Separation and partial characterization of proteinases with substrate specificity for basic amino acids from human MOLT-4 T lymphocytes: identification of those inhibited by variable-loop-V3 peptides of HIV-1 (human immunodeficiency virus-1) envelope glycoprotein

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The V3 loop of the HIV (human immunodeficiency virus)-1 envelope glycoprotein gp120 likely plays a role in HIV-1 infectivity. Although the amino acid sequence of the V3 loop is hypervariable, it contains a conserved region, Gly-Pro-Gly-Arg, that shows similarity to the active-site Gly-Pro-Cys-Arg sequence of inter- α -trypsin and trypstatin proteinase inhibitors. The purpose of the present work was to identify proteinases recognizing substrates with basic amino acids in the P1 substrate site that are present in MOLT-4 cells, a human CD4-positive T helper lymphocyte cell line, and to characterize these enzymes in terms of substrate, pH and ionic-strength preferences, size and susceptibility to various inhibitors, including 24- and 36-amino-acid-long V3 loop peptides. Extraction of MOLT-4 cells at low ionic strength solubilized nearly all of the trypsin-like activity, which was separable into five peaks of activity by chromatography on Mono-Q: Peaks 1, 2a, 2b, 3 and 4. All showed a neutral pH optimum, and all except Peak 4 showed optimal activity at high ionic strength. Peak 1 preferred Tos-Gly-Pro-

Arg, *p*-nitroanilide (-pNA) substrate; Peaks 2–4 preferred benzyloxycarbonyl-Val-Leu-Gly-Arg-pNA. Peak 1, a zinc-dependent enzyme with serine and histidine in the active site, exhibited an M_r of 75000 on Superose 12 and was poorly inhibited by V3 loop peptides. Peak 2 contained two overlapping peaks, called 2a and 2b, that exhibited properties of zinc-dependent metalloproteinases. Gel filtration of Peak 2 activities revealed a major peak of activity at 81 kDa and a shoulder centred at 240 kDa. Each was modestly inhibited by V3 loop peptides. Peak 3, a zinc-dependent proteinase, exhibited a molecular mass of 100 kDa by gel filtration and was particularly sensitive to inhibition by V3 loop peptides. Peak 4 exhibited a molecular mass of 1100 kDa by gel filtration and was not inhibited by V3 loop peptides. None of these enzymes could be classified as mast-cell tryptase, and material in MOLT-4 cells cross-reactive with anti-(human tryptase) antibodies was not detected. Whether any of the MOLT-4 proteinases described in this study play a role in HIV-1 infectivity remains to be examined.

INTRODUCTION

Human immunodeficiency virus-1 (HIV-1) binds to cells via an interaction between its envelope glycoprotein gp120 and a CD4 molecule present on the surface of T lymphocytes and monocytes (Maddon et al., 1986; Kowalski et al., 1987; Finbloom et al., 1991). One region of gp120 critical for its interaction with the CD4 receptor has been localized to amino acids 397–439 (Lasky et al., 1987). However, the principal neutralizing determinant of HIV-1 lies within a loop formed by a disulphide bridge between two invariant cysteine residues at positions 303 and 338. Antibodies against this variable V3 loop neutralize virus infectivity (Gorny et al., 1991; Palker et al., 1988; Skinner et al., 1988; La Rosa et al., 1990, 1991). In the case of monocytes, the critical determinant of gp120 for productive infection is a region which is upstream from the defined CD4-binding domain and encompasses the entire V3 loop domain (O'Brien et al., 1990; Hwang et al., 1991; Westervelt et al., 1991). There are also several reports available which demonstrate a CD4-independent pathway for HIV-1 infection, e.g. in human fibroblastoid cells (Tateno et al., 1989), neuronal cells (Harouse et al., 1989; Li et al., 1990) and hepatoma cells (Cao et al., 1990).

Although the amino acid sequence of the V3 loop of gp 120 is variable, the tip of this loop is highly conserved (Gly-Pro-Gly-

Arg) (La Rosa et al., 1990, 1991) and shows similarity to the sequence Gly-Pro-Cys-Arg that resides in the active site of inter- α -trypsin inhibitor and the rat mast-cell tryptase inhibitor called trypstatin (Hattori et al., 1989; Koito et al., 1989). Under certain cell culture conditions, cleavage of the V3 loop between Gly-Pro-Gly-Arg and Ala has been observed (Stephens et al., 1990), indicating its susceptibility to hydrolysis by enzymes with trypsin-like substrate specificity. Synthetic peptides corresponding to the V3 loop of gp120, human urinary trypsin inhibitor and trypstatin, have been shown to prevent syncytium formation of MOLT-4 cells caused by HIV-1 infection. In contrast, high concentrations (up to 3 mM) of *p*-aminodiphenylmethanesulphonyl fluoride, leupeptin, aprotinin and pepstatin were inefficient at preventing syncytium formation (Hattori et al., 1989; Koito et al., 1989), suggesting that proteinase(s) other than serine, cysteine and aspartic class proteinases are involved in HIV-1 infection. Proteolytic cleavage of virus surface components has been shown to augment infection by retroviruses other than HIV-1 (Andersen, 1987; Anderson and Skov, 1989; McClure et al., 1990), further elevating the possibility that this mechanism may play a role in HIV-1 infection.

Recently Kido et al. (1990) reported the purification of a proteinase named tryptase TL-2 from cell membranes of MOLT-4 cells (clone 8), a human CD4⁺ T lymphocyte line. The native

Abbreviations used: Boc, butoxycarbonyl; pNA, *p*-nitroanilide; HIV, human immunodeficiency virus; Bz, benzoyl; SBTI, soybean trypsin inhibitor; LBTI, lima-bean trypsin inhibitor; iPr₂P-F, di-isopropyl fluorophosphate; Tos-Lys-CH₂Cl, *N*-*p*-tosyl-L-lysylchloromethane ('TLCK'); DMF, *N,N*-dimethylformamide; pHMB, *p*-hydroxymercuribenzoate

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enzyme was extracted from membrane preparations by sonication at neutral pH in low-ionic-strength buffer. Tryptase TL-2 exhibited a molecular mass of 198 kDa by gel filtration and had two subunits of 32 kDa and four subunits of 28 kDa. The enzyme was strongly inhibited by 0.08 μ M gp120 and recognized the V3 loop of the molecule (Kido et al., 1991). Concentrations of synthetic peptides with the internal GPGR or GPCR (one-letter code) sequence approx. 100-fold higher were needed to cause the same magnitude of inhibition. Also, tryptase TL-2 was immunologically reactive with a polyclonal antibody raised in rabbits against rat tryptase. This antibody bound to the cell surface of MOLT-4 cells and inhibited syncytium formation caused by HIV-1. Tryptase TL-2 was classified as a serine proteinase (Kido et al., 1990). However, the failure of various serine-proteinase inhibitors to prevent HIV-1 infection argues against a crucial role for tryptase TL-2 in HIV-1 infection (Koito et al., 1989).

The purpose of the present study was to identify the proteases present in MOLT-4 cells capable of recognizing substrates at basic residues and to evaluate their susceptibilities to inhibition by V3 loop peptides containing the GPGR sequence. The results show that these cells contain low but detectable levels of at least five chromatographically separable proteinases that recognize Arg in the P1 position. One of these enzymes, a metalloproteinase, is particularly sensitive to inhibition by V3 peptides and may play a role in HIV-1 infectivity.

MATERIALS AND METHODS

Materials

Benzoyl-Arg *p*-nitroanilide (Bz-Arg-pNa), Bz-Ile-Glu-Gly-Arg-pNa, Tos-Gly-Pro-Lys-pNa, butoxycarbonyl (Boc)-Leu-Gly-Arg-pNa, α_1 -antitrypsin, soybean trypsin inhibitor (SBTI), lima-bean trypsin inhibitor (LBTI), phosphoramidon, aprotinin, leupeptin, di-isopropyl fluorophosphate (iPr₂P-F), *N*-*p*-tosyl-L-lysyl-chloromethane (Tos-Lys-CH₂Cl; 'TLCK'), Na₂EDTA, 1,10-phenanthroline, *p*-hydroxymercuribenzoate (pHMB), pepstatin A, E-64, cystatin (egg white), dithiothreitol, benzamidine, HIV-1 envelope protein (gp120) fragment 307–330 (Asn-Asn-Thr-Arg-Lys-Ser-Ile-Arg-Ile-Gln-Arg-Gly-Pro-Gly-Arg-Ala-Phe-Val-Thr-Ile-Gly-Lys-Ile-Gly, V3 peptide 24), Hepes, Tris, Mes, sodium acetate, glycerol, CaCl₂, ZnCl₂, BSA, cathepsin B and all cell-culture media and chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Boc-Val-Leu-Gly-Arg-pNa was a product of Bachem Feinchemikalien EG (Bubendorf, Switzerland). Tos-Gly-Pro-Arg-pNa and Bz-Pro-Phe-Arg-pNa were from Boehringer Mannheim G.m.b.H. (Mannheim, Germany). Recombinant HIV-1 gp120 (100 μ g/ml in PBS) was provided by American Bio-Technologies (Cambridge, MA, U.S.A.). A synthetic peptide corresponding to the V3 loop region from BH10 of HIV-1 (Ratner et al., 1985) (Cys-Thr-Arg-Pro-Asn-Asn-Asn-Thr-Arg-Lys-Ser-Ile-Arg-Ile-Gln-Arg-Gly-Pro-Gly-Arg-Ala-Phe-Val-Thr-Ile-Gly-Lys-Ile-Gly-Asn-Met-Arg-Gln-Ala-His-Cys, V3 peptide 36) was synthesized by SmithKline Beecham Pharmaceuticals (King of Prussia, PA, U.S.A.). Mono-Q anion-exchange column, Superose 12 gel-filtration column and Gel Filtration Calibration Kits (high and low molecular mass) from Pharmacia LKB Biotechnology (Uppsala, Sweden) and heparin-agarose from Gibco BRL (Gaithersburg, MD, U.S.A.) were purchased as indicated. Milli-Q (Millipore) water was used throughout this study. Goat anti-(human tryptase) IgG (Castells et al., 1987) and murine monoclonal anti-[active tryptase (B2)] (Schwartz et al., 1990) and anti-[inactive tryptase (G3)] (Irani et al., 1989) antibodies were prepared as described previously.

Human skin tryptase was purified by immunoaffinity chromatography as described previously (Schwartz et al., 1990).

Cell culture

MOLT-4 cells (T lymphoblast cell line, repository no. GM02219D) were purchased from American Type Cell Culture (CRL 1582) (Rockville, MD, U.S.A.). The cells were cultured in RPMI-1640 medium supplemented with 10% Controlled Process Serum Replacement-1 (CPSR-1; Sigma Chemical Co.), 2 mM L-glutamine, 100 units/ml penicillin G and 0.1 mg/ml streptomycin at 37 °C with 6% CO₂. Cells were harvested by centrifugation in a clinical centrifuge at room temperature, washed twice with 0.15 M NaCl containing 10 mM Tris/HCl buffer, pH 7.6, and then stored as a cell pellet at –70 °C.

Assays

The hydrolysis of each peptide pNa substrate was monitored with Bio-Tek EL-312 Bio-Kinetics Reader (Bio-Tek Instruments, Winooski, VT, U.S.A.) at a wavelength of 405 nm, using Falcon Pro-Bind 96-well assay plates (Beckton Dickinson, Lincoln Park, NJ, U.S.A.). Typically, 10–30 μ l of enzyme sample was mixed with 5 μ l of 20 mg/ml BSA (to prevent adsorption to plastic), 40 μ l of NaCl, buffer or inhibitor, as indicated, and 125–145 μ l of substrate, in a final volume of 200 μ l. Substrates were prepared as a 10 mM stock solution in 20% *NN*-dimethylformamide (DMF) and diluted 20-fold in 50 mM Hepes buffer prior to performing the enzyme-activity measurement. The linearity of the reactions for each enzyme was determined by monitoring the absorbance increase at intervals of 10–60 min for up to 3–5 h at room temperature, and reaction velocities were taken from the linear portion of the curve and at a time when less than 20% of substrate had been hydrolysed. Negligible spontaneous hydrolysis of substrates was detected over the time course of the reactions. Enzyme activities are expressed as units, where 1 unit of enzyme cleaves 1 μ mol of substrate/min. For determining apparent pH optima, sodium acetate, Mes, Hepes and Tris/HCl buffers were used at 50 mM in the range of pH 4.0–9.0. Inhibition assays were performed by incubating enzyme samples with substrate and inhibitor for about 30 min at room temperature, after which the linear reaction velocity was measured as described above. For assessment of inhibition of tryptase (15 ng/ml) by V3 peptides (0–20 μ M), 0.1 mM Tos-Gly-Pro-Lys-pNa was used as substrate in 50 mM Hepes/20 mM NaCl/heparin (10 μ g/ml)/BSA (0.2 mg/ml), pH 7.6. The rate of cleavage was assessed within 10 min of combining the peptides with tryptase. Protein concentrations were determined with the BCA Protein Assay Reagent (Pierce, Rockford, IL, U.S.A.), using BSA as the standard (Smith et al., 1985; Redinbaugh and Turley, 1986).

Isolation of proteinase activities

Frozen MOLT-4 cells (usually 1×10^9 cells) were thawed, suspended at 4 °C in 20 mM Tris/HCl buffer, pH 8.0, containing 5 mM CaCl₂ (10^8 cells/ml), and then sonicated on ice with a Model W-225R cell disruptor (Heat Systems–Ultrasonics, Plainview, NY, U.S.A.) having a 3 mm-diameter microtip attached, for 30 $\times$ 0.5 s pulses at power 2. The presence of CaCl₂ in the extract resulted in a large precipitate that was cleared by centrifugation in a Sorvall RC5C centrifuge (du Pont, Wilmington, DE, U.K.) equipped with a SS-34 rotor (40000 *g* for 60 min at 4 °C). The precipitate was washed with the same low-salt buffer, centrifuged, and then re-extracted with 1 M NaCl in 20 mM Tris/HCl buffer, pH 8.0, to determine whether

any proteinase activities were present. The supernatant of the low-salt sonicated material was adjusted to 80 mM NaCl and 10% glycerol in 20 mM Tris/HCl buffer, pH 8.0, and applied to a heparin-agarose column (1.4 × 6.0 cm) equilibrated with the same buffer. The heparin-agarose column was washed with 2 column vol. of loading buffer and bound material was eluted with 1 M NaCl in glycerol/Tris buffer. The effluent contained nearly all of each proteinase activity and was loaded directly on to the Mono-Q column.

The Mono-Q column was connected to a Perkin-Elmer (Norwalk, CT, U.S.A.) h.p.l.c. system (410 BIO pump, LC-95 UV/VIS detector and LCI-100 integrator), and equilibrated with 80 mM NaCl containing 10% glycerol and 20 mM Tris/HCl buffer, pH 8.0. Effluent fractions from the Heparin-agarose column were loaded and the bound protein was washed with 20 ml of equilibration buffer and eluted with a slightly concave gradient (program no. 1.5, Perkin-Elmer) from 80 mM to 1 M NaCl. Each collected fraction (0.25 ml) was tested for proteinase activity using 0.2 mM Tos-Gly-Pro-Arg-pNA and Boc-Val-Gly-Arg-pNA as the substrates at pH 7.6. Each peak of enzyme activity was pooled, concentrated by ultrafiltration with Centricon 10 microconcentrators (Amicon, Danvers, MA, U.S.A.) and injected into the calibrated Superose 12 gel-filtration column equilibrated with 1 M NaCl and 10% glycerol in 20 mM Tris/HCl buffer, pH 8.0, at a flow rate of 0.5 ml/min. Each eluted fraction (0.2 ml) was tested for proteinase activity as above. Molecular masses for each proteinase were estimated from their elution volume in relation to the calibration standard curve.

RESULTS

Extraction of proteinase activities

The supernatant of the low-salt-sonicated MOLT-4 cells was found to contain proteolytic activities (mean ± S.D.) of 8.7 ± 3.7 and 87 ± 21 μ -units/ 10^6 cells with the respective substrates Tos-Gly-Pro-Arg-pNA and Boc-Val-Leu-Gly-Arg-pNA, 0.2 mM each in 0.3 M NaCl/40 mM Hepes, pH 7.6. The subsequent high-salt extract contained less than 1% of the activity detected in the low-salt extract. The addition of CHAPS prior to sonication also failed to yield additional proteolytic activity. Consequently, the MOLT-4 cell pellets were routinely sonicated and extracted with 20 mM Tris/HCl buffer, pH 8.0, containing 5 mM CaCl_2 . This technique was similar to that used by Kido et al. (1990) to extract trypsin TL-2 from membrane preparations, but in the present case was applied to entire cell preparations.

Chromatography of MOLT-4 proteinases

Heparin-agarose chromatography was used as a negative selection step. Approx. 40% of the protein, including histones, was removed, whereas 82% of the activity cleaving Boc-Val-Leu-Gly-Arg-pNA and 72% of the activity cleaving Tos-Gly-Pro-Arg-pNA were recovered in the effluent. In the 1 M NaCl eluate less than 3% of the Tos-Gly-Pro-Arg-pNA- and less than 1% of the Boc-Val-Leu-Gly-Arg-pNA-cleaving activities were detected.

Figures 1(a) and 1(b) show typical elution profiles for Mono-Q, using effluent fractions from Heparin-agarose as the starting material. Total yields were 60% for Tos-Gly-Pro-Arg-pNA (Figure 1a) and 30% for Boc-Val-Leu-Gly-Arg-pNA (Figure 1b)-cleaving activities. No activity could be detected in effluent and wash fractions. The eluate showed five peaks of enzyme activity, labelled 1 (0.16 M NaCl), 2 (0.2 M NaCl), 3 (0.27 M

NaCl) and 4 (0.36 M NaCl). Essentially the same pattern of proteinase activities was observed if heparin-agarose chromatography was omitted, though specific activities were less. Peak 2, at 0.4 M NaCl, shows a shoulder. That portion eluted at lower ionic strength (0.19 M NaCl) will hereafter be called 'Peak 2a', whereas that portion eluted at higher ionic strength (0.21 M NaCl) will be called 'Peak 2b'.

Peak 1 enzyme activity was approx. 3-fold higher at 0.4 M NaCl than at 0.01 M NaCl. At 0.4 M NaCl this enzyme showed an apparent pH optimum of 7.4 (Figure 2). Of six substrates examined with Arg in the P1 position, only Tos-Gly-Pro-Arg-pNA was substantially hydrolysed (Table 1). Reactions proceeded for at least 4 h in a linear fashion or until 50% of the substrate had been consumed. Gel-filtration chromatography of Peak 1 enzyme on Superose 12 revealed a single peak of enzyme activity with a yield of 59% at an apparent molecular mass of 75 kDa that overlapped with two peaks of protein (Figure 3).

Figure 1 Mono-Q anion-exchange chromatography at pH 8.0

The effluent fractions from heparin-agarose (corresponding to 1×10^9 MOLT-4 cell-equivalents) were injected into the Mono-Q column at a flow rate of 0.5 ml/min. Eluted fractions (0.25 ml) were tested for tryptic enzyme activity using 0.2 mM Tos-Gly-Pro-Arg-pNA (GPR) (a) and Boc-Val-Leu-Gly-Arg-pNA (VLGR) (b) as substrates at pH 7.6. ●, Eluted with 0.4 M NaCl; ○, eluted with 0.02 M NaCl.

Figure 2 Determination of optimal pH for expression of Mono Q-derived Peaks 1, 3, and 4 of enzyme activity and for Mono Q/Superose-12-derived Peaks 2a and 2b

Tos-Gly-Pro-Arg-pNA was used at 0.4 M NaCl for Peak 1. Boc-Val-Leu-Gly-Arg-pNA was used at 0.4 M NaCl for Peaks 2a and 2b, 0.3 M NaCl for Peak 3 and 0.03 M NaCl for Peak 4.

Table 1 Substrate specificities of Peaks 1, 2b, 3 and 4 (see Figures 1 and 4)

Each activity peak was pooled as indicated in Figures 1 and 4. Enzyme activity was measured under optimal assay conditions, using 0.2 mM substrate. Activity is expressed as a percentage of that obtained with the best substrate. Bovine trypsin, as a control, hydrolysed all the substrates efficiently.

Substrate	Activity			
	Peak ... 1	2b	3	4
Bz-Arg-pNA	0	0	0	0
Bz-Pro-Phe-Arg-pNA	1	14	1	2
Bz-Ile-Glu-Gly-Arg-pNA	0	0	0	4
Tos-Gly-Pro-Arg-pNA	100	14	4	3
Boc-Leu-Gly-Arg-pNA	1	15	3	79
Boc-Val-Leu-Gly-Arg-pNA	4	100	100	100

Peak 2 (Figure 1) consisted of two overlapping peaks of activity. Optimal activity for Peak 2 occurred at 0.4 M NaCl, under which conditions the reaction velocities were constant for at least 5 h. The portion of Peak 2 activity eluted first was barely detectable under conditions of low ionic strength (0.01 M NaCl), whereas the later portion of Peak 2 activity was approx. 70 % as active under these conditions. Peak 2 fractions were pooled and subjected to gel filtration on Superose 12 (Figure 4). The total yield of enzyme activity measured at 0.4 M NaCl was 62 %. One major peak of activity with a molecular mass of 81 kDa (Peak 2b) was detected and accounted for approx. 85 % of the recovered activity. This enzyme demonstrated a marked preference for

Figure 3 Gel-filtration chromatography of Mono-Q Peak 1

Peak 1 after Mono-Q chromatography (Figure 1) was concentrated, adjusted to 1 M NaCl and injected into the Superose 12 column at a flow rate of 0.5 ml/min. Collected fractions (0.2 ml) were tested for tryptic activity, using 0.2 mM Tos-Gly-Pro-Lys-pNA as the substrate at pH 7.6. Elution positions are shown for molecular-mass markers as follows: 1, Blue Dextran (2000 kDa); 2, ferritin (440 kDa); 3, catalase (232 kDa); 4, aldolase (158 kDa); 5, BSA (67 kDa); 6, ovalbumin (43 kDa); 7, chymotrypsinogen (25 kDa) and ribonuclease (13.7 kDa).

Figure 4 Gel-filtration chromatography of Mono-Q Peak 2

Peak 2 after Mono-Q chromatography (Figure 1) was subjected to chromatography on Superose 12 as in Figure 3. Collected fractions (0.2 ml) were tested for tryptic activity, using 0.2 mM Boc-Val-Leu-Gly-Arg-pNA as the substrate at pH 7.6. The fractions combined to obtain Peaks 2a and 2b are shown. For molecular-mass markers, see Figure 3.

Boc-Val-Leu-Gly-Arg-pNA (Table 1) and exhibited an apparent pH optimum of 7.4 at 0.4 M NaCl (Figure 2). A second region of activity (Peak 2a) was observed as a broad shoulder with an apparent molecular mass centred at 240 kDa, and exhibited an apparent pH optimum of 7.8 at 0.4 M NaCl (Figure 2).

Peak 3 (Figure 1) activity consistently contained the major portion of Boc-Val-Leu-Gly-Arg-pNA-hydrolytic activity. Expression of activity was relatively insensitive to ionic strength and varied less than 20 % over a range of NaCl concentrations from 0.03 to 0.8 M. At 0.4 M NaCl, Peak 3 activity was linear for at least 6 h or until 30 % of the substrate was consumed and

Figure 5 Gel-filtration chromatography of Mono-Q Peak 3

Peak 3 after Mono-Q chromatography (Figure 1) was subjected to chromatography on Superose 12 and processed as in Figure 4. For molecular-mass markers, see Fig. 3.

Figure 6 Gel-filtration chromatography of Mono-Q Peak 4

Peak 4 after Mono-Q chromatography (Figure 1) was subjected to chromatography on Superose 12 and processed as in Figure 4. For molecular-mass markers see Figure 3.

exhibited an apparent pH optimum of 7.4 (Figure 2). Of the six substrates tested, only Boc-Val-Leu-Gly-Arg-pNA was substantially hydrolysed by this enzyme (Table 1). Simply removing Val from the P4 position of this substrate resulted in a decline of hydrolytic activity of more than 30-fold. Chromatography of Peak 3 on Superose 12 gave an overall yield of activity of 23%, approx. 95% of which resided in one major peak at an apparent molecular mass of 100 (Figure 5). A small shoulder of activity also was observed with an apparent molecular mass centred at 257 kDa.

Peak 4 activity showed a marked sensitivity to variations in ionic strength; increasing the NaCl concentration with 0.03 to 0.4 M decreased the activity at least 5-fold. The Peak 4 enzyme exhibited an apparent pH optimum of 7.8 at 0.03 M NaCl

Table 2 Inhibition specificities for Peaks 1, 2a, 2b, 3 and 4 tryptic activities (see Figures 1 and 4)

Enzyme activities were measured under optimal assay conditions, using 0.2 mM Tos-Gly-Pro-Arg-pNA (Peak 1) or Boc-Val-Leu-Gly-Arg-pNA (Peaks 2a, 2b, 3 and 4) as the substrates. Values shown are the averages of duplicate determinations.

Inhibitor	Concn.	Activity				
		Peak ... 1	2a	2b	3	4
Control		100	100	100	100	100
iPr ₂ P-F	2 mM	0	98	84	100	89
Tos-Lys-CH ₂ Cl	1 mM	1	nd†	nd	11	51
Aprotinin	100 μg/ml	100	87	87	104	97
SBTI	100 μg/ml	102	103	94	99	101
LBTI	100 μg/ml	103	nd	nd	101	103
α ₁ -Antitrypsin	100 μg/ml	106	nd	nd	95	102
1,10-Phenanthroline	1 mM	0	0	0	0	130
EDTA	1 mM	88	87	41	58	99
Pepstatin A	0.1 mM	94	100	104	102	97
Benzamidine	1 mM	75	97	88	88	97
E-64	1 mM	74	101	95	90	87
Cystatin	100 μg/ml	83	nd	nd	93	89
Dithiothreitol	3 mM	66	nd	nd	12	111
pHMB	1 mM	0	nd	nd	4	0
Leupeptin	1 mM	7	32	59	50	0
CaCl ₂	5 mM	109	nd	nd	102	43
V3 peptide 24	40 μM*	61	83	28	55	95
V3 peptide 36	10 μM†	60	49	44	7	100
ZnCl ₂	1 μM	99	nd	nd	108	nd
	10 μM	40	nd	nd	73	nd
	100 μM	0	nd	nd	0	nd

* 100 μg/ml.

† 40 μg/ml.

‡ nd, not determined.

(Figure 2) and showed a rate of substrate cleavage that was constant for 1–2 h. The Peak 4 enzyme hydrolysed Boc-Leu-Gly-Arg-pNA almost as well as Boc-Val-Leu-Gly-Arg-pNA, but showed negligible activity with the other four substrates tested (Table 1). Gel-filtration chromatography on Superose 12 resulted in a single peak of activity with an apparent molecular mass of 1100 kDa at a yield of 29% (Figure 6).

Evaluation of proteinase class

The capacities of various inhibitors to affect each of the five proteinase activities shown in Figure 1 (Peaks 1, 3 and 4) and Figure 4 (Peaks 2a and 2b) are summarized in Table 2. Only Peak 1 activity (Mono Q) was substantially inhibited by both iPr₂P-F and Tos-Lys-CH₂Cl, and thereby best fits classification as a serine proteinase. It was, however, inhibited also by the metal chelator, 1,10-phenanthroline and by leupeptin and pHMB, but not markedly by other cysteine- or aspartic-proteinase inhibitors.

Activity Peaks 2 (Superose 12; 81 kDa and 240 kDa) and 3 (Mono Q) revealed similar profiles of inhibition. None was inhibited by iPr₂P-F, even at 20 mM. All of them were strongly inhibited by 1,10-phenanthroline, but only partially by EDTA and leupeptin. Typical inhibitors of cysteine (E-64 and cystatin) and aspartic (pepstatin A) proteinases showed little, if any, activity against these enzymes, but completely inhibited cathepsin B (50 μg/ml) when tested for potency. However, non-specific thiol-group reactive reagents (pHMB and dithiothreitol) clearly inhibited Peak 3 activity, suggesting the possible importance of intramolecular disulphide bonds. Tos-Lys-CH₂Cl, a non-specific

Figure 7 Effect of V3 peptide 36 on the tryptic enzyme activities separated by chromatography on Mono-Q

Enzyme activities were measured in 0.4 M NaCl at pH 7.6, using 0.2 mM Tos-Gly-Pro-Arg-pNA (a) and Boc-Val-Leu-Gly-Arg-pNA (b).

inhibitor of serine and cysteine proteinases that alkylates histidine residues, strongly reduced Peak 3 activity.

Peak 4 activity showed a different inhibition profile. Only pHMB and leupeptin inhibited essentially all activity, which suggests a cysteine-proteinase classification. Dithiothreitol and the zinc chelator 1,10-phenanthroline tended to increase activity over buffer control. Typical serine-, cysteine-, aspartic- or metallo-proteinase inhibitors were inefficient inhibitors of Peak 4 activity.

Effect of V3 loop peptides of gp120 on MOLT-4 proteinase activities

Two V3 peptides (both from BH10 sequence of HIV), one corresponding to amino acids 307–330 and the other to amino

acids 302–337, were tested for inhibitory activities against the pooled peaks of activity eluted from Mono-Q (Figure 1) and Superose 12 (Figure 4) (Table 2). The 36-amino-acid peptide was a more potent inhibitor of Peaks 1, 2a and 3 than the 24-amino-acid peptide. In Table 2, activities of the MOLT-4 proteinases are shown in the presence of a 4-fold lower concentration of the V3 peptide 36 than the V3 peptide 24. Peak 1 activity was reduced to approximately the same extent by both peptides, whereas peak 2a and peak 3 activities were substantially lower with the lower concentration of the V3 peptide 36. No inhibition of Peak 4 and modest inhibition of Peaks 1, 2a and 2b were found. Peak 3 was particularly sensitive to inhibition by both V3 peptides.

Because some cross-contamination likely occurs in pooled fractions of Peaks 1, 2 and 3 (Figure 1), activity in the presence of 0, 3 or 30 μ M V3 peptide 36 was assessed in all fractions eluted from Mono-Q containing these activities, both with Tos-Gly-Pro-Arg-pNA (Figure 7a) and Boc-Val-Leu-Gly-Arg-pNA (Figure 7b). On the basis of the profiles of inhibition, the Peak-3 enzyme was most susceptible to inhibition by 3 μ M V3 peptide (72–87% inhibition), whereas Peak 2 showed 14–36% inhibition and Peak 1 only 12% inhibition. 1,10-Phenanthroline (0.5 mM) inhibited totally Peak 1, 2 and 3 enzyme activities, whereas phosphoramidon (0.5 mM) showed no effect (results not shown).

The inhibitory effect of V3 loop peptide 36 on the catalytic activity of human skin trypsin was examined for comparison. V3 peptides caused 15% inhibition at 1 μ M V3 peptide, 70% at 3 μ M, 94% at 5 μ M and 100% at 20 μ M. From these values, the inhibitory concentration that causes a 50% decrease in 0.1 mM Tos-Gly-Pro-Lys-pNA-hydrolysing activity is about 2.4 μ M. After incubation for 80 min the rate of substrate hydrolysis increased, suggesting that the inhibitory effect is reversible, perhaps reflecting utilization of V3 peptide as a competitive substrate. Thus V3 peptide probably functions as a substrate for mast-cell trypsin and, as such, competes with other substrates.

DISCUSSION

This study provides the first comparative characterization of the neutral proteinase activities with specificity for substrates with basic amino acids at the P1 site that reside in a human CD4-positive T cell line. Molecular mass, substrate preference, dependence on pH and ionic strength and susceptibility to inhibition by various proteinase inhibitors, including two GP120 V3 loop peptides, were examined. These peptides contain the conserved Gly-Pro-Gly-Arg internal sequence of V3 loop peptide (LaRosa et al., 1990). Relative to human mast cells, MOLT-4 cells contain 10000–30000-fold less trypsin-like activity (Schwartz et al., 1987). Five proteinase activities in MOLT-4 T lymphocytes were extracted and chromatographically separated. Although the precise identification of each of these proteinases is lacking, at least some may have been identified previously in other cells and tissues.

Peak 1 activity cleaved Tos-Gly-Pro-Arg-pNA and exhibited an apparent molecular mass of 75 kDa. It was the only proteinase inhibited by iPr_2P-F and Tos-Lys-CH₂Cl, suggesting serine and histidine in the active site, and could be destabilized or inhibited by 1,10-phenanthroline, but not EDTA, suggesting dependence on zinc. However, unambiguous classification of Peak 1, as for the other proteinase activities, must await analysis of purified proteinase.

Although human CD8-positive cytotoxic T lymphocytes contain several granule serine proteinases, called 'Granzymes' (Jenne and Tschopp, 1989), there is relatively little information concerning proteinases in CD4-positive T-cells. Granzyme A, a

50 kDa homodimer, cleaves Tos-Gly-Pro-Arg-pNA and is released from CD8-positive T cells (Gershenfeld et al., 1988; Fruth et al., 1988); a similar enzyme was reported in CD4-positive T-lymphocytes upon activation via ligand binding to the T-cell receptor-CD3 complex (Krähenbühl et al., 1988). The substrate preference of the 75 kDa enzyme activity (Peak 1), described in this study, resembles that of granzyme A, but inhibition properties, pH optima and subunit patterns were clearly different. More relevant to the current study is the observation that a serine proteinase immunologically and biochemically related to mast-cell tryptase resides in human MOLT-4 lymphocytes and is involved in HIV-1 infection. Called tryptase TL-2 (198 kDa), it was purified from a MOLT-4 T-cell line and, like mast-cell tryptase (Schwartz et al., 1981; Harvima et al., 1988b), classified as a serine proteinase, both enzymes being capable of cleaving Tos-Gly-Pro-Arg-pNA (Kido et al., 1990; Harvima et al., 1988b). Mast-cell tryptase also cleaves Z-Gly-Pro-Arg-4-methoxy-2-naphthylamine *in situ* (Harvima et al., 1988a). An enzyme having a similar subunit structure (four 28 kDa and two 32 kDa subunits) has not as yet been detected in naturally occurring CD4- and CD8-positive T-lymphocytes (Fruth et al., 1987). This subunit structure also differs from tetrameric human mast-cell tryptase (Schwartz et al., 1981; Harvima et al., 1988b). Nevertheless, a polyclonal antibody prepared in rabbits against rat mast-cell tryptase recognized one of the tryptase TL-2-subunits, stained the surface of human MOLT-4 cells and inhibited infection of MOLT-4 cells with HIV-1 (Kido et al., 1990). However, in the present study, staining of MOLT-4 cell cytopins by immunohistochemistry or of MOLT-4 cells in suspension by immunofluorescence flow cytometry (Facsan; Beckton Dickinson) was not detected using several anti-(human tryptase) antibodies, including goat anti-(inactive tryptase) IgG, murine monoclonal anti-[inactive tryptase G3] IgG and murine monoclonal anti-[active tryptase (B2)] (results not shown). Thus an enzyme closely related to human mast-cell tryptase does not appear to be present in MOLT-4 cells. Furthermore, Peak 1 activity exhibited low sensitivity for inhibition by V3 peptides and did not hydrolyse Xaa-Xaa-Gly-Arg-pNA substrates that mimic the putative cleavage site in the V3 loop. This information, along with published data showing that inhibitors of proteinases such as leupeptin are ineffective at preventing HIV-1 infection *in vitro* (Hattori et al., 1989; Koito et al., 1989), limits the likelihood that Peak 1 activity is involved in the HIV-1 infection process.

The enzyme activities under Peaks 2a and 2b (190 kDa to 400 kDa shoulder, 81 kDa) and Peak 3 (100 kDa), based on inhibition profiles and neutral to slightly alkaline pH optima, distinguish these enzymes from Peaks 1 and 4 and indicate their dependence on zinc. Each also exhibited a highly restricted substrate specificity. Although the tetrapeptide Boc-Val-Leu-Gly-Arg-pNA was efficiently cleaved, the corresponding tripeptide with only Val removed was not efficiently cleaved, at least by the 81 kDa and 100 kDa enzymes. The other tetrapeptide tested, Bz-Ile-Glu-Gly-Arg-pNA, also showed minimal activity as a substrate. These results suggest that the substrate-binding pocket of these enzymes is highly selective and recognizes the peptide sequences of at least four amino acids with the P3 amino acid having a non-polar side-chain. Of potential interest is the Gly-Pro-Gly-Arg sequence in the V3 loop of GP120.

Several zinc-dependent metalloproteinases have been described on the cell surface of human cells. CD10 (Knapp et al., 1989) is a 100 kDa membrane-bound zinc-metalloproteinase expressed on most acute-lymphoblastic-leukemia cells and several normal human cells. It cleaves numerous peptide hormones at the amino side of hydrophobic residues (e.g. the Gly-Phe amide bond), and is strongly inhibited by 1 μ M phosphoramidon (Letarte et al.,

1988; Malfroy et al., 1988; Shipp et al., 1988; Jongeneel et al., 1989). In the present study the zinc-dependent activities under Peaks 1, 2 and 3 were resistant to inhibition by 500 μ M phosphoramidon. In addition, immunofluorescence-flow-cytometric analysis of MOLT-4 cells with monoclonal antibody against CD10 (Gen Trak, Plymouth Meeting, PA, U.S.A.) was also negative, indicating that CD10 did not account for any of the enzyme activities under examination. CD13 (Knapp et al., 1989) is a 150 kDa zinc-dependent metalloproteinase found on the surface of human myeloid cells and on many other non-haematopoietic cells. However, it functions as an aminopeptidase liberating N-terminal amino acids from oligopeptides (Look et al., 1989; Ashmun and Look, 1990), which is not consistent with the properties of the enzyme activities in the present study.

The V3 loop of gp120 is thought to interact with a cell-surface trypsin-like enzyme (Koito et al., 1989). Thus the V3 loop of gp120 may function as a substrate or an inhibitor of such an enzyme. In the case of mast-cell tryptase, the V3 peptide serves as both a potent inhibitor and substrate, as shown previously (Clements et al., 1991) and in the present study. V3 loop peptides may mimic their activity in the parent molecule. For example, the 36-amino-acid-long V3 peptide used in the present study has been shown to be more potent at inhibiting syncytium formation caused by HIV infection than the smaller V3 peptide with 24 amino acids (Koito et al., 1989). Interestingly, V3 peptide 36 was a more effective inhibitor of the trypsin-like activities in Peaks 1, 2a and 3 than V3 loop peptide 24. V3 loop peptide 24, at the concentration used, was more effective against Peak 2b. On the basis of substrate preference and inhibition studies, the 100 kDa proteinase activity in Peak 3 represents the enzyme with the greatest potential to interact with the V3 loop in gp120. The subcellular distribution of this enzyme in MOLT-4 cells is unknown. One might anticipate that, for a proteinase to affect HIV infectivity, it must reside on the cell surface. However, a particular membrane proteinase may be distributed in intracellular as well as extracellular compartments. Alternatively, a role for endosomal proteinases in the infectious process has not been ruled out. Since the amino acid sequence of the V3 loop of gp120 is hypervariable, and the V3 peptide 36 used in the present study represents only one HIV-1 clone (BH10), it is possible that different membrane-associated metalloproteinases play a role in determining HIV-1 tropism in T-lymphocytes, monocytes and other human cell types.

The 1100 kDa enzyme activity (Peak 4) was resistant to inhibition by V3 peptides 36 and 24. It was sensitive only to non-specific cysteine-proteinase inhibitors. Preliminary results indicate a complex subunit structure having several bands on SDS/PAGE near 27–32 kDa, which resembles the enzyme from MOLT-4 cells called tryptase TL-1 (Katunuma and Kido, 1990). However, both the Peak 4 enzyme(s) and tryptase TL-1 may be components of the multicatalytic endopeptidase called proteasome (McDermott et al., 1991).

When considering proteinases likely to interact with the V3 loop of gp120, special attention should be given to metalloproteinases. Several types of normal human cells are known to have biologically active zinc-dependent metalloproteinases on their cell surface. Proteolytic cleavage of virus surface components has also been found to augment other retrovirus-induced cell fusions (Andersen, 1987; Andersen and Skov, 1989; Koito et al., 1989; McClure et al., 1990) supporting this kind of mechanism for HIV infection. Also, gp120 appears to undergo conformational changes when bound with CD4 that make the V3 loop more susceptible to cleavage by thrombin, another enzyme recognizing Gly-Pro-Arg substrates (Sattentau and Moore, 1991; Clements et al., 1991). Whether the proteinases identified in the

present study are involved in retrovirus infection, particularly in HIV-1 infection, will require further study.

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