Differential Sensitivity of Interleukin-1α and -β Precursor Proteins to Cleavage by Calpain, a Calcium-dependent Protease*

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In view of the observations that the calcium ionophores, A23187 and ionomycin, enhance the processing and secretion of interleukin-1 (IL-1α) and IL-1β from macrophages, and that IL-1α processing is mediated by calpain, a calcium-dependent protease, we evaluated the possibility that calpain might also play a role in the processing of IL-1β. Whereas calpain-containing P388D1 macrophage lysates and purified calpain processed precursor IL-1α to its mature 17-kDa form, precursor IL-1β was degraded by both sources of calpain. However, the activation of calpain in P388D1 cells that were transiently transfected with a cDNA expression vector encoding the precursor form of IL-1β did not result in the degradation of precursor IL-1β, but did result in the processing and secretion of IL-1α, implying that precursor IL-1β is protected from calpain degradation in vivo. Furthermore, calpain did not enhance the processing of the IL-1β precursor by the IL-1β-converting enzyme. These results indicate that calpain is not involved in the processing of precursor IL-1β in vitro or in vivo. The IL-1β precursor may be protected from calpain degradation by a sequestering mechanism that involves a cytoplasmic factor(s) that reduces the sensitivity of IL-1β to attack by calpain or localizes IL-1β to a site that precludes any interaction with the protease.

Although MDL 28,170, a calpain inhibitor, prevented the ionomycin-induced processing of precursor IL-1α to the mature protein in P388D1 cells, it did not inhibit the ionomycin-induced secretion of the mature IL-1α and -β proteins expressed in these cells. These results indicate that a calcium-dependent factor other than calpain is involved in the secretion of the mature IL-1 proteins.

Interleukin-1 (IL-1)† is an important inflammatory cytokine produced primarily by monocytes, macrophages, and polymorphonuclear leukocytes. By mediating the production of potent molecules such as prostaglandins, leukotrienes, platelet-activating factor, and nitric oxide, and by up-regulating endothelial cell-adhesion molecules, IL-1 affects processes as diverse as immune cell recruitment, blood pressure, vascular smooth muscle contraction, kidney function, cell proliferation, bone resorption, and central nervous system function (1). The two forms of IL-1, IL-1α and -β, are produced as precursor proteins of 31–33 kDa and are subsequently processed to mature proteins of 15–17 kDa (2–4). The precursor form of IL-1α is processed in vitro by calpain, a calcium-dependent protein; human IL-1α is cleaved between Phe118 and Leu119, whereas murine IL-1α is cleaved between Arg114 and Ser115 (5, 6). The precursor form of IL-1β is processed by the IL-1β-converting enzyme (ICE) between Asp116 and Ala117 (7–9). Although precursor IL-1α is biologically active, precursor IL-1β is biologically inactive due to weak receptor binding (10). Even though the amino acid sequence identity between the mature forms of IL-1α and IL-1β is low (26%), they bind to the same receptor and invoke the same set of biological responses (11, 12).

IL-1α and -β, basic fibroblast growth factor, histachodulin, and soy bean trypsin inhibitor belong to a family of proteins whose structure is comprised of 12 antiparallel β-strands connected by several loops and turns and arranged around a 3-fold axis of symmetry (13–17). These proteins are also characterized by the lack of an NH2-terminal secretory signal sequence. Examples of other proteins with a defined extracellular function but devoid of a secretory signal sequence include thioredoxin, transglutaminase, thymosin, parathymosin, and the soluble lectins L-14, L-29, and CBP30 (18–21). Drugs such as brefeldin A and monensin, which block the trafficking of proteins through the endoplasmic reticulum-Golgi pathway do not affect the secretion of several of these proteins, suggesting an alternative or “nonclassical” route to the cell exterior (20–24). This secretion of this group of proteins is enhanced by heat shock or calcium ionophores, suggesting that these proteins may share a common secretory pathway (20, 21, 23, 24).

Processing of the precursor forms of IL-1α and -β is also enhanced by calcium ionophores in monocytes and macrophages (5, 22, 25). When these cells are stimulated to synthesize and secrete IL-1 proteins, the mature forms of IL-1 are found only in the culture medium and not in association with cells, suggesting that processing and secretion may be so closely linked as to result in the rapid and efficient secretion of the mature IL-1 proteins. Analysis of IL-1 secretion in cell line macrophages as well as nonmacrophage cell lines expressing recombinant forms of the precursor and mature forms of IL-1α and -β (following transient transfection of an expression plasmid) revealed that the mature form is the preferred substrate for secretion (26); such cells secrete relatively low levels of precursor IL-1α and -β compared with the mature forms in the presence of ionomycin or LPS (26). Whereas transiently transfected cells secrete only 2% of the precursor IL-1 proteins following pulse labeling with [35S]methionine and incubation with ionomycin or LPS during a 6-h period, cells transiently transfected with an expression plasmid encoding mature IL-1 secrete essentially all of the radiolabeled IL-1 (26). The requirement for secondary stimuli such as ionomycin or LPS to induce the secretion of the mature IL-1 proteins indicates that processing by itself is not a sufficient stimulus for IL-1 secretion.
and that secretion, like processing, is also modulated by an increase in the intracellular level of calcium.

Calcium ionophore-induced processing and the subsequent release of mature IL-1α from monocytes and macrophages (5, 26) is not surprising given the role of calpain in the in vitro processing of precursor IL-1α (5, 6). Calpain is found in a wide variety of tissues, including monocytes, macrophages, lymphocytes, and fibroblasts. It is an heterodimer composed of an 80-kDa and a 30-kDa subunit, both of which contain calcium-binding EF-hand domains near the COOH termini (27). Calcium binding to these subunits induces a conformational change and the subsequent activation of the enzyme (27). The activity of calpain is regulated by calpastatin, a naturally occurring inhibitor of calpain. Thus the ability to process the precursor form of IL-1α is restricted to cell types in which the balance of calpain and calpastatin favors calpain (6). Processing of precursor IL-1α to the mature form on the other hand appears to be mediated by ICE. The importance of ICE in precursor IL-1β processing was demonstrated by the ability of ICE-specific inhibitors to prevent the secretion of mature IL-1β from activated monocytes and macrophages (8, 28). In addition, monocytes and macrophages derived from ICE-deficient mice do not secrete IL-1β when stimulated with LPS (29, 30). ICE is synthesized as an inactive 45-kDa precursor and is cleaved to a 20-kDa and 10-kDa peptides, presumably by autolysis (8). Unlike calpain, ICE does not require calcium to function. Thus it is not readily evident as to why calcium ionophores enhance the processing and secretion of mature IL-1β. Could calpain be involved in the processing and secretion of IL-1β? Calpain might, under certain conditions, convert precursor IL-1β to its mature form, thus representing a second processing enzyme for precursor IL-1β. Alternatively, calpain may enhance the activity of ICE or convert precursor IL-1β to a form that is more efficiently cleaved by ICE. The current study was undertaken to evaluate these possibilities.

MATERIALS AND METHODS

Cell Culture—The murine macrophage cell line P388D1 was maintained in suspension culture and the human monocyte cell line THP.1 was maintained in stationary culture in RPMI 1640 medium supplemented with 10% fetal bovine serum and 50 μg/ml gentamicin sulfate at 37°C. Cells were routinely tested for mycoplasma and found to be free of contamination.

Plasmid Construction—The construction of the plasmids encoding the human IL-1α and β precursor proteins (pRc/RSV-pL-1αFLAG and pRc/RSV-pL-1β) and the human IL-1α and β mature proteins (pRc/RSV-mL-1αFLAG and pRc/RSV-mL-1β) has been described previously (26). For in vitro translation of the human precursor and mature IL-1β proteins, the respective cDNA constructs were excised from the pRc/RSV vectors with BstXI and XbaI, gel-purified, and subcloned into the pCR-CMV expression vector (Invitrogen) containing the T7 promoter. The murine precursor IL-1α encoding plasmid, pGEM-3z/Pl-L-1α, was used for the in vitro translation of precursor IL-1α (6).

Transfections—P388D1 cells (1 x 10⁶) were transfected with 25 μg of DNA in 0.4-μm gene pulser cuvettes at 270 V and 960 microfarads using a Bio-Rad gene pulser (26). The transfected cells were split between two wells of six-well plates (5 x 10⁶ cells/well) and incubated at 37°C for 48 h in 5 ml of 10% fetal bovine serum-containing RPMI media. Experiments involving several wells of the same DNA transfection were plated at 5 x 10⁶ cells/well after pooling the transfected cells.

Radiolabeling of Cells and Analysis of IL-1 Secretion—Transfected cells were washed with warm RPMI medium and labeled with 250 μCi [35S]methionine in 0.1% fetal bovine serum-containing, methionine-free media for 6 h at 37°C. The labeled cells were washed, and 2 ml of RPMI was added back to each well. For the calpain inhibition experiments, cells were incubated with or without the calpain inhibitor MDL 28,170 (200 μM) (31) (a kind gift of Dr. Elkehard H. W. Bohme, Marion Merrell Dow Inc.) for 45 min prior to the addition of 1 μM ionomycin (dissolved in Me2SO; Sigma). Immediately following a 30-min incubation with ionomycin, the culture supernatants and cells were collected, cell lysates were prepared, and the IL-1 proteins were immunoprecipitated with anti-human IL-1α or β antiserum (generous gifts of Dr. Richard Chizzonite, Hoffmann-LaRoche) and run on 12.5% SDS-polyacrylamide gels as described previously (26). The dried gels were scanned in an AMBIS radioanalytic imaging system to quantitate the precursor and mature forms of IL-1α and β (26). The percent secretion for mature IL-1α and β proteins was determined by the following equation: (total radioactivity of secreted IL-1/total radioactivity of secreted IL-1 + total radioactivity of cell-associated IL-1) x 100%. The percent secretion for precursor IL-1α was determined by the following equation: (total radioactivity of secreted mature IL-1α/37 total radioactivity of cell-associated 35 and 33 kDa precursor IL-1α + total radioactivity of secreted mature IL-1α) x 100%. The 3/7 in this formula corrects for the difference in methionine content between precursor and mature IL-1α (precursor IL-1α has 7 methionines compared with mature IL-1α).

For the analysis of precursor IL-1β turnover, the labeled media was replaced with RPMI with or without 1 μM ionomycin, and culture supernatants and cells were collected during a 2-h period.

Preparation of Recombinant Radiolabeled IL-1α and IL-1β Proteins—One μg each of XbaI-linearized pRc/CMV-pL-1α, XbaI-linearized pRc/CMV-mL-1β, or unlinearized pGEM-3z/Pl-L-1α was transcribed, and the resulting mRNA was translated in the presence of approximately 50 μCi of [35S]methionine using an in vitro TNT rabbit reticulocyte lysate system (Promega). The reactions (total volume of 50 μl) were incubated at 30°C for 2 h. The percent incorporation and formation of protein/μl was determined using standard methods.

Preparation of THP.1 Cell Lysates—Cells were washed twice with cold phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 10 mM NaH2PO4, 1.8 mM KH2PO4, pH 7.4) and resuspended at 1 x 10⁶ cells/ml in a 0.5% Nonidet P-40 buffer containing 0.1 μg/ml each of aprotinin, elastase, and trypsin-chymotrypsin inhibitor, 100 μg/ml phenylmethylsulfonyl fluoride, 10 mM phenylmethylsulfonyl fluoride, and when noted, 100 μg/ml leupeptin. Cells were sonsed with ice for 20 min and Dounce-homogenized. After checking the cells for complete lysis, the samples were centrifuged for 15 min at 2 x 10³ g (4°C), and aliquots of the supernatants were frozen at -70°C. THP.1 cells were washed twice with phosphate-buffered saline and resuspended at 1 x 10⁶ cells/ml in a hypotonic buffer (10 mM KCl, 20 mM HEPES, pH 7.5, 1 mM MgCl2, 1 mM dithiothreitol, 2 mM EDTA, pH 8.0. Following a 10-min incubation at 37°C, the lysed cells were centrifuged for 10 min at 10³ rpm (4°C), and aliquots of the supernatants were frozen at -70°C. The百分率 secretion for precursor IL-1α proteins was determined using standard methods.

RESULTS

Cleavage of Precursor IL-1α by Calpain in Vitro—Earlier studies revealed that the calcium ionophores A23187 and i

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The calpain-mediated generation of multiple products with precursor IL-1β is in contrast to the situation with precursor IL-1α, in which only a single major product, mature IL-1α, is obtained (Fig. 1B, lanes 3–7; Ref. 6). Incubation of precursor IL-1β with increasing amounts of calpain in vitro resulted in a progressive decrease in the level of radioactivity associated with the IL-1β cleavage products (Fig. 2A) compared with the average total input radiolabeled precursor IL-1β (Fig. 1A, lanes 1 and 2). Thus at higher concentrations of calpain an increasing percentage of the precursor IL-1β was converted to very low molecular weight fragments that would be lost from the gels. The loss of IL-1β radioactivity at 5 and 10 units/ml purified calpain exceeded the expected 50% associated with the removal of 6 of 12 methionine residues as a result of the conversion of precursor IL-1β to mature IL-1β. These results are consistent with the notion that precursor IL-1β is degraded and not processed by calpain in vitro. In contrast to the results with precursor IL-1β, calpain did not degrade radiolabeled recombinant mature IL-1β (Fig. 2B) or precursor IL-1α (Fig. 2C) even at high concentrations, demonstrating a selective effect of calpain on precursor IL-1β. Conversion of murine precursor IL-1α to mature IL-1α involves a loss of 2 out of 6 methionines and therefore a theoretical loss of 33% of the total input radioactivity. Whereas an average of 18% of the input precursor IL-1α radioactivity is lost in experiments, most of the input precursor IL-1α radioactivity was recovered in the mature IL-1α protein in the experiment presented in Fig. 2C. However, in other experiments, the loss of radioactivity approached the expected value.

Cleavage of Precursor IL-1β by Calpain-containing Macrophage Lysates—In view of the sensitivity of precursor IL-1β to cleavage by purified calpain in vitro, we examined the effect of calpain-containing lysates prepared from P388D1 cells. Our earlier work established that such lysates readily convert precursor IL-1α to its mature form in the presence of calcium (6). Incubation of radiolabeled recombinant precursor IL-1β with an aliquot of the macrophage lysate in the presence resulted in the generation of two products, a major 27-kDa product, and a minor product (Fig. 3, lane 2) co-migrated with the mature IL-1β standard (lane 11). However, the lower molecular mass product was not mature IL-1β as evidenced by its sensitivity to proteinase K (data not shown). The 27- and 17-kDa products were not seen when precursor IL-1β was incubated with the lysates in the presence of EGTA (lane 1) or in the presence of any of the calpain inhibitors (lanes 3–5), indicating that they were specifically generated by calpain. The pattern of proteolysis of precursor IL-1β in the presence of the macrophage lysate is distinct from that generated with purified calpain (lane 7 and Fig. 1A) in that a substantially higher amount of the 27-kDa product and a 16–17-kDa product are obtained with the lysates. In addition, there was no loss of total IL-1β associated radioactivity (i.e. precursor IL-1β protein, the 27-kDa product, and the 16–17-kDa products), compared with the average total input radioactivity associated with the precursor IL-1β protein in the control lanes (lanes 1 and 6).

Since, unlike purified calpain, the calpain-containing macrophage lysate does not completely degrade precursor IL-1β, it was possible that the lysates might contain a much lower level of calpain. Thus it was important to relate the active calpain content in the macrophage lysates to the activity associated with the purified enzyme. Equal amounts of radiolabeled precursor IL-1α was incubated with increasing amounts of purified calpain or P388D1 lysates in the presence of calcium at 37 °C for 1 h and the percent of unprocessed precursor IL-1α remaining in each reaction was plotted against calpain concentration or lysate volume. 50% of the precursor IL-1α protein

**Fig. 1.** In vitro processing of precursor IL-1β and precursor IL-1α by purified calpain. Recombinant [35S]methionine-labeled precursor IL-1β or precursor IL-1α was incubated with increasing amounts of purified calpain at 37 °C for 1 h. A, precursor IL-1β. B, precursor IL-1α. Lane 1, 5 mM EGTA; lane 2, 1 mM CaCl₂; lanes 3–7, 1 mM CaCl₂ and 0.5, 1, 2.5, 5, or 10 units/ml purified calpain; lanes 8–10, 1 mM CaCl₂ and 5 units/ml purified calpain; lane 8, 200 μg/ml calpastatin; lane 9, 200 μg/ml E-64; lane 10, 100 μM MDL 28,170; lane 11, mature IL-1β standard.

likely that the calcium-ionophore-induced enhancement of precursor IL-1α processing in macrophages and monocytes is due to activation of this enzyme in cells. However, the conversion of precursor IL-1β to its mature form by I CE is not dependent on calcium (7–9). Thus the stimulatory effect of calcium ionophores on IL-1β processing must involve the action of a factor(s) other than I CE. Given the role of calpain in the processing of IL-1α, we explored the possibility that precursor IL-1β may also be susceptible to processing by calpain.

Our initial experiments focused on the effect of purified calpain on the in vitro cleavage of radiolabeled recombinant precursor IL-1β. When radiolabeled precursor IL-1β was incubated with increasing amounts of purified calpain in the presence of calcium, IL-1β was converted to several species with molecular masses (m) of 27, 17.4, 17.0, and 16.2 kDa (Fig. 1A, lanes 3–7). The 17-kDa band co-migrated with the mature IL-1β standard (lane 11). The appearance of these species was inhibited by the calpain-specific inhibitor calpastatin (lane 8) and the cysteine protease inhibitors E-64 and MDL 28,170 (lanes 9 and 10). When precursor IL-1β was incubated with calcium alone, there was no change in the molecular mass of the protein, indicating that the reticulocyte lysate used to translate the protein did not have any endogenous calpain activity (lane 2). To determine if any of the lower molecular mass calpain cleavage products were actually mature IL-1β, we evaluated their proteinase K sensitivity, since mature IL-1β is insensitive to proteinase K. Authentic recombinant mature IL-1β and the calpain-treated precursor IL-1β were treated with 10 μg/ml proteinase K (22 °C, 30 min) prior to analysis on SDS-polyacrylamide gels. Whereas proteinase K treatment of the calpain-treated samples resulted in a complete loss of all of the lower molecular mass species, there was no loss of the authentic mature IL-1β control sample (data not shown). Thus it is highly unlikely that any of these species is mature IL-1β.
was processed with approximately 1 unit/ml of the purified calpain (Fig. 4). Since the same amount of processing was obtained with approximately 4 μl of the P388D1 lysate (Fig. 4), we estimate that the lysate contains an equivalent amount of enzyme activity. Thus the calpain concentration in the lysate (as prepared from 10^8 cells) used in our experiments was approximately 7.5 units/ml. Although the sensitivity of precursor IL-1β to cleavage by purified calpain was not dramatically different from that of precursor IL-1α (Fig. 4, top), precursor IL-1β was markedly less sensitive to calpain cleavage in the presence of P388D1 lysate (Fig. 4, bottom). These results indicate that although P388D1 lysates possess a relatively high level of calpain activity and process precursor IL-1α as efficiently as purified calpain, precursor IL-1β is partially, but significantly, protected from cleavage by calpain in the lysate.

Is the 27-kDa Product Generated by Calpain a Better Substrate for ICE?—Having found that precursor IL-1β exhibits a differential sensitivity to purified calpain as opposed to calpain-containing macrophage lysates, we next considered the nature of the 27-kDa product generated in the presence of the P388D1 lysate. It was possible that this protein was simply an end point degradation product. On the other hand, it was also possible that the 27-kDa species represented a better substrate for ICE compared with precursor IL-1β. This latter possibility might provide an explanation for the stimulatory effect of calcium ionophores on processing of precursor IL-1β. If indeed the 27-kDa product is a better substrate for ICE, one would expect more mature IL-1β to be generated from the 27-kDa product than from precursor IL-1β in the presence of ICE. The observation that P388D1 lysates do not generate a substantial amount of mature IL-1β from the 27-kDa product raised the possibility that these cells do not express ICE activity. As shown in Fig. 5 (lane 1), lysates from P388D1 cells do not exhibit significant ICE activity. In contrast to P388D1 cells, THP.1 cell extracts were found, as expected (8, 32), to contain significant ICE activity (Fig. 5, lane 3). Inactive THP.1 extracts that were not incubated overnight did not process precursor IL-1β to mature IL-1β (data not shown). The absence of ICE activity in P388D1 extracts was due to the presence of an inhibitor of ICE, since incubation of activated THP.1 cell extracts with P388D1 cell extracts did not inhibit processing of radiolabeled precursor IL-1β to its mature form (data not shown). Thus the accumulation of the 27 kDa IL-1β species in reactions containing radiolabeled recombinant IL-1β and P388D1 lysate may be due to the lack of available ICE.

To test if the 27-kDa protein is a substrate for ICE, the 27-kDa species was initially generated by incubating precursor IL-1β with calpain-containing P388D1 lysates, the calpain in the lysates was inactivated using calpastatin, and THP.1 extract containing active ICE was added to the 27-kDa containing...
P388D1 lysate. As shown in Fig. 6, incubation of radiolabeled recombinant precursor IL-1α or precursor IL-1β proteins were incubated with increasing amounts of purified calpain or P388D1 lysate as described under "Materials and Methods." and the percentage of total input precursor IL-1α or precursor IL-1β protein remaining in each reaction was plotted against calpain concentration or lysate volume. Total input protein in each case was the average counts of precursor IL-1α or precursor IL-1β obtained from two different control reactions in which precursor protein remained intact.

P388D1 lysate. As shown in Fig. 6, incubation of radiolabeled precursor IL-1β with calpain-containing P388D1 lysate in the presence of calcium at 37 °C for 1 h generated the expected 27-kDa product (lane 3), the appearance of which was inhibited by calpastatin (lane 4). Further incubation of the 27-kDa product-containing lysate at 37 °C for 15 min with 200 µg/ml calpastatin resulted in a complete inactivation of the calpain content in the lysate as shown by the inability of a similarly incubated aliquot of P388D1 lysate to cleave precursor IL-1α in the presence of calcium (lane 1). Activated ICE-containing THP.1 extract which cleaves precursor IL-1β to the mature protein in the presence of EGTA (lane 6) was added to the 27-kDa product-containing P388D1 lysate and incubated for 1 h at 37 °C. The endogenous calpain content of the THP.1 extracts was inactivated by including leupeptin in the buffer used to make the extract. The leupeptin-treated extracts were unable to cleave radiolabeled precursor IL-1α protein (lane 2). Incubation of the 27-kDa product with active ICE-containing THP.1 extract resulted in its conversion to a slightly smaller product of 26 kDa (lane 5). This conversion was indeed due to ICE as it was inhibited by iodoacetamide, a general inhibitor of cysteine proteases (data not shown). These results indicate that although the 27-kDa product is susceptible to ICE, it is not converted to the mature IL-1β protein and very likely represents a degradation product of calpain cleavage.

Calpain Does Not Enhance the Activity of ICE—We next asked if the calcium ionophore-mediated enhancement of precursor IL-1β processing in cells might be due to an enhancement of ICE activity by calpain. THP.1 extracts were prepared without leupeptin so that they contained active calpain as well as ICE. The extracts were incubated overnight to activate ICE, and equal amounts of radiolabeled recombinant precursor IL-1β were incubated with these extracts 1) in the presence of calcium to ensure that both enzymes were active and 2) in the presence of EGTA to inhibit calpain activation to test if precursor IL-1β was converted to mature IL-1β to a greater extent in the calpain-containing ICE extracts as compared with the extracts in which calpain was not active. When precursor IL-1β was incubated with extracts containing only active ICE, 30–60% processing to the mature protein was obtained (Table I). When precursor IL-1β was incubated with extracts containing active ICE as well as calpain in the presence of calcium, the processing of precursor IL-1β to mature IL-1β was similar to that obtained when the precursor protein was incubated with
only active ICE (Table I). These results indicate that calpain does not enhance the activity of ICE.

Precursor IL-1β Is Not Degraded in Vivo by Calpain—Our in vitro results indicated that the calcium-mediated enhancement of precursor IL-1β processing to the mature protein in vivo is probably not due to a direct effect of calpain on precursor IL-1β or due to an indirect effect on ICE activity. Instead, it was possible that calpain may actually be involved in the degradation of precursor IL-1β in vivo. Siders et al. (26) found that P388D1 cells transfected with the precursor IL-1α DNA and treated with ionomycin completely processed and secreted the mature IL-1α protein within 2 h. However, cells transfected with the precursor IL-1β cDNA and treated with ionomycin did not process precursor IL-1β to mature IL-1β (26) or to the 27-kDa product that we have detected in the presence of P388D1 lysate (Fig. 3). Although the inability of P388D1 cells to process the precursor IL-1β protein to the mature form is due to a lack of ICE activity, the observation that a 27-kDa protein is not produced in vivo in cells containing active calpain implies that precursor IL-1β is protected from calpain-mediated degradation in vivo. In support of this conclusion, we found that precursor IL-1β is partially protected from degradation in calpain-containing lysates from P388D1 cells. To test if precursor IL-1β is stable in P388D1 cells containing active calpain, we examined the turnover rate of precursor IL-1β in P388D1 cells incubated in the presence or absence of ionomycin. As shown in Fig. 7, almost all of the radiolabeled precursor IL-1β initially present in the cells incubated in the presence or absence of ionomycin was recovered after 2 h. These results demonstrate that precursor IL-1β is not degraded under conditions that promote complete processing and release of IL-1α and imply that precursor IL-1β is protected in vivo from calpain.

Calpain Processes Precursor IL-1α in Vivo—Although it has been clearly demonstrated that calpain processes precursor IL-1α to mature IL-1α in vitro (5, 6), evidence that calpain is involved in the in vivo processing of precursor IL-1α has been lacking. To ensure that the ionomycin-mediated processing of precursor IL-1α in P388D1 cells is due to a member of the calpain family of proteases, we tested the effect of MDL 28,170, a cell-penetrating inhibitor of calpain (31), on ionomycin-induced IL-1α processing and secretion. In unstimulated cells, radiolabeled precursor IL-1α protein is not processed to mature IL-1α and secreted (26). In contrast, cells treated with ionomycin for 30-min process precursor IL-1α and the mature protein is recovered in the culture medium (Table I; Ref. 26). However, treatment of the cells with MDL 28,170 markedly inhibited the ionomycin-induced processing and secretion of IL-1α (Table I). These results indicate that a member of the calpain family of proteases is indeed involved in the processing of precursor IL-1α in vivo. Inhibition of calpain, however, did not prevent the ionomycin-induced secretion of mature IL-1α protein from cells transfected with a mature IL-1α-encoding expression vector (Table II), implying that whereas the calcium ionophore-mediated processing of precursor IL-1α is due to calpain, calcium ionophore-mediated enhancement of mature IL-1α secretion is due to another calcium-dependent factor. Inhibition of calpain also did not prevent the ionomycin-induced secretion of the mature IL-1β protein from cells transfected with a mature IL-1β expression vector, indicating that calpain is also not involved in the secretion of mature IL-1β (Table II).

Discussion

Since the precursor form of IL-1α is processed in vitro by calpain, a calcium-dependent protease, calcium ionophore-induced processing and secretion of IL-1α from monocytes and macrophages must be due, in part, to the activation of calpain and the subsequent accumulation of mature IL-1α, the form of IL-1α that is most efficiently secreted by cells (5, 6, 25, 26). In this study, we have demonstrated that calpain is indeed involved in the in vivo processing of precursor IL-1α, since MDL 28,170, a cell-penetrating inhibitor of calpain (31), prevented the ionomycin-induced processing of IL-1α in the P388D1 macrophage cell line (Table II). Since precursor IL-1β is processed by ICE, a protease that does not require calcium (7–9), it is not evident why calcium ionophores should also enhance the processing of precursor IL-1β. We were thus interested in determining if calpain was involved in precursor IL-1β processing and secretion. Although both purified calpain and calpain-containing P388D1 lysates cleave recombinant precursor IL-1α to a single major species, the 16-kDa mature IL-1α protein (Fig. 1B and Ref. 6), the pattern of proteolysis observed when recombinant precursor IL-1β is treated with the two calpain sources is substantially different (compare Fig. 1A with Fig. 3). Whereas purified calpain generated several species from the precursor IL-1β protein, calpain-containing lysates generated only two products, a major 27-kDa species and a minor 17-kDa species. In addition, whereas at higher concentrations of calpain, the cleavage of precursor IL-1β was almost complete, a substantial amount of precursor IL-1β remained intact in lysates containing equivalent amounts of calpain.

Table I

| Additions          | Percent processing<sup>a</sup> | Percent processing<sup>b</sup> |
|--------------------|-------------------------------|-------------------------------|
|                    | Exp. 1            | Exp. 2            |
| Inactive lysate<sup>c</sup> + EGTA | 2               | 2               |
| Activated lysate<sup>c</sup> + EGTA | 59              | 33              |
| - Calcium         | 35               | 35               |
| - Calcium + calpastatin | 38           | 30               |

<sup>a</sup> Radiolabeled recombinant precursor IL-1β was incubated with THP.1 cell extract at 37°C for 1 h. In the presence of 5 mM EGTA, 1 mM CaCl2, or 1 mM CaCl2 plus 200 μg/ml calpastatin as described under "Materials and Methods."<br><sup>b</sup> Percent processing = (mature IL-1β counts/6/12 (precursor IL-1β counts + 27-kDa species counts) * 100%.<br><sup>c</sup> Freshly prepared THP.1 cell extract that was not incubated overnight to activate ICE activity.
These results imply that the lysate contains a factor(s) that not only favors the generation of the 27-kDa protein, but also protects a significant amount of the precursor IL-1β protein from calpain proteolysis. Recombinant precursor IL-1β expressed in P388D1 cells was also protected from calpain-mediated proteolysis (Fig. 7), although the same conditions facilitate the complete processing and secretion of precursor IL-1α (26).

The estimated calpain concentration in the P388D1 lysate is approximately 7.5 units/ml. On a per cell basis there appears to be sufficient calpain to completely degrade precursor IL-1β. The in vivo stability of the precursor IL-1β protein in the presence of high levels of calpain is consistent with the notion that within the cell, precursor IL-1β, unlike precursor IL-1α, may be prevented from interacting with calpain. Where then are the precursor IL-1 proteins and their respective processing enzymes located within the cell? Both forms of precursor IL-1 lack an NH2-terminal as well as internal hydrophobic signal sequences and are believed to be synthesized on free ribosomes in the cytoplasm (4, 33). Agents such as brefeldin A and monensin do not block secretion of IL-1β from LPS-activated monocytes, indicating that IL-1 proteins do not traverse the classical secretory pathway (22). Immunoelectron microscopy studies have clearly shown that precursor IL-1β is present in the cytoplasmic ground substance of activated monocytes and absent in all other organelles such as the endoplasmic reticulum, the Golgi apparatus, plasma membrane, and lysosomal vesicles (34). The subcellular location of precursor IL-1α is not as well studied as that of precursor IL-1β, but it is also found mainly in the cytoplasmic fraction of activated monocytes (22). Although pro-ICE is found only in the cytoplasm of cells (32), the intracellular location of the active form of ICE is not known (35). Calpain, on the other hand, is a cytosolic enzyme that has been shown to translocate to the inner side of the plasma membrane under various stimulatory conditions (36, 37). Although calpain, ICE, and the precursor IL-1 proteins may be initially located within the cytoplasm in resting cells, the stimulation of macrophages with calcium ionophores may result in the translocation of the processing enzymes and precursor IL-1 proteins to the plasma membrane. Precursor IL-1α, as well as precursor IL-1β, have been shown to be myristoylated (38) and are theoretically capable of associating with membranes. The results of preliminary experiments indicate, however, that calpain is present only in the cytosolic fraction of ionomycin-treated P388D1 cells (data not shown). Nonetheless, our results are consistent with the hypothesis that precursor IL-1β is spared from calpain degradation in cells. However, the available evidence does not favor the view that IL-1β is sequestered in vesicles. It is more likely that in vivo, precursor IL-1β is associated with a factor(s) that protects it from cleavage by calpain. In support of this notion, Singer et al. (34) found that precursor IL-1β is present in the cytoplasm in the form of clusters, perhaps in association with a condensing molecule. Although our data suggest a physical separation between the two IL-1 species and their respective enzymes during processing, these barriers could break down under certain regulatory conditions, facilitating the down-regulation of IL-1β levels by calpain. Future studies regarding the intracellular location of the IL-1 substrates and their respective enzymes during resting and stimulatory conditions will allow us to evaluate this possibility.

The ability of ICE to process precursor IL-1β has been well established. Treatment of human peripheral blood monocytes with potent ICE-specific inhibitors prevents LPS and Staphylococcus aureus-induced processing and secretion of IL-1β (8, 28). In addition, monocytes and macrophages derived from mice carrying disrupted ICE alleles are unable to process and secrete IL-1β in response to LPS stimulation (29, 30). Although these observations favor ICE as the precursor IL-1β-processing enzyme, the THP-1 monocytic cells and human monocytes have been shown to contain surprisingly low levels of the p20/p10 form of ICE that is enzymatically active. In these cells, ICE is present predominantly as the inactive 45-kDa protein (32). Cytoplasmic extracts from these cells contain no detectable precursor IL-1β cleavage activity, even after LPS stimulation, and require overnight incubation at 4°C to generate enzyme activity (32). In light of the calcium ionophore-mediated enhancement of precursor IL-1β processing and secretion, it is possible that a calcium-dependent co-factor is involved in promoting the efficient autocatalysis of pro-ICE to active ICE. Alternatively, such a factor might enhance the activity of ICE, perhaps by stabilizing the enzyme or by converting precursor IL-1β to a form that is much more easily cleaved by ICE. Our results showed that in vitro, the activity of ICE is not enhanced by calpain, nor does ICE convert the calpain-generated 27-kDa species to the mature protein (Table I and Fig. 6). Although we cannot rule out the possibility that calpain may enhance ICE activity in vivo, our inability to detect a 27-kDa species in calpain-activated P388D1 cells argues against an indirect role of calpain in precursor IL-1β processing.

Calcium ionophores have been shown to enhance the secretion of the mature IL-1 proteins as well as several other proteins such as basic fibroblast growth factor, thioredoxin, L-29, and CBP30 that also lack an NH2-terminal secretory signal sequence (18, 20–24). Although the secretory mechanism for these proteins is unknown, several models have been proposed and include 1) the fusion of secretory vesicles containing these proteins with the plasma membrane, 2) extrusion of the proteins localized just below the plasma membrane in cytoplasmic blebs or podosomes which later release their contents extracellularly, and 3) direct traversal of these proteins across the plasma membrane assisted perhaps, by proteins belonging to the multidrug resistance family. Whatever the secretory mechanism for IL-1, it must be closely linked to the IL-1 processing.
machinery, since the mature forms of IL-1 have not been found in association with cells. Given the involvement of calpain in precursor IL-1α processing, we were interested in determining if calpain was also involved in the secretion of the mature IL-1α protein. Calpain might facilitate secretion by promoting podosome formation or by localized disruption of the cytoskeletal structure. Indeed, cytoskeletal proteins such as tubulin and microtubule-associated proteins have been shown to be susceptible to calpain digestion (27). Inhibition of calpain by MDL 28,170, however, did not inhibit ionomycin-induced secretion of recombinant mature IL-1α from P388D1 cells (Table II). This finding implies a calcium-dependent factor other than calpain in IL-1α secretion and underscores the independent nature of the processing and secretory events (26). Ionomycin-induced secretion of mature IL-1α from the P388D1 cells was also unaffected by MDL 28,170, indicating that calpain is not also involved in IL-1β secretion. If calpain is not involved in the secretion of the mature IL-1 proteins or in precursor IL-1β processing, what other calcium-dependent factors might regulate these two processes? Other members of the EF-hand family of calcium-binding proteins implicated in protein secretion are calmodulin and calcyclin. Calmodulin has been shown to be involved in the glucose-stimulated release of insulin from pancreatic β cells (39), catecholamine release from adrenal medullary cells (40), and acid secretion from oesophageal cells (41). Calcium-dependent insulin release from streptolydin O-permeabilized pancreatic β cells has also been shown to be enhanced by calcyclin and inhibited by calcyclin antibodies (42). The calmodulin antagonists trifluoperazine-dimaleate and W-7, however, did not inhibit ionomycin-induced secretion of the mature IL-1α and -β proteins from P388D1 cells in preliminary experiments. Members of the annexin family of calcium-binding proteins have also been shown to be involved in exocytosis, perhaps by promoting membrane contact between vesicles and the cytoplasmic face of the plasma membrane and also in endocytosis where they may facilitate the closing off of vesicles from the plasma membrane (43). In view of the observation that methylamine, an inhibitor of exocytosis and endocytosis prevents the secretion of IL-1β as well as several other proteins which lack an N-terminal secretory signal such as basic fibroblast growth factor (23), thioredoxin (24), and carbohydrate-binding protein 30 (21), it is possible that annexins may function in the IL-1 secretory pathway. Processing and secretion of IL-1β, like that of IL-1α, may involve two different calcium-dependent factors. Secretion of both the mature IL-1 species may even involve a common calcium-dependent factor. Development of a permeabilized cell system and the availability of compounds that inhibit the function of calcium-binding proteins should facilitate the analysis of the role of these proteins in IL-1 secretion.

Although it was well accepted that processing of precursor IL-1α and IL-1β involves two different enzymes, very little is known about where these processes occur within the cell. The data presented in this study indicate that the two IL-1 species and their respective enzymes must be differentially localized, at least during a portion of the processing and secretory pathway. Processing and secretion, which are clearly independent events, are nonetheless closely linked as evidenced by the absence of the mature IL-1α and -β proteins within cells. Clearly, further studies regarding the precise intracellular location of each of the two IL-1 species and their possible co-localization with calpain and ICE need to be done in order to better understand the processing and secretion of IL-1.

2 U. Kavita and S. B. Mizel, unpublished observations.
