Involvement of a Calpain-like Protease in the Processing of the Murine Interleukin 1α Precursor*

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Interleukin (IL) 1α is synthesized as a 33-kDa precursor that is enzymatically cleaved to the 15–17-kDa forms that are found in the culture supernatants of activated macrophages. We have explored the possibility that calcium might enhance IL-1 processing and secretion via the stimulation of a calcium-dependent protease. We have found that lysates prepared from human peripheral blood monocytes, the human histiocytic lymphoma cell line U937, and the murine macrophage cell line P388D1 contain a calcium-dependent IL-1α processing activity that cleaves the IL-1α precursor to its mature form. Although NIH 3T3 mouse fibroblast cell lysates also contain IL-1 processing activity, lysates from the murine thymoma EL-4, the human epidermoid cell line HEp-2, and the human foreskin fibroblast line FS-4 lack this activity. IL-1 processing activity is inhibited by leupeptin and exhibits a molecular mass of 80–110 kDa. The processing activity is also inhibited by a monoclonal antibody directed against calpain type I. These results indicate that the processing of the IL-1α precursor is mediated, at least in part, by a member of the calpain family of proteases. Mixing experiments revealed that lysates from EL-4 or HEp-2 cells contain an inhibitor(s) of the calpain-like protease in macrophage extracts. It is, therefore, likely that many non-macrophage cell types are unable to process the IL-1α precursor because the calpain present in these cells is only weakly active due to the presence of a specific inhibitor(s) such as calpastatin.

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1IL-1α is a peptide hormone produced by activated macrophages which stimulates a broad spectrum of immune and inflammatory responses including antigen and mitogen-dependent lymphocyte activation (1, 2), fever (3), and the synthesis of several acute-phase response proteins (4). Cloning studies revealed the existence of two forms of human and murine IL-1, termed IL-1α and IL-1β, that possess similar molecular masses but differ in isoelectric point and amino acid sequence (5–8).

Previous studies have shown that both forms of human and murine IL-1 are synthesized as 31,000–33,000-Da precursor proteins that are enzymatically cleaved to the lower molecular mass forms found in the culture supernatant of activated macrophages (7, 9). Sequence analysis of the IL-1α and IL-1β precursors revealed that they do not contain a conventional N-terminal hydrophobic signal sequence (7, 9). Furthermore, the presence of an internal signal sequence appears unlikely since the murine IL-1α precursor (pIL-1α) synthesized in an in vitro reticulocyte lysate system in the presence of dog pancreas microsomes does not translocate into the lumen of the microsomes (9). In addition, immunocytochemical studies on stimulated human monocytes demonstrated that newly synthesized pIL-1β is not localized in the Golgi apparatus but rather in the cytoplasm of these cells (10, 11). Taken together, these results are consistent with the notion that IL-1α and IL-1β might be processed and secreted from cells by a novel pathway that bypasses the Golgi apparatus. In a recent study we found that the processing and secretion of human and murine macrophage-derived IL-1α and IL-1β are dramatically enhanced by the addition of the calcium ionophores, ionomycin and A23187 (12). This finding raised the possibility that one or more calcium-dependent proteases might be involved in the secretory pathway(s) for IL-1. In the current study we have extended our observations on the effects of calcium on IL-1α processing and secretion and have characterized the involvement of a calpain-like protease in the maturation of murine pIL-1α.

MATERIALS AND METHODS

Cells—The murine macrophage cell line P388D1, the murine thymoma cell line EL-4, and the human histiocytic lymphoma cell line U937 were grown in suspension culture at 37 °C in RPMI 1640 (GIBCO) supplemented with 10% fetal bovine serum (KC Biologicals) and 50 μg/ml gentamicin sulfate (U. S. Biochemical Corp.). The human foreskin fibroblast cell line FS-4, NIH 3T3 mouse fibroblasts, and the human epidermoid carcinoma cell line HEp-2 were grown as monolayers in RPMI 1640 supplemented with 10% fetal bovine serum and gentamicin sulfate. Cells were routinely tested for mycoplasma contamination and were found to be negative.

Preparation of Radiolabeled Murine pIL-1α—The plasmid pIL20MIL-1 containing a 1.1-kilobase cDNA for murine pIL-1α was kindly provided by Dr. Peter Lomedico (Hoffmann-LaRoche, Nutley, NJ). The 1.1-kilobase pIL-1α cDNA was excised and inserted into the HincII site of pGEM-3a (Promega) and amplified in Escherichia coli using standard methods (13). Purified plasmid was linearized with HindIII or EcoRI and then transcribed using SP6 or T7 polymerase in in vitro transcription system (Promega). The mRNAs were processed according to the manufacturer’s instructions. In vitro translations were performed using micrococcal nuclease-treated rabbit reticulocyte extracts (Promega Biotech) supplemented with in vitro synthesized mRNA and 25 μCi/ml [35S]methionine.

Preparation of Cell-free Extracts—Cell-free extracts were prepared...
by hypotonic lysis of cells. Adherent cells were washed twice with phosphate-buffered saline (10 mM phosphate, pH 7.4, 150 mM NaCl) and once with lysis buffer (20 mM HEPES, pH 7.4, 10 mM KCl, and 1.5 mM MgCl₂) prior to scraping with a rubber policeman. Adherent and nonadherent cells were harvested by centrifugation at 500 g and then swelled in lysis buffer for 20 min on ice. Lysis of cell suspensions was accomplished by 35 strokes in a Dounce homogenizer. Lysates were then spun in a Beckman table-top ultracentrifuge at 100,000 × g for 15 min. The supernatants (termed lysates) were frozen at -70 °C until used in cleavage assays.

In Vitro Cleavage Assay for pIL-la-The pIL-la cleavage assays were performed using 50 or 100 μl of a 1:4 dilution of rabbit reticulocyte extract containing [³⁵S]methionine-labeled pIL-la with 50 or 100 μl of lysate in the presence or absence of 1 mM CaCl₂. Reactions were incubated at 37 °C for 1 h. In some experiments, cell lysates from different cell types were mixed with macrophage-derived lysate to determine if the absence of processing activity in some cell types was due to the presence of an inhibitor(s) of processing activity. Specifically, U937 lysate (20 μl) was incubated with varying amounts of HeP-2 or EL-4 cell lysates (0-80 μl), and the cleavage of pIL-la was assessed. The protein concentration of the mixtures was held constant by the addition of bovine serum albumin. As a control in these experiments, we evaluated the effect of the nonprocessing HeP-2 lysate (lane 2) on trypsin-catalyzed pIL-la cleavage. pIL-la was added to each mixture and incubated as described above.

The effect of calcium-activated neutral protease (CANP) on pIL-la processing was assessed by incubating pIL-la in lysis buffer containing 1 unit/ml CANP in the presence or absence of 1 mM CaCl₂. To test for a protease inhibitor (specific for CANP) present at a concentration of 5 units/ml. The calpain inhibitor trans-epoxysuccinyl-1-leucylamido-(4-guanidino)butane (E-64) was used at a concentration of 50 μg/ml. A murine ascites containing a monoclonal antibody against human type I calpain (the gift of Dr. J. Elce, Queen's University) was used at a dilution of 1:500. This antibody was capable of blocking 1 unit/ml CANP activity. In related experiments using trypsin the antibody was ineffective in blocking cleavage of pIL-la. After incubation, all samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described previously (9). For quantitative analysis (percent conversion), autoradiographs were scanned using an LKB Bromma Ultra-Scan XL laser densitometer (LKB).

Gel Filtration Chromatography of the pIL-la Processing Activity in Lysate of U937 Cells—U937 cell lysate (300 μl, 1 × 10⁶ cells/ml of lysate) was loaded onto a 20-ml Sephadex G-150 column equilibrated in lysis buffer and chromatographed at a flow rate of 4 ml/h. Fractions (0.5 ml) were collected and individually assayed for cleavage activity by incubation with pIL-la as described above.

Western Blot Analysis of Cell Lysates—Lysate and column fractions were assayed for the presence of calpain using Western blots. Briefly, 10 μl of cell lysate or column fractions was subjected to SDS-PAGE on 7.5% gels, and the proteins were blotted to nitrocellulose membranes using the Bio-Rad Trans-Blot apparatus. The membranes were washed with Tris-buffered saline (TBS) (pH 8.0, 150 mM NaCl) containing 5% nonfat dry milk to block nonspecific binding and then washed with TBS prior to incubation with a mouse anti-human monoclonal against human type 1 calpain. Following overnight incubation at 4 °C, the membranes were washed with TBS containing 0.05% Tween-20 and incubated for 2 h with a 1:1000 dilution of a rabbit anti-mouse IgG alkaline phosphatase-conjugated antibody. The membranes were washed and incubated with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (Promega) in alkaline phosphatase buffer (0.1 M Tris, pH 9.5, 0.1 mM MgCl₂, 5 mM MgCl₂). The reaction was stopped by the addition of TE (10 mM Tris-HCl, pH 8.0, and 1 mM EDTA).

Site-directed Mutagenesis of pIL-la—The pIL-la CDNA was excised from plasmid with BamHI and then ligated into the HincII site of the β-galactosidase gene in the M13 cloning vector M13mp19. Point mutations were made at desired sites using M13 site-directed mutagenesis as described by Kunkel et al. (14). Oligonucleotides (18mers) encoding specific amino acid substitutions were synthesized on an Applied Biosystems DNA according to established protocols. Mutations were confirmed by dideoxy sequencing (15) using the Sequenase Version 2.0 kit (U. S. Biochemical Corp.).

RESULTS

Calcium-dependent in Vitro Processing of pIL-la—In a recent study we found that calcium ionophores such as ionomycin and A23187 dramatically enhanced the processing and secretion of newly synthesized IL-1α from human and murine macrophages (12). This observation prompted us to suggest that a calcium-dependent protease might be involved in the processing and secretion of IL-1α. To test this possibility formally we sought to establish an in vitro system for the processing of pIL-la to its mature form (15-17 kDa). Radiolabeled murine pIL-la was prepared by in vitro translation of pIL-la mRNA in the presence of [³⁵S]methionine and used as a source of substrate. When radiolabeled pIL-la was added to lysates prepared from the murine macrophage cell line P388D1 and incubated in the absence of calcium, no detectable cleavage of the pIL-la was observed (Fig. 1, lane 1). However, if calcium was added to the cleavage reaction (lane 2), the pIL-la was completely converted to its mature form. Calcium-dependent processing of pIL-la was inhibited in the presence of 5 mM EGTA (lane 4). Cleavage activity was also inhibited by the serine protease inhibitor, leupeptin (lane 5), but not by aprotinin, phenylmethylsulfonyl fluoride, benzamidine, or α-macroglobulin (lanes 6-9). Trypsin (lane 3), in the absence of calcium, cleaved the pIL-la to a form that migrated with the cleavage product produced by incubation of pIL-la with P388D1 cell lysate.

In addition to P388D1 cells, the human histiocytic lymphoma cell line U937, human peripheral blood monocytes, and NIH 3T3 mouse fibroblasts contained pIL-la processing activity (Fig. 2). However, FS-4 cells, a human foreskin fibroblast line that can synthesize but not secrete IL-1 (12, 17), did not contain detectable pIL-la processing activity (Fig. 2, lane 5). Lysates prepared from the murine thymoma cell line EL-4 and the human epidermoid carcinoma cell line HeP-2 also lacked pIL-la processing activity as measured in the presence or absence of calcium (Fig. 3). These results indicate that although the ability to process pIL-la is not macrophage-specific, it does not appear to be a universal property of all mammalian cells.
Fig. 2. Evaluation of pIL-1α processing activity in various cell lines. Macrophage and non-macrophage cell lysates were prepared as described under "Materials and Methods" and incubated with wild-type pIL-1α. Lane 1, P388D1 lysate; lane 2, NIH 3T3 lysate; lane 3, U937 lysate; lane 4, human peripheral blood monocyte lysate; and lane 5, FS-4 lysate.

Fig. 3. Lack of pIL-1α processing by lysates from EL-4 and HEp-2 cells. Lysates were prepared from HEp-2 (lane 1) and EL-4 (lane 2) cells as described under "Materials and Methods" and incubated with pIL-1α.

Fig. 4. Gel filtration chromatography of the pIL-1α processing activity in U937 cells. Lysate (300 μl; 1 x 10⁶ cells/ml) from U937 cells was chromatographed on a Sephadex G-150 column in equilibrated in lysis buffer and run at 4 ml/h. Fractions were tested for cleavage activity as described under "Materials and Methods." IgG (150 kDa) eluted at 8.25 ml and bovine serum albumin (68 kDa) at 11.75 ml. Peak activity was seen in fractions 34-41. Aliquots of each fraction were also tested for the presence of calpain using Western blots.

Characterization of the Protease(s) Responsible for the in Vitro Processing of pIL-1α—Having established that the pIL-1α processing activity is calcium dependent and leupeptin sensitive, we used Sephadex gel filtration chromatography to establish a molecular mass for the protease(s). A concentrated lysate prepared from U937 cells was chromatographed on a Sephadex G-150 column, and the fractions were assayed for cleavage activity against pIL-1α. As shown in Fig. 4, the cleavage activity eluted with molecules with molecular masses in the range of 80-110 kDa.

To gain additional insights into the properties of the calcium-dependent protease(s), we sought to define the site(s) at which it cleaves the pIL-1α. Site-directed mutagenesis was used to create mutations in the pIL-1α protein at selected sites, and the mutant proteins were then tested for susceptibility to calcium-dependent cleavage. Previous studies on the murine IL-1α purified from the culture supernatant of stimulated P388D1 cells demonstrated that the mature protein exhibits microheterogeneity (18). Six species of IL-1α were detected which exhibited slight differences in size and isoelectric point. Sequence analysis of the mature form of IL-1α revealed that serine 115 of the precursor served as the N-terminal amino acid. Since residue 114 in the pIL-1α is an arginine, we decided to generate a mutant pIL-1α lacking an arginine at this position and test if it was susceptible to cleavage by the calcium-dependent protease(s) found in macrophage lysates. Site-directed mutagenesis was used to convert Arg to an isoleucine in the pIL-1α cDNA. In a number of instances, endoproteases involved in hormone processing have been found to cleave at di-, and occasionally tetra-basic residues, the cleavage occurring on the carboxyl side of these basic residues (19-22). Since the pIL-1α contains a tetra-basic region (Lys-Lys-Arg-Arg) at residues 85-89, the suggestion was made that this region might be a primary processing site (6). Thus, a second set of mutant pIL-1α proteins was produced in which Lys was converted to a threonine, and Arg was changed to an leucine. Finally, a "triple" mutant was created with all three substitutions described above (Thr, Leu, and Ile). The mutant cDNAs were transcribed in vitro, and the resultant mutant pIL-1α mRNAs were translated in a rabbit reticulocyte lysate in the presence of [35S] methionine. As shown in Fig. 5, all five mutant pIL-1α proteins were cleaved to the same molecular mass as the wild-type pIL-1α in the presence of P388D1 lysate. As expected, calcium was required for the cleavage of the mutant proteins; only background levels of cleavage occurred in the absence of calcium (data not shown). Thus, neither the tetra-basic region at residues 85-89 nor Arg is required for processing of pIL-1α in this in vitro system. Radiosequencing analysis of the mature form of the wild-type pIL-1α generated by in vitro processing revealed that the amino-terminal sequence was Gln-Ser-Asp, thus establishing the preferred site of cleavage between Tyr-120 and Gln-121.

Involvement of a Calpain-like Protease in pIL-1α Processing in Vitro—In view of the fact that members of the calpain family of proteases are calcium dependent, leupeptin sensitive, and possess a molecular mass in the range of 80-110 kDa (23), we examined the possibility that the pIL-1α processing...
activity in macrophage lysates was a member of this family of proteases. The 80-110-kDa range of the pIL-1α processing activity (Fig. 4) corresponds to the molecular mass of the large subunit (80 kDa) and heterodimeric (110 kDa) forms of calpain. As a first step, we used a monoclonal antibody against the 80-kDa subunit of human calpain type I in Western blots to determine if this enzyme was present in the Sephadex G-150 column fractions that contained pIL-1α cleavage activity. We did indeed find that those fractions with cleavage activity were positive for calpain (data not shown). Furthermore, the fractions possessing the highest level of cleavage activity (Fig. 4) also contained the highest levels of calpain. It is important to note that in SDS gels, the molecular mass of the calpain in each column fraction was 80 kDa. This observation may provide an explanation for the relatively broad peak of pIL-1α processing activity. During chromatography, a portion of the 80-kDa large subunit of calpain may have dissociated from the 30-kDa subunit, giving rise to the observed rather broad elution profile.

Additional evidence for the involvement of a calpain-like protease in the in vitro processing of pIL-1α was obtained in two types of experiments in which the anti-calpain monoclonal antibody was used to inhibit calpain activity present in the lysates of U937 cells, and calpain itself was tested for pIL-1α processing activity. As presented in Table I, the anti-calpain monoclonal antibody significantly diminished the cleavage activity present in U937 lysates. Furthermore, a protease inhibitor (specific for CANP) also reduced the conversion of pIL-1α to its mature form. E-64, another specific calpain inhibitor, also reduced the conversion of pIL-1α by U937 lysate. When purified CANP was added to pIL-1α in the absence of any cell lysate, a mature form corresponding in size to cell lysate-generated mature IL-1α was obtained. The activity of CANP was blocked by the addition of the anti-calpain monoclonal antibody (data not shown). Thus, on the basis of its requirement for calcium, leupeptin and E-64 sensitivity, and inhibition by a calpain inhibitor as well as an anti-calpain monoclonal antibody, it is quite likely that the pIL-1α processing activity in macrophage lysates is mediated by a member of the calpain family of proteases. Radiosequence analysis of mature IL-1 generated by incubation with CANP revealed two possible amino-terminal species of the molecule at Gin-Ser-Asp and Ser-Ala-Pro. The first sequence corresponds to the amino terminus generated by the U937 lysate cleavage reaction. The second sequence corresponds to a species of mature IL-1 seen in the culture supernatant of activated macrophages (9, 12, 18). The results of this sequence analysis are consistent with a calpain-like enzyme being involved in the processing of IL-1.

Although our findings are consistent with the notion that a calpain protease is responsible for the processing of pIL-1α, we were confronted by the initially puzzling problem of why a cell such as FS-4 that possesses calpain (as determined by Western blot analysis; data not shown) is unable to process pIL-1α in vitro. It has recently been shown that the calpain activity in a particular cell may be tightly regulated by the natural inhibitor of calpain, calpastatin (24, 25). Thus, it was possible that cell lines such as FS-4, EL-4, and HEp-2 might contain a higher level of a calpain inhibitor than P388D1, U937, human monocytes, or NIH 3T3 cells and thus would have insufficient levels of active calpain to process pIL-1α in vitro. To examine this possibility, mixing experiments were performed to test the effect of HEp-2 and EL-4 cell lysates on the processing activity present in lysates from U937 cells. When increasing amounts of either Hep-2 or EL-4 lysate (both nonprocessors) were mixed with a constant amount of U937 lysate, a concentration-dependent inhibition of pIL-1α cleavage was observed (Table II). A dilution of the lysate with lysis buffer alone had no effect. Furthermore, the processing of pIL-1α by CANP was also blocked, in a concentration-dependent manner, by HEp-2 lysates. HEp-2 lysate had no effect on trypsin-mediated cleavage of pIL-1α (Table II). Thus, it is quite likely that the ability to process pIL-1α is dependent upon a favorable balance between the levels of calpain and one or more specific inhibitors of calpain activity.

**DISCUSSION**

The results from several recent studies indicate that IL-1 may not follow a conventional secretory pathway that involves cotranslational processing and transit through the Golgi apparatus (10-12, 26). The mature forms of IL-1 (15-17 kDa) are not detectable in lysates of stimulated, IL-1-secreting macrophages, suggesting that processing is a co- or postsecretory event. However, it is possible that IL-1 may be processed intracellularly and secreted in such a rapid and efficient manner that little, if any, mature IL-1 remains in the cell. Although most of the IL-1 secreted by macrophages is in the mature form, the presence of intact IL-1 precursor in the

**TABLE I**

| Additions | % conversion |
|-----------|--------------|
| U937 lysate Ca²⁺ | 15 |
| + | 93 |
| + + 5 mM EGTA | 2 |
| + + anti-CANP | 12 |
| + + E-64 (50 μg/ml) | 12 |
| + Protease inhibitor (inhibitor of CANP) | 45 |
| - + CANP (1 unit/ml) | 87 |

**TABLE II**

| Additions | % conversion |
|-----------|--------------|
| U937 lysate HEp-2 lysate | 8 |
| 20 μl | 80 μl |
| 20 μl | 40 μl |
| 20 μl | 20 μl |
| 20 μl | 10 μl |
| 20 μl | 0 μl |
| U937 lysate EL-4 lysate | 21 |
| 20 μl | 80 μl |
| 20 μl | 40 μl |
| 20 μl | 20 μl |
| 20 μl | 10 μl |
| 20 μl | 0 μl |
| CANP HEp-2 lysate | 36 |
| 1 unit/ml | 50 μl |
| 1 unit/ml | 40 μl |
| 1 unit/ml | 30 μl |
| 1 unit/ml | 0 μl |
| Trypsin HEp-2 lysate | 99 |
| 10 ng/ml | 80 μl |
| 10 ng/ml | 40 μl |
| 10 ng/ml | 20 μl |
| 10 ng/ml | 10 μl |
| 10 ng/ml | 0 μl |
