Human Mast Cell Carboxypeptidase

Purification and Characterization

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Abstract

A carboxypeptidase activity was recently identified in highly purified human lung mast cells and dispersed mast cells from skin. Using affinity chromatography with potato-tuber carboxypeptidase inhibitor as ligand, mast cell carboxypeptidase was purified to homogeneity from whole skin extracts. The purified enzyme yielded a single staining band of ≈ 34,500 D on SDS-PAGE. Carboxypeptidase enzyme content estimated by determination of specific activity, was 0.5, 5, and 16 μg/10^6 mast cells from neonatal foreskin, adult facial skin, and adult foreskin, respectively.

Human mast cell carboxypeptidase resembled bovine carboxypeptidase A with respect to hydrolysis of synthetic dipeptides and angiotensin I, but was distinguished from carboxypeptidase A in its inability to hydrolyze des-Arg^9 bradykinin. The amino acid composition of human mast cell carboxypeptidase was similar to the composition of rat mast cell carboxypeptidase. The amino-terminal amino acid sequence of mast cell carboxypeptidase demonstrated 65% positional identity with human pancreatic carboxypeptidase B, but only 19% with human carboxypeptidase A. Thus, human mast cell carboxypeptidase is a novel member of the protein family of zinc-containing carboxypeptidases, in that it is functionally similar but not identical to bovine carboxypeptidase A, but has structural similarity to bovine and human pancreatic carboxypeptidase B.

Introduction

Human mast cells contain a variety of preformed secretory granule mediators, including two enzymes designated tryptase (1, 2) and chymase (3–5). A carboxypeptidase (CP)[1] activity, thought to be carboxypeptidase A (CPA) or CPA-like, has been identified in rodent mast cells (6, 7) and purified from rat peritoneal mast cells (6) and rat skeletal muscle (8). A CP activity was recently identified in highly purified human mast cells from lung (9). The carboxypeptidase is ≈ 30,000–35,000 D, is active at neutral to alkaline pH, is inhibited by potato tuber carboxypeptidase inhibitor (PCI), EDTA, phenanthroline, and 8-hydroxyquinoline, and is released from mast cells by immunologic challenge. These characteristics suggest that the CP is a mast cell secretory granule zinc-metalloexopeptidase, related to the CPs purified from pancreas (10). A CP activity identical with respect to pH optimum, molecular mass, inhibitor profile, and enzymatic activity was also identified in preparations of dispersed skin mast cells and in extracts from whole human skin (9). In this article we report the purification and physicochemical characterization of the human mast cell CP, and demonstrate that the enzyme closely resembles carboxypeptidase B (CPB) with regard to primary structure, but functionally acts similarly although not identically to CPA.

Methods

Materials

Angiotensin I (AI), angiotensin II (AII), des-Leu^{10} AI, bradykinin, and des-Arg^9 bradykinin (Peninsula Laboratories, Inc., San Carlos, CA); polyethylene glycol (PEG) 3350, $NaClO_4$, 85% H_3PO_4, and CH_3CN (HPLC grade) (Fisher Scientific Co., Medford, MA); phenylisothiocyanate (PITC), trifluoroacetic acid (TFA) (Pierce Chemical Co., Rockford, IL); *N*-carbobenzoxy-glycyl-L-phenylalanine (N-CBZ-Gly-Phe), *N*-carbobenzoxy-glycyl-L-arginine (N-CBZ-Gly-Arg), bovine pancreatic CPB, bovine pancreatic CPA, soybean trypsin inhibitor (SBTI), PCI, collagenase type 1A (Sigma Chemical Co., St. Louis, MO), EDTA, 1-10 phenanthroline, DNAse, (Calbiochem Behring Corp., La Jolla, CA); crystallized human serum albumin (Miles Laboratories, Inc., Elkhart, IN); RPMI 1640 medium with 25 μM Hepes, Hanks' solution (Gibco, Grand Island, NY); Percoll, Sephacryl S-200 (Pharmacia Fine Chemicals, Piscataway, NJ); lysylendopeptidase (Wako, Dallas, TX); and vinylpyridine (Aldrich Chemical Co., Milwaukee, WI) were obtained as noted.

Assay of human mast cell CP

CP activity was measured by hydrolysis of the carboxy-terminal His^9-Leu^{10} bond of AI to form the nonapeptide des-Leu^{10} AI. Enzymes or enzyme sources were incubated with 50 μM AI for 30 min at 37°C in 500 μl of 0.01 M Tris-HCl, pH 7.4, 0.15 M NaCl. Reactions were stopped by addition of an equal volume of HPLC solvent, and samples were then stored on ice. To determine that the hydrolysis of AI was due to CP and not a contaminating enzyme, aliquots were preincubated at 37°C for 15 min with and without 1 μM PCI, before initiation of the reaction with substrate. The reaction was assayed by HPLC as described (11, 12). CP activity is expressed as the amount of des-Leu^{10}-AI generated from AI substrate; (1 U = 1 nmol des-Leu^{10} AI generated/min). This assay detects 5–10 pmol of each angiotensin peptide (0.004–0.008 U), and when mast cells from skin were used, the assay routinely detected activity in the equivalent of 65–200 mast cells per assay in a preparation of dispersed skin cells.

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1. *Abbreviations used in this paper:* AI, angiotensin I, AII, angiotensin II, CP, carboxypeptidase; CPA, carboxypeptidase A; CPB carboxypeptidase B; N-CBZ-gly-phe, *N*-carbobenzoxyglycyl-L-phenylalanine; N-CBZ-Gly-Arg, *N*-carbobenzoxyglycyl-arginine; PCI, potato tuber carboxypeptidase inhibitor; PEG, polyethylene glycol; PITC, phenylisothiocyanate; SBTI, soybean trypsin inhibitor; TFA, trifluoroacetic acid.

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Isolation of dispersed human skin mast cells

Foreskins from circumcisions of neonates and adults, and adult facial skin from cosmetic surgical procedures were stored in Dulbecco's modified Eagle's medium with 1.5% fetal bovine serum overnight at 4°C. Dispersed mast cells were obtained by enzymatic digestion as described (9). Percoll gradients were made by layering 3-ml fractions of 50%, 70%, and 80% in a 15-ml conical tube. Cells were suspended in media and layered over the gradient, which was centrifuged at 500 *g* for 15 min. Mast cells were identified by Alcian blue staining. Viability of mast cells was greater than 99% as assessed by trypan blue dye exclusion.

To measure carboxypeptidase activity, dispersed mast cells from skin were sonicated on ice in 250 μl of 0.01 M Tris, pH 8.0, 1 M NaCl, and 50 μM SBTI with a sonic dismembrator (model 300; Oscar Fisher Co., Inc., Newburgh, NY) using a microtip at power 35, twice for 15 s. SBTI was included to inhibit mast cell chymase, which degrades mast cell CP (6). The sonicate was assayed for CP activity as described above.

Assay of peptide hydrolysis by CP

CPA and CPB hydrolysis of AI to des-Leu^{10} AI was measured using a protocol identical to that used for the mast cell CP. For these enzymes, activity was expressed as 1 U = 1 nmol des-Leu^{10} AI generated/min. Equivalent amounts of enzyme, in terms of des-Leu^{10} AI generation, were used to examine the hydrolysis of other substrates. The hydrolysis of des-Leu^{10} AI and bradykinin by mast cell CP, bovine CPA, and CPB was assayed by incubating an appropriate amount of each enzyme with 25 nmol of each peptide in 0.01 M Tris-HCl, pH 8.0, 0.15 M NaCl per 500-μl reaction. Each reaction was stopped after 15 min at 37°C by the addition of an equal volume of HPLC solvent used for the assay of peptide hydrolysis. Hydrolysis of des-Leu^{10} AI was measured by the HPLC assay used for the AI assay described above. Hydrolysis of bradykinin to des-Arg^{9} bradykinin was measured by a modification of a previously described HPLC assay (13) using an Ultrasphere ODS column and a linear gradient from 0.1% TFA in water to 0.1% TFA in 60% acetonitrile over 40 min at a flow rate of 1 ml/min. Peptides were monitored at 210 nm, and identified by retention time using external standards. CPA-like activity of mast cell carboxypeptidase was measured using a synthetic substrate, N-CBZ-Gly-Phe, by a kinetic spectrophotometric assay as described (6). As controls, bovine pancreatic CPA (0.3–2.5 U, Sigma Chemical Co.) hydrolyzed the peptide, and CPB (1–10 U, Sigma Chemical Co.) did not. CPB-like activity was measured by a spectrophotometric assay using the synthetic substrate N-CBZ-Gly-Arg (14). Bovine pancreatic CPB hydrolyzed this peptide, and CPA did not.

Procedure for purification of mast cell CP

Extraction of CP from skin. CP activity was extracted from freshly obtained human skin specimens after abdominoplasty or reduction mammoplasty using a modification of a previously described procedure (9). Subcutaneous fat was manually removed with a No. 10 blade. Pieces of skin (≈ 30 cm^2) were frozen in liquid nitrogen, and pulverized at top speed in a blender (Waring Products, New Hartford, CT) with a steel container. To solubilize the CP activity, the powdered skin was incubated on ice for 2 h in 10 mM Tris-HCl, pH 7.4, 4 M NaCl, 10 μM SBTI, 0.1% PEG, and intermittently vortexed. The mixture was centrifuged at 4°C at 10,000 *g*, the pellet was extracted for another hour in the same solution, and the supernatants were combined and stored at −70°C.

PCI affinity chromatography. Extracts were subjected to PCI affinity chromatography using a modification of the procedure described by Everitt and Neurath (6) and Petra (15). The PCI affinity column was prepared by linking 5–10 mg of PCI per 1.0 ml of CNBr-activated Sepharose 4B as described by the manufacturer. Extracts were applied at room temperature to a 1-ml or 5-ml column previously equilibrated in the skin extraction buffer. The column was then washed with 20 bed volumes of 10 mM Tris, pH 7.4, 1 M NaCl, 10 μM SBTI, 0.1% PEG, and then with 20 bed volumes of the identical buffer without SBTI. The column was eluted with 100 bed volumes of 0.1 M Na_2CO_3, pH 11.4, 2 M NaCl, 0.1% PEG, and collected in 5-ml fractions for the 1-ml column, or in a single volume. The eluate was immediately neutralized in 1 vol of 1 M Hepes, pH 6.9, 2 M NaCl, 0.1% PEG per 9 vol of eluate. Samples were assayed for carboxypeptidase activity in the presence and absence of PCI, and for protein by absorbance at 280 nm and by Coomassie Brilliant Blue dye binding (Bio-rad Laboratory, Richmond, CA), standardized using bovine serum albumin. The active eluate was concentrated at 4°C using a 50-ml concentrating chamber (Amicon Corp., Danvers, MA) with a PM 10 membrane.

Procedures for physicochemical characterization

HPLC and spectral analysis. Affinity-purified material was analyzed by HPLC (5-ml system, Rainin Instrument Co., Inc., Woburn, MA) using a reverse-phase system with a C-8 RP 300 2.1 × 30 mm column (Brownlee Labs, Santa Clara, CA), employing a linear gradient of 0.1% TFA in water to 0.1% TFA in 70% acetonitrile over 45 min at 0.2 ml/min. Protein in the eluate was monitored at 215 nm with a model 1040A Diode Array HPLC detection system (Hewlett Packard Co., Palo Alto, CA). Spectra were collected and analyzed by a model 300 series computer (Hewlett Packard Co.).

Sephacryl S200 gel filtration. The concentrated eluate was filtered through a 1.6 × 91 cm Sephacryl S-200 column run at 4°C in 0.01 M Tris-HCl, pH 7.4, 1M NaCl, 0.1% PEG at 15 ml/h. Fractions of 2-ml vol were collected, screened for protein by OD_{280}, and Coomassie Brilliant Blue dye binding, and assessed for activity.

PAGE. Aliquots of reduced and alkylated, and unreduced purified CP were subjected to SDS-PAGE as described (16). Gels were stained with Coomassie Blue.

Amino-terminal amino acid sequence analysis. The NH_2-terminal amino acid sequence of 100 pmol of protein was determined at the Biomolecular Resource Center at University of California, San Francisco. The protein sample was bound electrostatically to a polybrene treated glass fiber filter and automated Edman degradation was performed on a model 470A gas phase protein sequencer (Applied Biosystems, Inc., Foster City, CA), equipped with an on-line 120A PTH Analyzer (17). The average repetitive yield was 95%, and the theoretical initial yield was 43 pmol. Lysylendopeptidase digestion was performed by incubating 15 μg of purified human mast cell CP with 4.4 × 10^3 U of lysylendopeptidase in 64 mM Tris-HCl, pH 8.85, 1.2 M guanidine-HCl for 16 h at 30°C. Peptides were separated by HPLC and sequenced as described above. The NH_2-terminal and internal sequences were compared to an updated Dayhoff protein data base using the Wilber and Lipman fast search computer algorithm (18).

Amino acid analysis was performed at the Biomolecular Resource Center at University of California, San Francisco by ninhydrin analysis consisting of vapor phase hydrolysis (6 N HCl, 24 h, 108°C) and separation of amino acids using an amino acid analyzer (model 121MB; Beckman Instruments, Inc., Palo Alto, CA) (19). The picotag method was used to quantitate cysteine residues (20). Approximately 15 μg of CP was dried to ≈ 10 μl in a Speed Vac Concentrator (Savant Instruments, Inc., Hicksville, NY) and 50 μl of 250 mM Tris-HCl, pH 8.5, 1 mM EDTA, 6 M guanidine-HCl was added. The reaction was incubated for 2 h in the dark under argon. Vinylpyridine, 2.5 μl neat, was added and the reaction was incubated for 2 h. The sample was desalted by HPLC using a reverse-phase system using a 2.1 × 30-mm C-8, RP 300 column (Rainin Instrument Co., Inc.) The recovered derivatized protein was dried and hydrolyzed as described above. The hydrolysate was derivatized with PITC, and PTC-amino acid separation was performed on an ABI 130A separation system, employing a sodium acetate/acetonitrile buffer elution system with a 2.1 × 220-mm C-18 column. Amino acid analysis was also performed by the picotag method without vinylpyridine derivatization, consisting of vapor phase hydrolysis (6 N HCl, 24 h, 108°C) on a "workstation," and PTC-amino acid separation on a model 840 HPLC system (Waters Associates, Milford, MA). Derivatization and separation protocols were obtained from the manufacturer (20). A computer algorithm that

yields a best fit total amino acid composition based on a given molecular weight range was used to generate the amino acid compositions.

Results

Localization of CP activity to mast cells from skin. Mast cell CP has been localized to human mast cells from lung, but detected only in crude skin extracts and in partially purified dispersed populations of human mast cells from skin (9). Dispersed skin cells contain multiple cell types, notably keratinocytes, fibroblasts, and endothelial cells (21). To confirm the mast cell association of CP activity in skin, the distribution of mast cells and CP activity was examined in discontinuous Percoll gradient fractions of dispersed skin cells (n = 3). The results of one experiment are shown in Table I. Dispersed skin cells from adult foreskins containing 2% mast cells (1.7×10^6) were applied to a Percoll gradient, and 1.2×10^6 mast cells were recovered. The gradient fractions were analyzed morphologically for mast cells by Alcian blue staining and for carboxypeptidase activity. The results (Table I) demonstrate a correlation between the percentage of CP activity and the percentage of mast cells in each fraction, and suggest that the CP identified in whole skin extracts is a component of human skin mast cells.

Purification of human mast cell CP from skin extracts. Since the CP activity in skin extracts is mast cell associated and is similar to the human lung mast cell CP with respect to function, molecular weight, and inhibition profile, human skin served as the source for the purification of this CP. Whole-skin extracts obtained from 50–80 g skin (wet weight) were each subjected to PCI affinity chromatography (Fig. 1). The CP activity present in each fraction could be 100% inhibited by PCI, and protein in the eluate was undetectable. The results of one preparation of purified CP are shown in Table II. A 1,348-fold purification was achieved with a specific activity of 36.4 U/μg protein. The specific activities of the two other preparations were 29 and 25 U/μg protein.

The affinity-purified CP was examined for purity by HPLC, spectral analysis, S-200 gel filtration, and SDS-PAGE. PCI affinity-purified material (5 μg) was analyzed by HPLC, and a single peak eluted at 36.75 min at $\approx$ 43% acetonitrile (Fig. 2 *A*). Spectral analysis (A_{210}–A_{400}) of three areas across the peak exhibited complete spectral superimposition (Fig. 2 *B*), a result consistent with a single protein.

Next, PCI affinity-purified protein (840 μg) was subjected to Sephacryl S200 gel filtration. CP activity was detected in fractions corresponding to an apparent molecular mass, of 30,000–35,000 D (not shown). After pooling and concentration of active fractions, 21% and 38% of the activity was recovered in each of two preparations.

Table I. Distribution of CP Activity and Mast Cells in Percoll Gradients

	Mast cells			CP	
	Purity	No./Fx*	Portion of total	U/Fx	Portion of total activity
	%		%		%
0/50% interface	3	4.0	35	193	31
50% Percoll	17	1.3	11	67	11
50%/70% interface	26	3.8	33	187	30
70% Percoll	43	1.4	12	113	18
70%/80% interface	56	1.0	8	55	9

* Number of mast cells $\times 10^5$ per fraction (Fx).

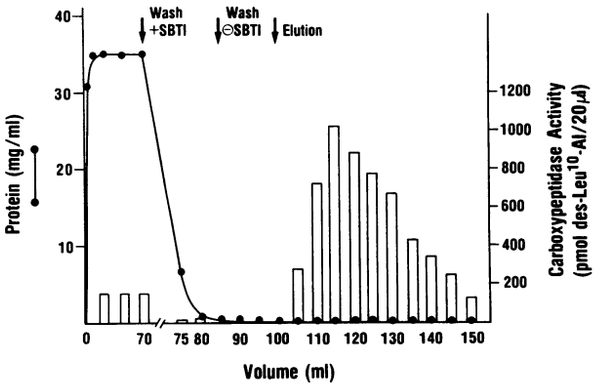

Figure 1. PCI affinity chromatography of human mast cell CP from whole-skin extracts using a 1-ml column. 5-ml fractions were collected and assayed for protein and enzyme activity.

Affinity-purified material (2 μg) was also subjected to SDS-PAGE. Reduced material demonstrated a single staining protein band of an apparent molecular mass of 34,500 D (Fig. 3), consistent with the molecular weight of the affinity-purified material as determined by S-200 gel filtration. A faint band, not visible in Fig. 3, of $\approx$ 57,000 D was seen only in the absence of reducing agent, and was not seen in reduced enzyme preparations even on overloaded gels.

Partial structural characterization. The amino-terminal amino acid sequence of 100 pmol of affinity-purified carboxypeptidase was determined. The first 28 residues of mast cell carboxypeptidase were unambiguously identified and are depicted along with published sequences of human and bovine pancreatic CPA and CPB (22–24) (Table III). These sequences were examined for identity with the homologous NH_2-termini of other CPs. There is 54% positional identity with bovine CPB, but only 18% with bovine CPA over these 28 residues. In this region the extent of identity between bovine CPA and CPB is 21%. When the aminoterminal residues are compared to the known sequences for human pancreatic CPA and CPB (22), the extent of identity of mast cell carboxypeptidase is 19% with CPA and 65% with CPB. Human pancreatic CPA and CPB share 15% positional identity in this region.

A second PCI-purified preparation was subjected to lysyl-endopeptidase digestion and HPLC separation, which yielded multiple peptides. The major peak eluted at 31 min and was sequenced, demonstrating a single amino acid sequence homologous with residues 61–78 of bovine CPA (not shown). This peptide had 78% identity with bovine CPB and 55% identity with bovine CPA. Bovine CPA and CPB demonstrate 50% positional identity in this region.

The amino acid composition was determined for 100 pmol of a preparation of affinity-purified human mast cell carboxypeptidase which had been used for NH_2-terminal amino acid sequencing (Table IV). This composition is compared to published amino acid compositions of bovine pancreatic CPA and CPB, and rat mast cell CP. The overall amino acid composition does not characterize the enzyme as CPA or CPB-like.

Table II. Purification of Human Mast Cell CP from Skin

Purification step	Volume	Protein	Activity	Specific activity	Recovery	Purification
	ml	*mg*	*U*	*U/μg*	*%*	*-fold*
Skin extract	150	525	14,000	0.027	100	1
Concentrated affinity eluate	1.5	0.23	8,370	36.4	60	1,348

* 1 U is defined as the amount of enzyme activity generating 1 nmol des-Leu AI/min.

Functional relationship to CPA and CPB. Initial studies demonstrated that mast cell CP and bovine CPA hydrolyzed AI and suggested that the mast cell enzymes's activity was A-like with respect to this substrate. Since the structural data indicated that the mast cell enzyme is more similar in sequence to CPB than CPA, additional substrates known to be hydrolyzed by CPA or CPB were examined to determine whether the mast cell CP was A-like with respect to the cleavage of these substrates. The activities of the human mast cell enzyme, bovine CPA, and bovine CPB were standardized with respect to hydrolysis of AI, in terms of the amount of des-Leu^{10} AI generated as product (see Methods), and the amount of each enzyme which yielded 0.07 U of AI hydrolyzing activity was utilized in the following experiment. Human mast cell CP and CPA generated only des-Leu^{10} AI from AI, and 0.14 and 0.35 U of each enzyme did not further hydrolyze des-Leu^{10} AI.

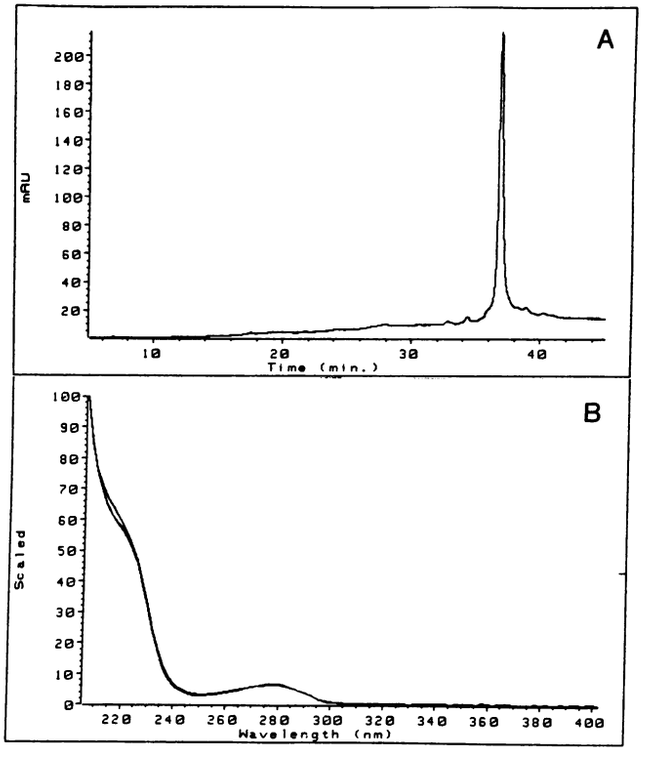

Figure 2. (*A*) HPLC chromatogram of PCI affinity-purified human mast cell CP. The single peak had a retention time of 36.746 min. (*B*) Spectral analysis from 210–400 nm taken at three points within the HPLC peak at 36.603, 36.740, and 36.873 min. The figure represents a composite of the three spectra which completely superimpose to appear as one curve.

However, in the case of CPB, 0.035 and 0.07 U of enzyme activity also generated 9 and 16 pmol AII per minute. Since des-Leu^{10} AI was a possible substrate for CPB because it has a basic COOH-terminal amino acid, histidine, its hydrolysis by each enzyme was examined under identical conditions used for AI. Human mast cell CP and bovine CPA caused no detectable hydrolysis of des-Leu^{10} AI. However, CPB generated 82 and 159 pmol AII/min, indicating that CPB activity hydrolyzed the carboxyterminal Phe^{8}-His^{9} bond of des-Leu^{10} AI. To further characterize human mast cell CP using a substrate with another basic carboxy terminus (arginine) known to be hydrolyzed by CPB, each enzyme was incubated with 50 μM bradykinin for 30 min. CPB completely hydrolyzed 25 nmol of bradykinin to a single product, des-Arg^{9} bradykinin. However, CPA (110 U) and human mast cell carboxypeptidase (330 U) caused no detectable hydrolysis of bradykinin. Since des-Arg^{9} bradykinin has a COOH-terminal phenylalanine, and is therefore a possible substrate for CPA and mast cell CP, it was evaluated as a possible substrate for these enzymes. CPA (56 U) hydrolyzed 30% of des-Arg^{9} bradykinin, but mast cell CP (70 and 175 U) caused no detectable hydrolysis of this substrate. Next, the hydrolysis by mast cell carboxypeptidase of two synthetic NH_2-substituted dipeptides, N-CBZ-Gly-Phe and N-CBZ-Gly-Arg, substrates of CPA and CPB, respectively, was examined using 0.5 mM substrate. The hydrolysis of N-CBZ-Gly-Phe was dose dependent, with initial velocities of 0.005–0.31 A_{224}/min using 25–125 U of enzyme. Equivalent amounts of mast cell carboxypeptidase caused no hydrolysis of N-CBZ-Gly-Arg. Thus, human mast cell carboxypeptidase is A-like with respect to its ability to hydrolyze AI and N-CBZ-Gly-Phe, but under the conditions of these experiments is distinguished from CPA by its inability to hydrolyze des-Arg^{9} bradykinin.

Functional quantitation of CP in skin mast cells. The association of CP with mast cells from skin, and determination of

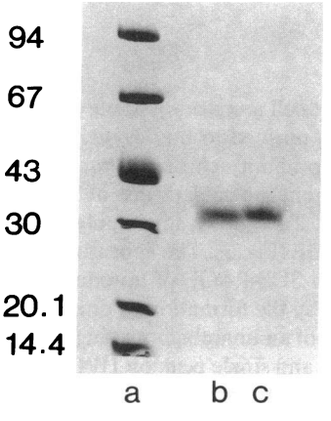

Figure 3. SDS-PAGE of 2 μg of PCI affinity-purified human mast cell carboxypeptidase. Lane *a*, molecular mass markers in kilodaltons; lane *b*, unreduced CP; lane *c*, reduced CP. Not visible in this figure (lane *b*) is a faint ≈ 57,000-D band seen only in unreduced material.

Table III. NH_2-Terminal Amino Acid Sequences of Human Mast Cell, and Human and Bovine Pancreatic CPA and CPB

Residue no.	1 10
	* * * * *
Human mast cell‡	Ile-Pro-Gly-Arg-His-Ser-Tyr-Ala-Lys-Tyr-
Bovine CPB	Thr-Thr-Gly-His-Ser-Tyr-Glu-Lys-Tyr-
Human CPB	Ala-Thr-Gly-His-Ser-Tyr-Glu-Lys-Tyr-
Human CPA	Ser-Thr-Asp-Thr-Phe-Asn-Tyr-Ala-Thr-Tyr-
Bovine CPA	Ala-Arg-Ser-Thr-Asn-Thr-Phe-Asn-Tyr-Ala-Thr-Tyr-
Residue no.	11 20
	* * * * * * * * *
Human mast cell	Asn-Asn-Trp-Glu-Lys-Ile-Val-Ala-Trp-Thr-
Bovine CPB	Asn-Asn-Trp-Glu-Thr-Ile-Glu-Ala-Trp-Thr-
Human CPB	Asn-Asn-Trp-Glu-Thr-Ile-Glu-Ala-Trp-Thr-
Human CPA	His-Thr-Leu-Asp-Glu-Ile-Tyr-Asp-Phe-Leu-
Bovine CPA	His-Thr-Leu-Asp-Glu-Ile-Tyr-Asp-Phe-Met-
Residue no.	21 28
	* *
Human mast cell	Glu-Lys-Met-Met-Asp-Lys-Tyr-Pro
Bovine CPB	Glu-Gln-Val-Ala-Ser-Glu-Asp-Pro
Human CPB	Gln ..... §
Human CPA	..... §
Bovine CPA	Asp-Leu-Leu-Val-Ala-Glu-His-Pro

Previously published sequences for human CPA and CPB (22), bovine CPA (23) and CPB (24). * Identical amino acid residues between human mast cell CP and CPB. ‡ The NH_2-terminal amino acid of mast cell CP (Ile) is labeled residue 1. This is a tentative assignment as there was a free amino acid background (mostly Thr) in the first cycle. § Published sequences end at the last amino acid noted above.

the specific activity of highly purified preparations of mast cell CP, permit initial estimates of the amount of enzyme in human mast cells from skin. Dispersed mast cells from adult facial skin, and adult and neonatal foreskins were obtained and examined for CP activity. The amount of CP activity was 17 U±8 (SD) per 10^6 neonatal foreskin mast cells (n = 5), 173 U±45/10^6 mast cells from adult facial skin (n = 3), and 564 U±101/10^6 adult foreskin mast cells (n = 2). Using the specific activity of our most active PCI affinity-purified preparation (36.4 U/μg protein), the carboxypeptidase enzyme content in these preparations is estimated to be ~ 0.5, 5, and 16 μg/10^6 mast cells from neonatal foreskin, adult facial skin, and adult foreskin, respectively.

Discussion

Human mast cell CP is a mast cell secretory granule enzyme (9). Human mast cell CP was purified to homogeneity from human skin using a single-step affinity chromatography procedure (Fig. 1). It has an apparent molecular mass of 30,000–35,000 D by gel filtration (Fig. 2), and is a single polypeptide chain of 34,500 D by SDS-PAGE (Fig. 3). The appearance of a minor band of ≈ 57,000 d on SDS-PAGE of unreduced enzyme is most likely explained by the formation of dimers and not by a contaminant, in light of an unambiguous single NH_2-terminal amino acid sequence and single peak on HPLC analysis. This enzyme appears to be a zinc-metalloexopeptidase based on its inhibition by *O*-phenanthroline, EDTA, and 8-hydroxyquinoline (6, 9). It is active at neutral to alkaline pH, and is inhibited by PCI (9). Studies comparing the kinetic constants of human mast cell CP with bovine pancreatic CPA and CPB, using a series of synthetic di- and tripeptide substrates substituted at the COOH-terminal amino acid, will be required to quantitatively compare the substrate specificities of the three enzymes. However, like CPA, human mast cell carboxypeptidase hydrolyzes AI and N-CBZ-Gly-Phe, and does not hydrolyze des-Leu^{10} AI, bradykinin, and N-CBZ-Gly-Arg. It is unlike CPA under the conditions used in these experiments in its inability to hydrolyze des-Arg^9 bradykinin. Therefore, these studies indicate that the activity of mast cell CP is similar but not identical to pancreatic CPA.

Amino acid sequence data for pancreatic CP show that CPA and CPB arose from a common ancestor by gene duplication (24, 25). The amino acid sequences of two portions of mast cell CP demonstrate significant positional identity with CPB (Table III). However, the functional and structural differences between mast cell carboxypeptidase and the well-characterized pancreatic CPs demonstrate that the human mast cell enzyme is neither CPA nor CPB, and should be designated mast cell CP. While the data suggest that human mast cell CP may be homologous to the pancreatic CPs, CPA and CPB, further amino acid and nucleotide sequence data will be required to conclusively confirm this, and to elucidate the basis of the substrate specificities of these enzymes (25).

Table IV. Amino Acid Compositions of Human and Rat Mast Cell, and Bovine Pancreatic CPs

Amino acid	Mast cell CP		Bovine pancreatic	
	Human*	Rat‡	CPA‡	CPB‡
	mol amino acid/mol protein			
Alanine	18	17	21	22
Arginine	18	16	11	13
Aspartic/NH_2	36§	30	29	28
Glutamic/NH_2	24	18	25	25
Glycine	17	19	23	22
Cysteine	4‖	5	2	7
Histidine	8	9	8	7
Isoleucine	25	20	21	16
Leucine	22	21	23	21
Lysine	30§	27	15	17
Methionine	7	7	3	6
Phenylalanine	14	13	16	12
Proline	15	15	10	12
Serine	20	28	32	27
Threonine	20	19	26	27
Tryptophan	—¶	9	7	8
Tyrosine	18	14	19	22
Valine	16	15	16	14

* Determined by ninhydrin analysis.
‡ Reported by Everitt and Neurath (6).
§ The ninhydrin and picotag data differ markedly for aspartic acid and lysine with values of 25 and 23, respectively by picotag.
‖ Determined by picotag analysis as described in Methods.
¶ Residues destroyed during acid hydrolysis.

Human mast cell CP is likely to be orthologous to mast cell CPs of other species. A CP activity was first identified in rat mast cells (6, 8), was thought to be CPA-like, and was recently identified in mouse mast cells (7). Functionally CPA-like CP has been purified from rat peritoneal mast cells (6), and from rat skeletal muscle mast cells (8). While the amino acid sequences of the rodent mast cell CPs are not known, these enzymes have a similar molecular mass, pH optimum, inhibitor profile, and amino acid composition to the human mast cell enzyme. The purified rat peritoneal mast cell CP was CPB-like with respect to half-cystine and methionine content, and differed from CPA with regard to K_m and K_{cat} values for N-CBZ-Gly-Phe (6). The purified rat skeletal muscle CP differed from bovine pancreatic CPA in thermal lability and stability of the purified holo and apoenzyme (8).

Quantitative studies using a functional standard demonstrate that the content of CP in adult foreskin mast cells (16 $\mu g/10^6$ cells) falls between those of tryptase (35 $\mu g/10^6$ cells) and chymase (4.5 $\mu g/10^6$ cells) (26). Human mast cell CP activity is higher in mast cells from human skin vs. lung (9), and in adult vs. neonatal skin. This distribution of CP is similar to that of chymase, a marker for a subset of tryptase-positive, chymase-positive mast cells (TC type) abundant in human skin (26, 27). TC type mast cells are relatively uncommon in human lung, which contains primarily tryptase-positive, chymase-negative (T type) mast cells (27). Therefore, one may speculate that CP, like chymase, may also be a biochemical marker for the TC type of mast cell abundant in human skin. Recent observations (7) suggest that mast cell CP may be a differentiation marker in the mouse. The biologic role for CP and other mast cell enzymes is unclear. It is possible that these enzymes confer a unique biochemical potential to mast cells in skin which may be reflected in disorders mediated by mast cells in skin in which tissue destruction is a significant aspect (28).

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