A Pre-aspartate-specific Protease from Human Leukocytes That Cleaves Pro-interleukin-1β

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Interleukin-1β is a 17.4-kilodalton hormone derived from a 33-kilodalton inactive precursor produced by monocytes. We used the precursor as a substrate to detect proteolytic activities in peripheral blood mononuclear cell-conditioned medium that might be involved in interleukin-1β processing. We found that the conditioned medium, following passage through DEAE-Sephacel, generates a biologically active fragment from the precursor that runs slightly higher than the mature hormone in sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The responsible activity behaved as a single protein in ion exchange chromatography. It was completely inhibited by metal ion chelators and not by inhibitors of serine, cysteine, or aspartate proteases, and it was dependent on both calcium (or magnesium) and zinc. The enzyme was not inhibited by three substrate-based metalloprotease inhibitors, phosphoramidon, benzoylcarboxyl-Gly-Leu-NH₂, and N-(2-carboxy-3-phenylpropionyl)-Leu-NH₂-terminal sequence analysis showed that cleavage of the precursor occurred between a histidine and an aspartate residue, and digestion of synthetic peptides indicated that the protease is specific for pre-aspartate cleavages.

EXPERIMENTAL PROCEDURES

Cell Culture and Preparation of Conditioned Medium—Human blood was obtained, and serum-free PBMC cultures were set up as described previously (10), except that the medium used was RPMI 1640 buffered with 10 mM Hepes (pH 7.4). The cells were stimulated with 1% phytohemagglutinin (Gibco or Sigma) for 18 h. The conditioned medium was harvested as described previously (10) and concentrated 10-20-fold on an Amicon DC-10 L concentrator. The concentrated material was diluted 10-fold with 10 mM Tris-Cl (pH 8.1) to reduce the molarity and then re-concentrated 10-fold. The final concentrate was then centrifuged at 4 °C for 30 min at 7,500 × g, and the supernate was passed through a 0.45-μm filter. For the experiment shown in Fig. 1, the material was then concentrated 20-fold further with an Amicon YM-10 membrane.

Protease Assay—Five microliters of pro-IL-1β (10–50 μg/ml, prepared as in Ref. 3) were incubated with 10 μl of the material being tested for protease activity. The incubation was carried out at 37 °C for 1 h and was terminated by addition of 15 μl of 2 × SDS sample buffer (11) followed by boiling for 5 min. SDS-polyacrylamide gel electrophoresis (11) was carried out with 14% polyacrylamide gels, and Western blots were carried out as described previously utilizing the IL-1β COOH-terminal-specific monoclonal antibody 16F5 (3). Mature IL-1β was prepared as described previously (10), and approximately 20 ng was used for Western blots.

Chromatography—All chromatographic procedures were carried out at 4 °C. The ion exchange resins were pretreated with 0.1% Triton X-100 and 10% fetal calf serum to reduce nonspecific adsorption of protein. Fractions were assayed for protease activity as described above, for protein concentration with the Bio-Rad protein assay and ovalbumin as standard, and for salt concentration with a conductivity meter. PBMC conditioned medium was prepared as described above and applied at 50 ml/h to a 10 × 5-cm column of DEAE-Sephacel (Pharmacia LKB Biotechnology Inc.) which had been equilibrated in 10 mM Tris-Cl buffer (pH 8.1). Material that flowed through the DEAE column was loaded at 30 ml/h onto a 30 × 1.6-cm column of blue agarose (Bethesda Research Laboratories) which had been equilibrated in 10 mM Tris-Cl buffer (pH 8.1). The column was washed with 3 column volumes of the equilibration buffer, and material was then eluted with an increasing linear gradient of NaCl ranging from 0 to 800 mM in 10 mM Tris-Cl buffer (pH 8.1) (3 column volumes). Thirty 5-ml fractions were collected.

Inhibitor Studies—Five microliters of a solution of the inhibitor, of the inhibitor plus divalent cation, or of an appropriate control solution (i.e. buffer or solvent without inhibitor) was added to 10 μl of protease, and the mixture was incubated for 10 min at 37 °C. Five microliters of pro-IL-1β was then added and the incubation was continued as described above. All inhibitors were obtained from Sigma except Z-Gly-Leu-NH₂, which was obtained from Vega. The protease used in these experiments had been purified through the DEAE and blue agarose steps described above. It was then diluted 5-fold in 10 mM Tris-Cl (pH 8.1) to reduce the salt concentration, and the diluted material was concentrated 20-fold in an Amicon Centricon 10 concentrator.

NH₂-terminal Sequencing—One milliliter of the material purified sequentially through DEAE and blue agarose (see above) was incu-

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‡ The abbreviations used are: IL-1β, interleukin-1β; PBMC, peripheral blood mononuclear cell(s); SDS, sodium dodecyl sulfate; PMSP, phenylmethylsulfonyl fluoride; HPLC, high pressure liquid chromatography; Z-, benzyloxycarbonyl; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; E-64, trans-epoxysuccinyl-L-leucylamido-(4-guanidino)buthane.
bated with 0.5 ml of pro-IL-1β (50 μg/ml) at 37 °C for 2 h. The sample was then dialyzed three times against 1 liter of H2O at 4 °C. After dialysis, the material was concentrated to dryness in a Speed-Vac and then dissolved in SDS sample buffer. Following SDS-polyacrylamide gel electrophoresis, proteins were transferred to a polyvinylidene difluoride membrane and stained with Coomassie Blue (12). The product of interest was cut out and analyzed for sequence in an Applied Biosystems model 470A protein sequencer.

Preparation of Peptides—Peptides were synthesized with an Applied Biosystems model 430A peptide synthesizer. The NH2 termini of the peptides were modified by the addition of either an acetyl group or 9-fluorenylethoxycarbonyl (Fmoc), and the COOH termini were amidated. After cleavage from the resin by HF, the peptides were applied to a Vydac C8 HPLC column (25 × 1.0 cm) and eluted with a gradient of acetonitrile in 0.1% trifluoroacetic acid.

Digestion of Peptides and Analysis of Products—Generally, 0.5 ml of peptide solution (approximately 5 mg/ml in 10 mM Tris-HCl (pH 8)) was mixed with 0.5 ml of protease solution, and the mixture was incubated at 37 °C. The protease used in these experiments was purified through the DEAE and blue agarose steps described above, and was pre-incubated for 15 min with 1 mM PMSF and 1 μg/ml leupeptin (E-64, 0.1 mM) to eliminate contaminating proteolytic activities that might have complicated the cleavage pattern. Fifty-microliter aliquots were removed from the incubation mixture periodically for analytical HPLC runs to monitor the progress of the reaction. When a substantial portion of the starting peptide had been cleaved (5–24 h into the incubation), the reaction was stopped by adding an equal volume of 10% (v/v) trichloroacetic acid. Precipitated protein was removed by centrifugation at 4°C and 10,000 × g.

RESULTS

Cleavage of Pro-IL-1β by PBMC Conditioned Medium—Conditioned medium from an 18-h phytohemagglutinin-stimulated PBMC culture was concentrated as described under “Experimental Procedures,” and a portion of it was applied to a DEAE-Sephacel column. Both the crude material and the DEAE flow-through were incubated with pro-IL-1β, and the incubation mixtures were then analyzed by Western blot, utilizing an antibody specific for the COOH terminus of IL-1β (3). The crude material generated a number of products including one that migrated close to but distinctly above mature IL-1β, while the DEAE flow-through produced a different set of fragments including one that ran slightly above the mature protein (Fig. 1). The remainder of this communication focuses on the analysis of the DEAE flow-through responsible for generating the product slightly larger than mature IL-1β (the “DEAE flow activity”).

Chromatography of DEAE Flow Activity—To determine if the DEAE flow activity was due to a single protease or to a combination of enzymes, we subjected the flow-through material to a number of chromatographic procedures. Fig. 2 shows the results with a blue agarose column: all the activity was retained and was subsequently eluted with 0.2–0.3 M NaCl in a single symmetrical peak. Similar results were obtained with other chromatographic materials, including phenyl-Sepharose, Polybuffer Exchanger 118 (Pharmacia LKB Biotechnology Inc.), and Procion Red-agarose. Gel filtration with Sephadex G-75 indicated a molecular mass of between 20 and 30 kilodaltons.

Inhibitor Sensitivity—Further characterization was carried out with a pool of the active fractions eluted from a blue agarose column. PMSF (1 mM), E-64 (0.1 mM), and pepstatin (5 μg/ml) had no effect on the activity, while 1 mM EDTA and 1 mM 1,10-phenanthroline completely inhibited it (Fig. 3). Full activity was restored to EDTA-treated material by the addition of 1.25 mM CaCl2 (Fig. 4); MgCl2 also fully restored activity at 1.25–2.5 mM (data not shown). CaCl2 was inhibitory at concentrations above 1.25 mM. Neither CaCl2 nor MgCl2 restored activity to the phenanthroline-treated protease, but ZnCl2 did so, at 23 μM; higher concentrations were inhibitory (Fig. 4). Three commonly used substrate-based inhibitors of metalloproteases, phosphoramidon, Z-Gly-Leu-NH2, and N-(2-carboxy-3-phenylpropionyl)-Leu, had little or no effect on the protease (Fig. 3).

Specificity of Cleavage—To determine where this protease cleaves the precursor to produce the fragment slightly larger than mature IL-1β, we sequenced the NH2-terminal amino acids of this product. This analysis indicated that cleavage occurred between histidine 115 and aspartate 116 of pro-IL-1β (see Fig. 6A for adjoining amino acids). This product was highly active in the EL4 6.1 C10 bioassay (3): incubation of the precursor with a preparation of the protease purified as shown in Fig. 2 resulted in a 14-fold increase in activity, and
we know from our earlier work that little of this activity was due to the larger products generated during the incubation (3).

To characterize further the specificity of the enzyme, two peptides were synthesized, composed of residues 109–121 and 103–113 of the precursor (with modified ends as described under "Experimental Procedures"). Each peptide was incubated with the partially purified protease, and the resulting fragments were separated by HPLC and analyzed for amino acid composition (Fig. 5). This analysis indicated that all the significant products resulted from cleavages at the amino side of the two aspartic acid residues in each peptide (Fig. 6, A and B).

DISCUSSION

We have identified a previously undescribed proteolytic activity from human peripheral blood mononuclear cells that appears to cleave proteins and peptides prior to aspartate residues. Most if not all known mammalian metalloendoproteases cleave before or after hydrophobic amino acids (13). The recovery of this activity in a single peak upon ion exchange chromatography suggests that it is due to a single protease. This conclusion is supported by the finding that metal chelators eliminate all of the activity, while inhibitors of non-metalloproteases have no effect. The failure of common substrate-based metalloprotease inhibitors to affect the activity further indicates a unique enzyme and is consistent with the unusual cleavage specificity indicated by the protein and peptide products of the protease.

We detected this activity by its ability to cleave pro-IL-1β near the mature IL-1β NH₂ terminus, and it is interesting that this enzyme is much more active with respect to pro-IL-1β than other proteases we have tested. Based on a maximal estimate of how much of the protease is present, it converts the precursor to lower molecular weight products at one-tenth of the concentration required with the proteases used in our previous work (3). The apparent inhibition of the protease in crude PBMC conditioned medium is also consistent with a role in generating mature IL-1β, because processing by mono-

**FIG. 3. Inhibitor sensitivity.** Aliquots from a pool of the active fractions eluted from a blue agarose column were incubated with an appropriate control solution or the inhibitor for 10 min prior to the addition of pro-IL-1β. After a further 60-min incubation, samples were analyzed by Western blot. Lanes 1 and 2, and + 1 mM PMSF; lanes 3 and 4, and + 0.1 mM E-64; lanes 5 and 6, and + 5 μg/ml pepstatin; lanes 7 and 8, and + 1 mM EDTA; lanes 9 and 10, and + 1 mM 1,10-phenanthroline; lanes 11 and 12, and + 0.75 mM phosphoramidon; lanes 13 and 14, and + 2.5 mM Z-Gly-Leu-NH₂; lanes 15 and 16, and + 1 mM N-(2-carboxy-3-phenylpropionyl)Leu. PRO, pro-IL-1β; MAT, mature IL-1β.

**FIG. 4. Restoration of activity by divalent cations.** Aliquots from a pool of active fractions eluted from a blue agarose column were incubated with an appropriate control solution, with the inhibitor, or with the inhibitor plus divalent cation for 10 min prior to the addition of pro-IL-1β. After a further 60-min incubation, samples were analyzed by Western blot. Lanes 1 and 2, and + 1 mM EDTA; lane 3, 1 mM EDTA plus 5 mM CaCl₂; lane 4, 1 mM EDTA plus 2.5 mM CaCl₂; lane 5, 1 mM EDTA plus 1.25 mM CaCl₂; lane 6, 1 mM EDTA plus 0.625 mM CaCl₂; lane 7, 1 mM EDTA plus 0.313 mM CaCl₂; lanes 8 and 9, and + 1 mM 1,10-phenanthroline; lane 10, 1 mM 1,10-phenanthroline plus 375 μM ZnCl₂; lane 11, 1 mM 1,10-phenanthroline plus 94 μM ZnCl₂; lane 12, 1 mM 1,10-phenanthroline plus 23 μM ZnCl₂; lane 13, 1 mM 1,10-phenanthroline plus 6 μM ZnCl₂; lane 14, 1 mM 1,10-phenanthroline plus 3 μM ZnCl₂. PRO, pro-IL-1β; MAT, mature IL-1β.

**FIG. 5. Peptide fragments generated by the DEAE flow activity.** As described under "Experimental Procedures," peptides representing pro-IL-1β (β-Ala₁-(109–121) (NH₂)) (A) and pro-IL-1β (acetyl-)-(103–113) (NH₂) (B) were digested with blue agarose-purified DEAE flow activity, and the resulting fragments were then separated by HPLC. Amino acid analysis indicated the following identification of these fragments: A, peak 1, Asp₁₀⁶-Ser₁₁₀ (NH₂); peak 2, Asp₁₀⁶-His₁₁₀; peak 3, (β-Ala₁-) Asp₁₀⁶-Ser₁₁₀ (NH₂); peak 4, no amino acids present; B, peak 1, Asp₁₀⁶-Tyr₁₁₀ (NH₂); peak 2, no amino acids present; peak 3, Asp₁₀⁶-Tyr₁₁₀; peak 4, Asp₁₀⁶-Tyr₁₁₀ (NH₂); peak 5, (acetyl-) Ile₁⁰²-Phe₁⁰⁶; peak 6, (acetyl-) Ile₁⁰²-Tyr₁₁₀ (NH₂).

**FIG. 6. Identification of cleavage sites.** A, peptide representing pro-IL-1β-(109–121). B, peptide representing pro-IL-1β-(103–113). Numbering is from the NH₂-terminal methionine residue of the precursor. Mature IL-1β begins with alanine 117. Arrows indicate sites of cleavage.
cytes appears to occur at the cell surface, where the protease could be shielded from inhibitors. (Since cleavage could occur during or immediately after secretion, an extracellular protease is as likely to be involved as a cell-associated one.) Apart from monocytes, keratinocytes also produce pro-IL-1β, but these cells are unable to convert it to the mature form (14). In this case, a circulating protease would have to be involved in activating the hormone. It should be noted, however, that the protease we have described generates a form of IL-1β one amino acid longer than the form purified from monocytic cultures. There is an aminopeptidase in human blood that removes NH₂-terminal aspartate residues and thus could complete the processing (15). Another issue requiring further investigation is whether there are intermediate forms of IL-1β in vivo that correspond to the larger fragments of the precursor apparently produced by the enzyme.

If the protease is not involved in generating mature IL-1β, it will be interesting to determine what function it does perform. The highly restricted cleavage specificity observed, with no cleavage even at glutamate or asparagine residues, suggests a role in processing rather than in general protein degradation. The development of a specific, nontoxic inhibitor will be required to pursue the function of this protease.

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