotech) connected in series to a Superose 12 gel filtration column (Amersham Pharmacia Biotech) and eluted with Buffer B containing 0.1 M NaCl. Active fractions were diluted 1:2 with Buffer B and then eluted over 20 min with a linear gradient from 0 to 1 M NaCl at a flow of 0.5 ml/min. Proteins in active fractions were subjected to PAGE and then stained with Coomassie Blue.

**DNasel Cloning**—MC-DPPI cDNA was obtained by PCR amplification of cDNAs from a previously prepared Br mastocytoma library (24). The cDNA was amplified by PCR using Taq polymerase and the following primers, which were designed to include conserved regions of human and rat DPPIs (25, 26): 5′-CGACACACTGCGAATGCTA-5′ and 5′-CCTCTGGGATCCGGTCCAC-3′. A second set of primers corresponding to nucleotides 1139–1163 of dog MC-DPPI (5′-CCTGGTGCTGTATGATCGTACTG-5′) and a portion of sggt10 (5′-GAAGTTGCTTATGAGTACTTTCTCAGG-3′) near the insertion site were used to amplify the 3′-end of MC-DPPI, which was subcloned into plasmid or PCR (Stratagene, La Jolla, CA) and sequenced in both directions. Amino acid sequence was deduced from the cDNA sequence and aligned to published sequences from other species using Gene Works software (Oxford Molecular Group, Campbell, CA).

**Deglycosylation**—Deglycosylation of MC-DPPI activity was measured spectrophotometrically by monitoring hydrolysis of L-Ala-Ala-p-nitroanilide and Gly-L-Phe-p-nitroanilide at 37 °C (27). 1–50 μl of enzyme solution were incubated for 5 min in 500 μl of activation buffer (100 mM Na2HPO4 buffer, 20 mM NaCl, 1 mM EDTA, 4 mM cysteine (pH 6)), followed by the addition of 400 μl of substrate buffer (100 mM Na2HPO4, 20 mM NaCl, 1 mM EDTA, 125 μM L-Ala-Ala-p-Na, 1% dimethyl sulfoxide). Tryptase activity was measured using hz-Val-Gly-Arz-p-nitroanilide. 15 μl of enzyme solution were incubated in 0.2 ml of 0.06 M Tris-HCl (pH 7.8) containing 0.4% dimethyl sulfoxide and 80 μg/ml of substrate at 37 °C. In both enzyme assays, release of free nitroanilide was measured at 410 nm for 5–10 min.

**pH Profile of DPPI Activity**—The pH dependence of t-ALA-P-nitroanilide hydrolysis was determined at intervals of 0.5 pH units from pH 3.0 to 8.0 in citrate-phosphate buffers as described previously (27).

**Mast Cell Degranulation**—Mast cell exocytosis was stimulated as described previously (21). Briefly, mastocytoma cells were harvested by centrifugation, washed twice in Ca2+- and Mg2+-free phosphate-buffered saline, and then resuspended in serum-free Dulbecco’s modified Eagle’s H16 medium to a final concentration of 15 × 106 cells/ml. Cells were then incubated alone, with 1 μM z-pheniramine, 20 mg/ml of substrate buffer (100 mM Na2HPO4, 20 mM NaCl, 1 mM EDTA, 125 μM L-Ala-Ala-p-Na, 1% dimethyl sulfoxide). Tryptase activity was measured using hz-Val-Gly-Arz-p-nitroanilide. 15 μl of enzyme solution were incubated in 0.2 ml of 0.06 M Tris-HCl (pH 7.8) containing 0.4% dimethyl sulfoxide and 80 μg/ml of substrate at 37 °C. In both enzyme assays, release of free nitroanilide was measured at 410 nm for 5–10 min.

**Data Representation**—Data represent a typical purification. Activity was measured using t-ALA-P-nitroanilide as substrate for MC-DPPI.

| Step | Total protein | Total activity | Specific activity | Enrichment |
|------|--------------|---------------|------------------|------------|
| Extract | 30.4 | 10.2 | 0.34 | 1.0 |
| Acid precipitate | 3.39 | 0.5 | 1.28 | 3.8 |
| Lecitin column | 0.50 | 3.8 | 7.60 | 22.4 |
| Mono Q column | 0.214 | 2.1 | 9.50 | 38.8 |
| Mono-Q column | 0.098 | 1.0 | 10.2 | 30.0 |

bestatin, 500 μM; phenylmethylsulfonyl fluoride, 1 mM; aprotinin, 100 μM; cystatin, 50 μg/ml; Gly-Phe diazomethyl ketone (Enzyme Systems Products, Dublin, CA), 20 μM; E-64, 10 μM; or iodoacetate, 1 mM. After a 15-min preincubation with inhibitor, 400 μl of substrate buffer with the corresponding inhibitor were added, and t-ALA-P-nitroanilide hydrolytic activity measured as above. Activity with inhibitor was compared with that without inhibitor. Antiprotease Production and Immuno blotting—100 μg of purified dog MC-DPPI plus Freund’s adjuvant were injected into two rabbits. At 2-week intervals, rabbits were injected with 100 μg of dog MC-DPPI. At week 12, the rabbits were bled. Serum was collected and analyzed by ELISA for MC-DPPI immunoreactivity. Antibodies were produced by Immune-Dynamics (La Jolla, CA). For immunoblot analysis, samples were separated by SDS-PAGE under reducing conditions and then transferred to polyvinylidine difluoride membrane (Bio-Rad) in 10 mM CAPS containing 10% methanol for 1 h at 4 °C. The membrane was washed with 50 mM Tris-HCl, 0.5 mM CaCl2, 0.01% Tween-20 (TBS) (pH 7.5), incubated with a 1:1000 dilution of MC-DPPI antiserum in TBS for 1 h, washed with TBS, incubated with a 1:2000 dilution of goat anti-rabbit antiserum (Sigma) in TBS for 1 h, and washed again. Immunoreactivity was detected using the Fast Red TR/naphthol AS-MX detection system (Sigma).

**RNA Blotting**—Poly(A)+ RNA was isolated from mastocytoma cells, incubated alone or in the presence of phorbol ester, c-kit ligand (a gift from Amgen, Thousand Oaks, CA), tumor necrosis factor-α (R & D Systems, Minneapolis, MN), interleukin-1β (R & D Systems), using the Micro-fast track kit (Invitrogen, Carlsbad, CA). Degraded poly(A)+ RNA was size-fractionated by agarose gel electrophoresis and transferred to Hybond Plus nylon membrane (Schleicher and Schuell) (28). Vacuum-baked membranes were prehybridized at 42 °C for 2 h and hybridized with an [α-32P]dCTP (Amersham Pharmacia Biotech) random prime-labeled dog MC-DPPI probe at 42 °C overnight. After two washes, the membranes were air-dried and the membrane was exposed to film for 19–48 h and then developed. Following removal of previously bound probe, the membrane was hybridized with a γ-actin-labeled probe to account for possible variations in signal intensity due to differing amounts of mRNA loaded per lane.

**RESULTS**

**Purification of MC-DPPI**—DPPI enzymatic activity was readily measured in dog BR and C2 mastocytoma cell sonicates using Gly-L-Phe-p-nitroanilide and t-ALA-P-nitroanilide. t-ALA-P-nitroanilide was used in all subsequent experiments because the specific activity was higher for this substrate. Hydrolysis of t-ALA-P-nitroanilide was 7-fold greater in C2 than in BR cell extracts; therefore, C2 cells were used as the source for MC-DPPI. Hydrolysis of t-ALA-P-nitroanilide was completely inhibited by Gly-L-Phe-diazomethylketone, a specific DPPI inhibitor (5), indicating that the activity is entirely due to MC-DPPI. Repeated iterations of the purification protocol yielded 90–200 μg of pure MC-DPPI per g of cells. The 6-fold enrichment provided by Lentil lectin chromatography was the single most effective step of the purification protocol (Table I). The Mono-Q chromatographic step, although yielding only a small boost in specific activity, was used because it separated MC-DPPI from a few proteins of high M. Using a specific activity determined by hydrolyzing t-ALA-P-nitroanilide with a known quantity of purified MC-DPPI and back-calculating, we estimate that C2 cells contain 1–2 μg of MC-DPPI/cell.
SDS-PAGE migrates as a single band with an \( M_r \) of \(-175,000\) in the absence of reducing agents (Fig. 1A). This is approximately the size one expects for a tetrameric assembly of enzymatic units, with each unit consisting of a heavy chain, a light chain, and a portion of propeptide. The faint band of \( M_r \) 45,000 may be a small amount of single enzymatic units. Under reducing conditions (Fig. 1B), MC-DPPI migrates as three bands: a broad band with \( M_r \) of 22,000–25,000, a closely-spaced doublet of \( M_r \) \(-16,000\), and a band that migrates with the dye front. After N-deglycosylation, the broad, high \( M_r \) band resolves into three bands with estimated \( M_r \) values of 19,000, 20,000, and 22,000 (Fig. 1B), suggesting that the heavy chain is heterogeneously processed to three different major species. The \( M_r \) 16,000 double band resolves into a single band of greater intensity after treatment with PNGaseF.

**Protein Sequencing**—Edman degradation of the three highest molecular weight bands was performed to determine whether variable NH2-terminal cleavage explains the heterogeneity observed on SDS-PAGE. Major and minor sequences were obtained for bands with the following \( M_r \) values: 19,000, LPTSW (major) and RLPTS (minor); 20,000, ISRLP (major) and LPTSW (minor); 21,000, ISRLP (major) and RLPTS (minor). Alignment of these sequences to those of human DPPI (25, 26) and of the cDNA-derived dog MC-DPPI sequence (see below) confirms that they correspond to sequences in the NH2-terminal region of the heavy chain, with the exception that there are multiple NH2 termini extended by 1, 3, and 5 amino acids compared with the human enzyme. NH2-terminal sequencing of the \( M_r \) 16,000 band reveals two sequences, the predominant one, DTPADXTH, corresponding to the pro-region of human DPPI. The minor sequence, DFPNFLKEL, represents a small amount of light chain. The propeptide \( M_r \) 16,000, as determined by SDS-PAGE, is less than the \( M_r \) of 23,000 predicted by nucleic acid sequence (see below) and suggests that the propeptide is truncated at its COOH terminus by proteolysis.

**MC-DPPI cDNA**—To achieve a better understanding of the nucleotide and predicted amino acid sequence of dog MC-DPPI, we cloned a DPPI cDNA fragment from a dog mastocytoma cDNA library using reverse transcription-PCR (Fig. 2). The cloned 1539-base pair cDNA encodes the propeptide, heavy chain, and light chain. A 224-base pair 3'-untranslated region ends in a poly(A) tail. A consensus polyadenylation signal (AATAAA) resides 19 base pairs upstream of the poly(A) tail. NH2-terminal amino acid sequences obtained by Edman degradation agree with those predicted by the cDNA, suggesting that the cDNA in fact corresponds to the purified enzyme. The nucleotide sequence and predicted amino acid sequence are 86 and 84% identical, respectively, to the corresponding sequences of human DPPI (26). There are four consensus sites for N-glycosylation in the propeptide and one in the heavy chain. All cysteines are conserved among the dog, human, mouse, and rat species (25, 26, 29), as are the putative catalytic amino acid residues Cys234 and His381 (Cys25 and His159 by papain numbering (30)). Two other amino acids believed to be important for catalysis by cysteine proteases, Glu228 and Trp399 (31), (Glu19 and Trp177 by papain numbering), are also conserved. There is strict conservation of 60% of the propeptide (residues 1–203) and 80% of both heavy chain (residues 204–370) and light chain (residues 371–413) among the four species of DPPI analyzed (Fig. 3).

**pH Profile of MC-DPPI Activity**—The pH dependence of MC-DPPI activity for hydrolysis of L-Ala-Ala-p-nitroanilide (Fig. 4) reveals that MC-DPPI has activity over a broad pH range and that its optimum activity is at pH 6.5. MC-DPPI retains >80% of its activity at pH 7.5, a pH similar to that in the extracellular compartment.

**Secretion of MC-DPPI**—Incubation of C2 cells with 1 \( \mu \)mol ionophore stimulates time-dependent release of MC-DPPI and tryptase activities (Fig. 5A). After a 15-min incubation with ionophore, 35% of total active MC-DPPI, 20% of total active tryptase, and 35% of total histamine (data not shown) is released by mastocytoma cells. Similarly, a significant amount of DPPI and tryptase is released by C2 cells after a 60-min incubation with 100 \( \mu \)mol substance P (Fig. 5B). Control, ionophore, and substance P-stimulated cells are >90% viable after 15 min as determined by trypan blue exclusion and LDH release, indicating that enzyme release is not due to cell death and lysis.

**Inhibition of MC-DPPI**—Inhibitor profiles for hydrolysis of L-Ala-Ala-p-nitroanilide by purified MC-DPPI and degranulation supernatants were determined to confirm that activity measured in degranulation supernatants was from MC-DPPI and not from another protease. The relative percentage of inhibition by specific inhibitors is similar for purified MC-DPPI and degranulation supernatants (Table II), confirming that activity measured in the degranulation supernatants is entirely due to MC-DPPI.

**Immunoblotting**—Presence of MC-DPPI immunoreactivity in the supernatants from control (unstimulated) and degranulated mast cells is detected using a polyclonal antibody generated against MC-DPPI (Fig. 6). More immunoreactive material is present in supernatants generated by ionophore-stimulated cells than in those generated by unstimulated cells. This material co-migrates with purified MC-DPPI, confirming its identity and its secretion under the influence of ionophore. The visualized band corresponds to the heavy chain of MC-DPPI, suggesting that epitopes recognized by the antibody are exclusively on the heavy chain.

**DPPI mRNA Regulation**—Due to the greater amounts of MC-DPPI protein in C2 cells compared with BR cells, we sought to determine whether this is, in part, due to a difference in mRNA levels. Fig. 7 shows that the basal level of MC-DPPI mRNA is greater in C2 cells than BR cells. Phorbol increases by 6-fold the steady state MC-DPPI mRNA levels in BR cells. C2 cells, however, have a high baseline level of MC-DPPI that is not increased by phorbol stimulation. These results indicate that DPPI mRNA levels can be regulated and that the high levels of mRNA in C2 cells may explain the high-level expression of active enzyme. Levels of MC-DPPI mRNA in BR cells are not changed by incubation of BR cells with 100 ng/ml of e-kit ligand, 5 ng/ml of tumor necrosis factor-\( \alpha \), or 10–10 \( \mu \)l interleukin-1\( \beta \).

**DISCUSSION**

This report describes the purification and characterization of DPPI from dog mastocytoma cells, which are the richest source of the enzyme yet described. Dog MC-DPPI is similar to human DPPI in oligomeric structure, activity at acidic pH, inhibition by inactivators of cysteine proteinases, and inducibility of mRNA expression (15, 27, 32). Possibly unique features are variable processing of its heavy chain, preservation of activity...
at a neutral to alkaline pH, and capacity for exocytic secretion. These features raise the possibility that MC-DPPI levels vary when mast cells are exposed to different external stimuli and that MC-DPPI enacts extracellular roles in addition to its previously described intracellular actions.

The strategy we used to purify MC-DPPI is a modification of the techniques used to purify DPPI from other sources (15, 27). The purification protocol takes advantage of the stability of MC-DPPI at acidic pH, N-glycosylation (resulting in binding to lentil lectin), and large size relative to other mast cell proteins. Using our purification scheme, we purified up to 200 μg of MC-DPPI per g of mastocytoma cells, as compared with 2 (27) and 1 (15) μg/g from human kidney and spleen, respectively. These results suggest the possibility that mast cells, because of their wide distribution, are the major source of DPPI in many tissues. From the specific activity determined for purified MC-DPPI, we estimate that C2 mastocytoma cells contain 1–2 pg of MC-DPPI per cell, an amount similar to that of chymase (23), another major mast cell protease.

As reported for human kidney DPPI (27), purified MC-DPPI separates into three bands with Mr values of ~24,000, 16,000, and ~10,000 on reducing SDS-PAGE. Resolution of the heavy chain into three smaller bands after N-deglycosylation shows that the heavy chain of MC-DPPI is N-glycosylated and is heterogeneously processed. Alternative splicing of mRNA does not explain the size differences because there is only one mRNA species noted on our RNA blots. Our data indicate that MC-DPPI has multiple heavy chain NH2 termini. The LPTSW...terminus is identical to that reported for human DPPI (27); however, the ISRLP...RLPTS...and EE...NH2 termini are novel and appear to result from proteolysis at sites proximal to the LPTSW...sequence. Unlike the LPTSW...heavy chain terminus of human DPPI, our ISRLP...NH2 terminus is the predominant site of cleavage as estimated by SDS-PAGE. Alternative NH2-terminal processing does not fully explain the Mr...heterogeneity of the heavy chain. Therefore, variable COOH-terminal proteolytic processing and glycosylation, as well as other modifications, may contribute to the size difference.

The propeptide and light chain of MC-DPPI are processed in a manner similar to those of human DPPI. Their estimated Mr...
and NH2-terminal sequences correspond exactly to those reported for human DPPI (27). Resolution of the propeptide doublet into a single band after N-deglycosylation suggests that it is N-glycosylated. NH2-terminal sequencing of deglycosylated material reveals an Asp instead of the predicted Asn at the fifth residue of the propeptide. This is consistent with glycosylation of the first predicted glycosylation site because an Asp is generated by N-deglycosylation. The blank in the sixth cycle of the propeptide sequence results from oxidation of cysteine.

There are several possible explanations for the variable NH2 termini identified of the MC-DPPI heavy chain. The ISRLP...NH2 terminus may result from processing by a granzyme B-like protease, and the LPTSW...NH2 terminus may result from cleavage by a tryptic-like protease (possibly tryptase). Alternatively, there may be only one major site of endoproteolytic cleavage of the proenzyme, with further exoproteolytic processing of the NH2 terminus by aminopeptidases or dipeptidyl peptidases, possibly MC-DPPI itself. An initial major endoproteolytic cleavage may generate the EEISRLPT...heavy chain isoform, which is then autoprocessed by MC-DPPI itself, yielding the ISRLPT... and RLPTS... NH2 termini, or processed by tryptase, yielding the LPTSW... NH2-terminal isoform identified by Edman degradation. Processive hydrolysis by mast cell monoaminopeptidase cleavage (33) of the EEISRLP...NH2 terminus could explain some or all of the subsequent NH2 termini identified; however, one might have expected to detect EISRL... and SRLPT... NH2 termini if aminopeptidase processing had occurred exclusively.

**FIG. 3. DPPI alignment.** The deduced amino acid sequence for dog MC-DPPI was aligned with published sequences for human, mouse, and rat DPPIs. Boxes enclose residues identical in all four species. The propeptide, heavy chain, and light chain correspond to residues 1–203, 204–370, and 371–438, respectively. Solid bars highlight conserved consensus N-glycosylation sites. Open bars highlight consensus N-glycosylation sites unique to dog MC-DPPI. Arrowheads point to the catalytic dyad residues amino acids Cys25 and His159 (Cys259 and His381 papain numbering) critical for catalysis of cysteine proteases. Additional conserved amino acids, Gln228 and Trp399, thought to be critical in the active site of cysteine proteases, are indicated by a pound sign (#). Other cysteines are indicated by an asterisk (*).
represent the mean net percentage of release ± S.E. for three experiments for A and for five experiments for B.

### Table II

| Inhibitor | Percentage of control activity of purified MC-DPPIa | Percentage of control activity of degranulation supernatantb |
|-----------|-----------------------------------------------------|-------------------------------------------------------------|
| Bestatin (500 μg/ml) | 100 | 100 |
| Phenylmethylsulfonyl fluoride (1 mM) | 100 | 100 |
| Aprotinin (100 μM) | 83 | 75 |
| Cystatin (50 μg/ml) | 7 | 0 |
| Gly-Phe diazomethylketone (20 μM) | 7 | 0 |
| E-64 (10 μM) | 0 | 0 |
| Iodoacetic acid (1 mM) | 0 | 0 |

a 1 μg of purified MC-DPPI or 30 μl of degranulation supernatant (15-min time point) were preincubated with the stated inhibitors for 15 min. Residual activity was determined by hydrolysis of L-Ala-Ala-p-nitroanilide in assay buffer.

The functional significance of variable heavy chain processing is unknown. It is possible that differences in processing affect substrate specificity, pH optimum, and specific activity. Identification of the proteases involved in MC-DPPI processing and the sites of such processing (endoplasmic reticulum, Golgi apparatus, secretory granules, or cell extracts) require future study.

As seen in Fig. 3, much of dog MC-DPPI is conserved in DPPIs of other species. The most conserved region of DPPI is the heavy chain, suggesting that many heavy chain residues, and the heavy chain itself, are critical for enzyme activity and specificity. Conservation of the catalytic amino acids Cys234 and His381, as well as two amino acids, Gln228 and Trp399, which are believed to be important for stabilizing the catalytic dyad and its intermediates (31), suggests that the deduced amino acid sequence is that of catalytically active MC-DPPI. Specific residues critical for determining exopeptidase activity of MC-DPPI have yet to be determined.

The function of the propeptide of MC-DPPI specifically and of DPPIs in general is unknown. The propeptides of cathepsin B and cathepsin L, two of the nearest relatives of DPPI, inhibit enzymatic activity by binding to the substrate binding cleft (34, 35). Following a proteolytic event, the propeptide is released, and the enzyme is thereby activated. This scenario probably differs for MC-DPPI because the propeptide is 2–3 times longer than that of cathepsins B and L, and a portion of its propeptide remains associated with the active enzyme. The propeptide of MC-DPPI may initially function similarly to that of cathepsins B and L by occupying the MC-DPPI active site and inhibiting catalytic activity. A COOH-terminal portion of the propeptide maybe released by proteolysis, leaving a smaller fragment of the propeptide associated with the active enzyme. Alternatively, the propeptide may confer a specific conformation to MC-DPPI, which then changes after removal of the propeptide fragment. The remaining portion of the propeptide is probably disulfide-linked to the mature enzyme, as suggested by the lack of a propeptide fragment on nonreducing SDS-PAGE. Although the function of the portion of propeptide that remains linked with the active enzyme is unknown, it may help to maintain structural

![FIG. 5. Time-dependent release of MC-DPPI and tryptase by stimulated mastocytoma cells.](Image 5)

C2 mastocytoma cells (15 × 10^6 cells/ml) were incubated with 1 μM ionophore A23187 (A) or 100 μM substance P (B). At the designated time intervals, an aliquot of medium was removed and separated from cells by centrifugation. Cells pellets were resuspended in an equal volume of medium and lysed by sonication. Supernatants were assayed for MC-DPPI and tryptase activity with L-Ala-Ala-p-nitroanilide and Bz-L-Val-Gly-Arg-p-nitroanilide substrates, respectively. Values represent the mean net percentage of release ± S.E. for three experiments for A and for five experiments for B.

![FIG. 6. Immunoblot of degranulation supernatants for MC-DPPI.](Image 6)

10 μl of a 10-fold concentrate of supernatant collected from control (lane 1) or A23187-stimulated (lane 2) cells at the 15-min time point (lane 1) (Fig. 5) and 125 ng of purified dog MC-DPPI (lane 3) were separated under reducing conditions by SDS-PAGE, blotted to polyvinylidene difluoride membrane, and probed with a polyclonal anti-dog MC-DPPI antibody raised in rabbits. Immunoreactivity was identified using a goat anti-rabbit alkaline phosphatase detection system.

![FIG. 7. Regulation of MC-DPPI mRNA expression by phorbol ester in BR and C2 mastocytoma cells.](Image 7)

3.5 μg of mRNA isolated from BR or C2 cells were separated by agarose gel electrophoresis, blotted to Nytran nylon membrane, and probed sequentially for MC-DPPI and actin with the corresponding α-[32P]dCTP-labeled cDNA probes. Lanes with mRNA isolated from cells incubated for 18 h with (+) and without (−) 25 ng/ml 12-O-tetradecanoylphorbol-13-acetate are shown in the two panels.
surprising because other lysosomal enzymes, such as cell secretory products tryptase and histamine. Finding DPPI, tured mast cells in parallel with tryptase and histamine. This by degranulating mast cells. Activity at neutral to alkaline pH is significant because we showed that MC-DPPI can be released by degranulating mast cells. Activity at neutral to alkaline pH raises the possibility of extracellular roles following release.

In this report, we showed that MC-DPPI is secreted by cultured mast cells in parallel with tryptase and histamine. This observation indicates that MC-DPPI is functionally, and possibly physically, in the same compartment as the classic mast cell secretory products tryptase and histamine. Finding DPPI, a lysosomal protease, in mast cell secretory granules is not surprising because other lysosomal enzymes, such as β-hexosaminidase, also reside in these granules (22). The observation that DPPI and tryptase activity coexist in a cytoplasmic granular fraction in extracts of mouse mastocytoma cells further supports the possible colocalization of DPPI and tryptase (5).

Secretion of MC-DPPI by cells stimulated with substance P is significant because it identifies a physiological stimulus whereby MC-DPPI may be released extracellularly. Mast cells have been found in close association with nerve terminals of neurons from the nonadrenergic, noncholinergic nervous system, including those that store substance P (38, 39). Depolarization of these neurons may result in release of substance P, which may then act on the nearby mast cell, stimulating secretion of MC-DPPI.

The role of extracellular MC-DPPI is unknown at this time. Individually or in cooperation with other proteases, MC-DPPI may degrade targets such as neutrophilic mucus, cytokines, or extracellular matrix proteins. In doing so, MC-DPPI may influence cellular functions or matrix remodeling. These effects would probably be limited to the local region around the mast cell because circulating protease inhibitors, such as the cystatin (40), should inhibit MC-DPPI as it diffuses away from the cell. However, high concentrations of MC-DPPI in the immediate vicinity of the degranulating mast cell may overwhelm inhibitor defenses temporarily, allowing MC-DPPI to encounter and hydrolyze extracellular substrates.

The polyclonal antibody raised against dog MC-DPPI recognizes predominantly the heavy chain of MC-DPPI, and not the propeptide or light chain, although each of these components is present in the purified material used to immunize rabbits. A possible explanation for this observation is that dog MC-DPPI propeptide and light chain are more homologous to the corresponding fragments of rabbit DPPI than is the heavy chain.

The steady-state quantity of MC-DPPI mRNA and corresponding protein levels rise in cells incubated with phorbol ester. This suggests that MC-DPPI protein expression may be controlled by varying the amount mRNA via transcriptional or posttranscriptional mechanisms. Regulation of MC-DPPI has several implications. First, quantities of enzyme may vary depending on the tissue location of the mast cell. An example of this is the variable expression of tryptase and chymase depending on whether the mast cell is of skin or mucosal origin (20). Second, MC-DPPI may be the rate-limiting step in production of active tryptase and chymase because the amount of active tryptase or chymase will be the rate-limiting step in production of active tryptase and chymase depending on whether the MC-DPPI is secreted by mast cells or released extracellularly. Mast cells have a role in allergic and inflammatory diseases, such as asthma (41). Inflammatory mediators in these diseases may alter quantities of MC-DPPI and influence its roles in these diseases. The finding that c-kit ligand, tumor necrosis factor-α, and interleukin-1β do not alter MC-DPPI mRNA levels suggests that MC-DPPI may not be subject to regulation by these cytokines.

In conclusion, dog mastocytoma cells produce a DPPI that has structural and enzymatic similarities to and differences from DPPIs purified from other tissues and species. Unique features of MC-DPPI are the heterogeneous processing of its heavy chain, enzymatic activity over a broad pH range, and secretion by mast cells. These findings invoke the possibility that MC-DPPI acts on extracellular targets.
Regulated Expression, Processing, and Secretion of Dog Mast Cell Dipeptidyl Peptidase I
Paul J. Wolters, Wilfred W. Raymond, John L. Blount and George H. Caughey

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