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Immunology

Identification of calcium-activated neutral protease as a processing enzyme of human interleukin 1α

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ABSTRACT **We describe here the involvement of calcium-activated neutral protease (CANP or calpain, EC 3.4.22.17) in calcium-dependent proteolytic processing of the precursor of human interleukin 1α (IL-1α) into mature IL-1α. Calcium ionophore ionomycin enhanced proteolytic processing of pre-IL-1α and the release of mature IL-1α either from lipopolysaccharide (LPS)-activated human adherent mononuclear cells or from a human bladder carcinoma cell line (HTB9 5637) that constitutively produces human IL-1α and -β. The proteolytic processing of pre-IL-1α was completely inhibited by EGTA. Similar calcium-dependent proteolytic processing of pre-IL-1α was also observed with lysates of either LPS-activated human adherent mononuclear cells or HTB9 5637 cells. Since the optimal pH for processing was between 7 and 8, and E-64 (a cysteine protease inhibitor) and leupeptin (a serine and cysteine protease inhibitor) both inhibited this processing by cell lysates, we hypothesized that a calcium-activated neutral protease, CANP, might be responsible for this processing. This hypothesis was supported by data showing that the specific CANP inhibitor peptide inhibited this proteolysis in cell lysates in a dose-dependent fashion (IC_{50} = 0.05 μM) and that treatment of pre-IL-1α with purified CANP yielded the 17-kDa mature form of IL-1α, which has an amino terminus identical with that reported for mature human IL-1α. Taken together, these findings indicate that calcium-dependent proteolytic processing of pre-IL-1α is selectively mediated by CANP.**

Interleukin 1 (IL-1), produced by a variety of cells, is widely known to manifest multiple biological activities on various types of target cells (1). Two biochemically distinct forms of IL-1, IL-1α and -β, have been genetically cloned (2–4). Although both types of IL-1 are lacking in a signal peptide, they have been reported to be present not only in the cytosol (5) but also in the cell membrane (6) and extracellularly. Since mature IL-1 has a molecular mass of 17 kDa, precursor IL-1 (pre-IL-1; molecular mass of 33–35 kDa) presumably is processed by an as-yet-unidentified proteolytic enzyme(s) to generate mature IL-1.

A calcium ionophore has been reported to augment production of extracellular IL-1 by lipopolysaccharide (LPS)-stimulated human adherent mononuclear cells through the entry of exogenous calcium into cells (7). LPS also has been shown to increase intracellular calcium in macrophages (8). On the other hand, there are some cell types, such as HTB9 5637, which constitutively produce pre-IL-1 but do not generate and release mature IL-1 effectively (ref. 9; unpublished results). We, therefore, decided to test the possibility that calcium plays some roles in release and/or processing of IL-1 by comparative studies of this cell line and human adherent mononuclear cells. We examined the effects of calcium ionophore on the release and processing of IL-1 in detail, and we identified a major calcium-dependent proteolytic processing enzyme of pre-IL-1α as calcium-activated neutral protease (CANP or calpain; EC 3.4.22.17).

MATERIALS AND METHODS

Materials. L-[^{35}S]Methionine (Tran^{35}S-label, 1037 Ci/mmol; 1 Ci = 37 GBq) was purchased from ICN. Recombinant human IL-1α and -β were generous gifts from Dainippon Pharmaceutical (Osaka) and Otsuka Pharmaceutical (Tokushima, Japan), respectively. Rabbit antibodies to human IL-1α or -β were made by immunizing rabbits with each recombinant material. These two antibodies were found to be specific for IL-1α or -β as evidenced by Western blotting analysis, neutralization assays, and immunoprecipitation (10). All the protease inhibitors, including 3,4-dichloroisocoumarin (DCL), E-64, leupeptin, pepstatin, phenylmethanesulfonyl fluoride (PMSF), and phosphoramidon, as well as ionomycin, were purchased from Boehringer Mannheim. LPS from *Escherichia coli*, 055:B55, was obtained from Difco. CANP active at micromolar calcium (μCANP) was purified from rabbit skeletal muscle (11). One unit of CANP activity was defined as the amount of enzyme that catalyzes casein as substrate to increase by 1.0 absorbance unit at 280 nm in 1 hr as described in ref. 11. Specific CANP inhibitor peptide (domain I, residues 184–217) was synthesized by a peptide synthesizer (Applied Biosystems; model 430A) and purified by HPLC (12).

Radiolabeling of Human Adherent Mononuclear Cells and Human Bladder Carcinoma Cells (HTB9 5637) with L-[^{35}S]Methionine. Subpopulations enriched in human monocytes were obtained by adherence of peripheral blood mononuclear cells to plastic (nearly 90% of adhering cells were positive for nonspecific esterase), cultured at 37°C for 2 hr in the presence of LPS (10 μg/ml), and labeled by incubation at 37°C for an additional 2 hr with 0.1 mCi of L-[^{35}S]methionine (Tran^{35}S-label) in 1 ml of methionine-free RPMI 1640 medium containing 1% dialyzed fetal calf serum. The human bladder carcinoma cells (HTB9 5637) (American Type Culture Collection) were labeled by incubation at 37°C for 2 hr with 0.1 mCi of L-[^{35}S]methionine. After incubation, adherent mononuclear cells or HTB9 5637 cells were treated with ionomycin (1–6 μM) or an equal volume of vehicle [dimethyl sulfoxide (Me_2SO)] for 30 min. The resultant culture supernatants were immunoprecipitated by rabbit antibodies to human IL-1α or

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Abbreviations: IL-1, interleukin 1; LPS, lipopolysaccharide; CANP, calcium-activated neutral protease; CHAPS, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate; DCL, 3,4-dichloroisocoumarin; PMSF, phenylmethanesulfonyl fluoride; Me_2SO, dimethyl sulfoxide.

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-β and staphylococcal protein A-Sepharose (Pharmacia) as previously described (10). In some cases, these cells were treated with 9 mM 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) in 20 mM Tris·HCl buffer (pH 8.0) at room temperature for 10 min to obtain cell lysates.

SDS/PAGE. Radiolabeled samples were analyzed by SDS/PAGE (13). Labeled proteins were detected by fluorography using Kodak XAR film, and relative intensity was estimated by densitometry tracing. Because pre-IL-1α and mature IL-1α contain seven and three methionine residues respectively, the percentage of maturation from pre-IL-1α to mature IL-1α was calculated by the following formula:

$$\left(\frac{\text{intensity of 17-kDa}}{\frac{3}{7}\text{ (intensity of 33-kDa)} + \text{intensity of 17-kDa}}\right) \times 100.$$

Sequencing of Cleavage Product. Pre-IL-1α was purified by immunoaffinity column chromatography as previously described (10), starting from HTB9 5637 cell lysate labeled with L-[^{35}S]methionine or L-[^{3}H]leucine. Labeled pre-IL-1α was cleaved by incubating with purified CANP (90 units/ml) at 37°C for 30 min. Mature labeled IL-1α was purified from this incubation mixture by SDS/PAGE followed by extraction from gel slices with 0.5% SDS twice at 37°C for 1 hr. Amino-terminal sequencing of the labeled cleavage product was done by automated Edman degradation on a Shimadzu model PSQ-1 gas-phase sequencer.

RESULTS

Calcium Ionophore-Induced Proteolytic Processing of Pre-IL-1α. Since calcium ionophores have been reported to augment production of extracellular IL-1 (7), we hypothesized that calcium may affect proteolytic processing of IL-1, its release, or both. We, therefore, treated LPS-activated human adherent mononuclear cells with ionomycin at various concentrations, 1–6 μM, for 30 min and then immunoprecipitated the resultant supernatants to examine whether ionomycin induced the cells to release pre- and/or mature IL-1. As shown in Fig. 1*A*, lanes 2 and 3, ionomycin (6 μM) could enhance proteolytic processing of pre-IL-1α to mature IL-1α. In contrast, ionomycin did not enhance proteolytic processing of pre-IL-1β to mature IL-1β (Fig. 1*A*, lane 6). This proteolytic processing of pre-IL-1α was completely blocked by 1 mM EGTA (Fig. 1*A*, lane 4), suggesting that the entry of calcium from outside of the cells is necessary for this proteolysis. Ionomycin also induced proteolytic processing of pre-IL-1α to mature IL-1α in HTB9 5637 cells, which are constitutive producers of IL-1α and -β, and induced their release of both pre-IL-1α and mature IL-1α (Fig. 1*B*, lanes 1–6). Again, in the case of IL-1β, there was no formation of mature IL-1β, although pre-IL-1β was released (Fig. 1*B*, lanes 8–13).

Calcium-Dependent Proteolytic Processing of Pre-IL-1α in Cell Lysates. Next, we examined whether calcium-dependent proteolytic processing of pre-IL-1α also occurs in lysates of HTB9 5637 cells. As shown in Fig. 2, pre-IL-1α could be cleaved into 17-kDa IL-1α in cell lysates of HTB9 5637 cells treated with CHAPS in the presence of 1 mM calcium (lane 1). When 2 mM EGTA was added to the cell lysates, this proteolytic processing was completely blocked (lane 2), indicating that this proteolysis absolutely requires calcium. Again, pre-IL-1β could not be cleaved into 17-kDa IL-1β in cell lysates in the presence of calcium (data not shown), confirming the results obtained by using ionomycin-treated human adherent mononuclear cells. The same results were obtained when lysates of LPS-activated human adherent mononuclear cells were used (data not shown).

We then determined how much calcium is required for this proteolysis. As shown in Fig. 3*A*, half-maximal production of

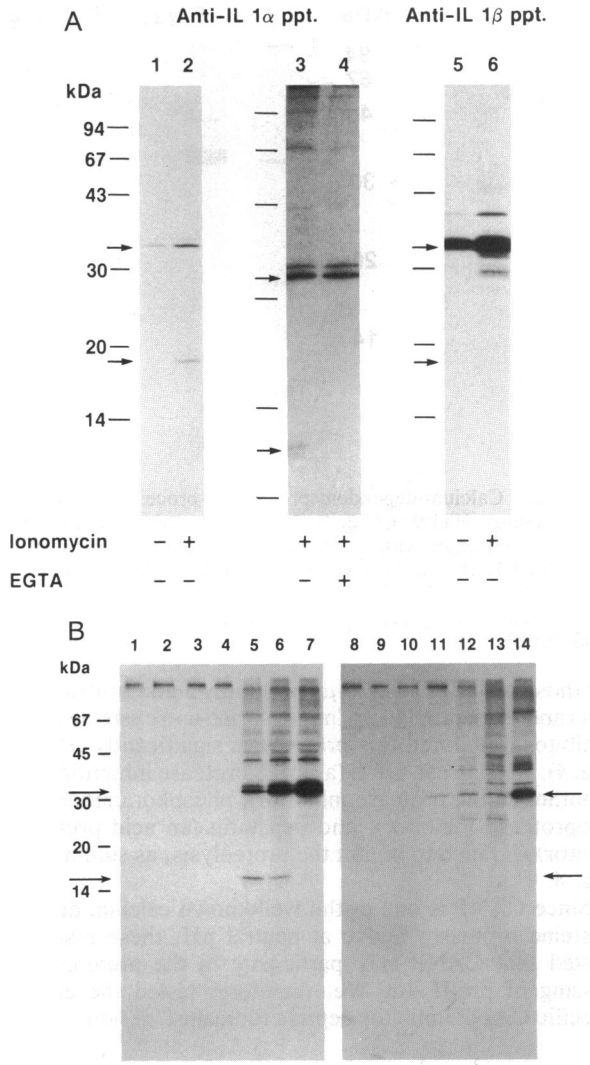

FIG. 1. Calcium ionophore-induced proteolytic processing of pre-IL-1α and -β. (*A*) LPS-activated human adherent mononuclear cells (10^6 cells per ml per well in six-well plates) were labeled with L-[^{35}S]methionine and then treated with 6 μM ionomycin (lanes 2, 3, 4, and 6) or with an equal amount of Me_2SO (lanes 1 and 5) for 30 min at 37°C. In the case of lane 4, EGTA (1 mM) was added together with ionomycin. Each supernatant was harvested just after incubation with ionomycin or Me_2SO. The resultant supernatants were then immunoprecipitated (lanes 1, 2, 3, and 4 by anti-IL-1α antibodies; lanes 5 and 6 by anti-IL-1β antibodies) and analyzed by SDS/PAGE. Lanes 1 and 2, lanes 3 and 4, and lanes 5 and 6 show the results of different experiments. (*B*) HTB9 5637 cells (10^6 cells per ml per well in six-well plates) were labeled with L-[^{35}S]methionine and then treated with ionomycin at 0.75 μM (lanes 2 and 9), 1.5 μM (lanes 3 and 10), 3 μM (lanes 4 and 11), 6 μM (lanes 5 and 12), or 12 μM (lanes 6 and 13) or with an equal amount of Me_2SO (lanes 1 and 8) for 30 min at 37°C. Each supernatant was harvested just after incubation with ionomycin or Me_2SO. The resultant supernatants or cell lysates (lanes 7 and 14) were then immunoprecipitated (lanes 1–7 by anti-IL-1α antibodies; lanes 8–14 by anti-IL-1β antibodies) and analyzed by SDS/PAGE.

mature IL-1α was obtained between 1 and 100 μM calcium. It was possible to examine the time course of this proteolysis either by adding calcium to cell lysates containing EGTA to initiate the proteolysis or by adding EGTA to stop the reaction. As shown in Fig. 3*B*, the reaction reached its maximum after 30 min at 37°C. Next, the optimal pH of this calcium-dependent proteolysis of pre-IL-1α was determined. As shown in Fig. 3*C*, it was found to be between 7 and 8.

We then examined the effects of various kinds of protease inhibitor on the calcium-dependent proteolysis of pre-IL-1α.

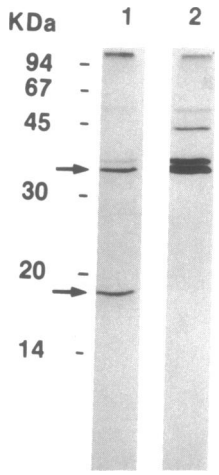

FIG. 2. Calcium-dependent proteolytic processing of pre-IL-1α in cell lysates. HTB9 5637 cells (10^6 cells per ml per well in six-well plates) were labeled with L-[^{35}S]methionine and then lysed with 1 ml of 9 mM CHAPS in 20 mM Tris·HCl buffer (pH 8.0) with 1 mM calcium (lane 1) or 2 mM EGTA (lane 2). After incubation for 30 min at 37°C, each cell lysate was immunoprecipitated and analyzed by SDS/PAGE.

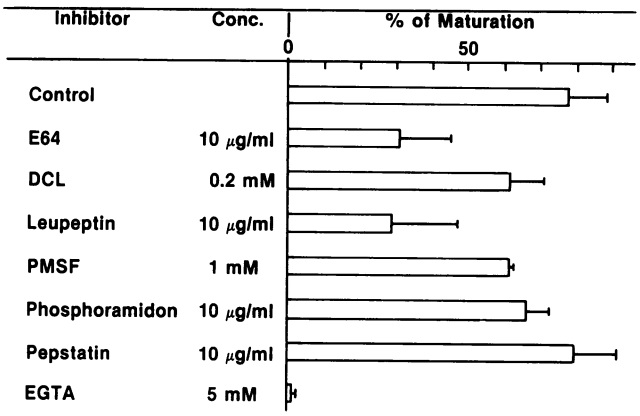

FIG. 4. Inhibition of proteolytic processing of pre-IL-1α by various protease inhibitors. Labeled HTB9 5637 cell lysates were prepared with 9 mM CHAPS in 20 mM Tris·HCl buffer. Reaction was initiated by adding 1 mM calcium and various protease inhibitors. The final concentration of each inhibitor is given. The mixtures were incubated for 30 min at 37°C and then each cell lysate was immunoprecipitated and analyzed by SDS/PAGE. The experiment was performed three times in exactly the same way, and the results are expressed as the mean ± SEM.

Of those tested, E-64 (10 μg/ml) (a cysteine protease inhibitor) and leupeptin (10 μg/ml) (a serine and cysteine protease inhibitor), inhibited this proteolysis significantly ($P < 0.01$; Fig. 4). In contrast, DCL (a serine protease inhibitor), PMSF (another serine protease inhibitor), phosphoramidon (a metalloprotease inhibitor), and pepstatin (an acid protease inhibitor) all failed to inhibit the proteolysis, as summarized in Fig. 4.

Since CANP is one of the well-known calcium-dependent cysteine proteases active at neutral pH, these results suggested that CANP may participate in the proteolytic processing of pre-IL-1α. We, therefore, tested the effects of specific CANP inhibitor peptide (domain I, residues 184–217 of CANP inhibitor or calpastatin) on this proteolysis, because this peptide has been reported to selectively inhibit CANP but not other cysteine proteases (12). The results shown in Table 1 clearly demonstrated that this proteolysis was inhibited by this peptide in a dose-dependent manner (IC_{50} = 0.05 μM). Furthermore, pre-IL-1α processing mediated by purified CANP was also inhibited by this peptide in a similar dose-dependent manner (Table 1). In addition, monoclonal antibody to CANP (P-1) kindly provided by Ronald L. Mellgren (Ohio University) could inhibit calcium-dependent processing of pre-IL-1α in cell lysates to an expected extent (30%).

We attempted to digest pre-IL-1α immunoprecipitated with protein A-Sepharose-bound anti-IL-1α antibody by pu-

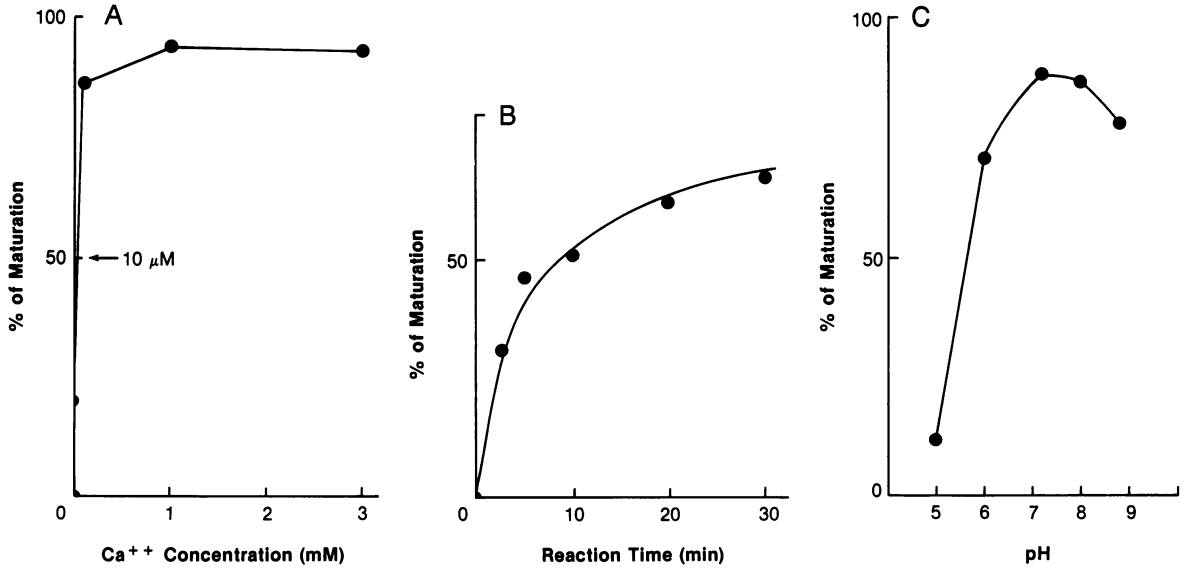

FIG. 3. (*A*) Dependency of the proteolytic processing of pre-IL-1α in cell lysates on calcium concentration. Labeled HTB9 5637 cells were lysed with 9 mM CHAPS in 20 mM Tris·HCl buffer (pH 8.0) containing various concentrations of calcium for 30 min at 37°C. Each cell lysate was immunoprecipitated and analyzed by SDS/PAGE. (*B*) Time course of proteolytic processing of pre-IL-1α in cell lysates. Labeled HTB9 5637 cell lysates were prepared in the presence of 2 mM EGTA. Proteolytic processing was initiated by adding 5 mM calcium and was stopped by adding 10 mM EGTA after various time periods. Each cell lysate was immunoprecipitated and analyzed by SDS/PAGE. (*C*) pH dependency of calcium-dependent proteolytic processing of pre-IL-1α. Labeled HTB9 5637 cell lysates were prepared with 9 mM CHAPS in 20 mM sodium acetate buffer (pH 5.0) or Tris·HCl buffer (pH 6.0, 7.2, 8.0, 8.8) in the presence of 2 mM EGTA. Reaction was initiated by adding 5 mM calcium. The mixture was incubated for 30 min at 37°C, and then 10 mM EGTA and 0.1 mM Tris·HCl buffer (pH 8.0) were added to stop the reaction. Each cell lysate was immunoprecipitated and analyzed by SDS/PAGE.

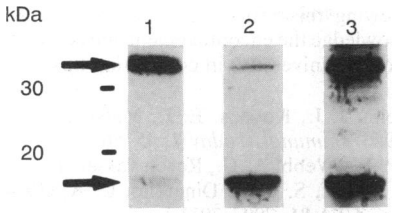

FIG. 5. Proteolysis of pre-IL-1α by CANP or trypsin. Labeled HTB9 5637 cell lysates were prepared with 9 mM CHAPS in 20 mM Tris·HCl buffer (pH 8.0) and immunoprecipitated. Each immunoprecipitate bound to protein A-Sepharose was digested by buffer only (lane 1), trypsin (0.01 μg/ml; lane 2, or CANP (10 units/ml; lane 3) for 30 min at 37°C and then analyzed by SDS/PAGE.

rified CANP or by trypsin as a control. As shown in Fig. 5, lane 3, CANP generated a 17-kDa peptide from pre-IL-1α with an efficiency of around 50%. The molecular mass of the digestion product was found to be nearly identical with that of a trypsin product (lane 2) and also that of naturally occurring mature IL-1α (data not shown). However, the cleavage site of pre-IL-1α is known to be distinct from the cleavage site for trypsin (14).

Finally, the sequence of the amino terminus of the 17-kDa peptide, generated by incubation of immunoaffinity-purified [^{3}H]leucine- or [^{35}S]methionine-labeled pre-IL-1α with CANP, was determined by automated Edman degradation. Peaks for labeled leucine and methionine were detected at cycles 1 (and possibly 18) and 9 as shown in Fig. 6. This result as well as molecular mass of the cleavage product was compatible with cleavage occurring between Phe-118 and Leu-119 (14). When we used the 17-kDa peptide generated by incubation of labeled cell lysates in the presence of calcium, identical sequence data were obtained (data not shown), further confirming that calcium-dependent processing of pre-IL-1α is mediated by CANP.

Table 1. Inhibition of processing of pre-IL-1α by synthetic CANP inhibitor peptide

Exp.	Peptide, μM	EGTA, mM	% maturation*	% inhibition†
1. Ca^{2+}, 1 mM	0	0	63 ± 2	0
	5	0	2 ± 2	97 ± 3
	0.5	0	15 ± 5	76 ± 8
	0.05	0	38 ± 5	40 ± 8
	0.005	0	67 ± 5	−6 ± 8
	0	2	0 ± 0	100 ± 0
2. CANP, 5 U/ml	0	0	87 ± 15	0
	5	0	5	94
	0.5	0	11	87
	0.05	0	72	17
	0.005	0	90	−3
	0	2	2	98

In exp. 1 the lysates of HTB9 5637 cells were incubated in 20 mM Tris·HCl (pH 8) containing 1 mM Ca^{2+} and various concentrations of synthetic CANP inhibitor peptide (0–5 μM) or EGTA (2 mM) for 30 min at 37°C. Immunoprecipitation and analysis by SDS/PAGE followed. In exp. 2 pre-IL-1α immunoprecipitated with anti-IL-1α antibodies and protein A-Sepharose was digested by CANP (5 units/ml) in the presence of various concentrations of synthetic CANP inhibitor peptide (0–5 μM) or EGTA (2 mM) for 30 min at 37°C. Boiling with SDS/PAGE sample buffer and analysis by SDS/PAGE followed.

*Determined according to the method described in *Materials and Methods*.

†Calculated by the following formula, in which "control" is 1 mM Ca^{2+} or CANP at 5 units/ml:

$$\left(\frac{\%\text{ maturation (control)} - \%\text{ maturation (experimental)}}{\%\text{ maturation (control)}}\right) \times 100.$$

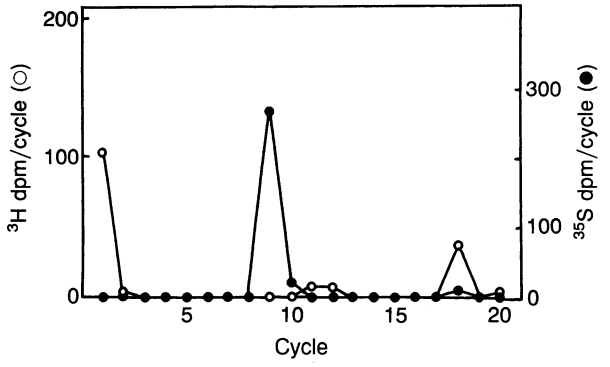

FIG. 6. Sequence analysis of 17-kDa cleavage product. [^{3}H]Leucine- or [^{35}S]methionine-labeled pre-IL-1α was cleaved with CANP. Each 17-kDa cleavage product was isolated, mixed with carrier, and subjected to Edman degradation. Fractions from each cycle were counted for radioactivity.

DISCUSSION

This paper presents several lines of evidence indicating that calcium-dependent proteolytic processing of pre-IL-1α is mediated by CANP. First, this processing absolutely required the entry of calcium into the cells in the case of ionomycin-treated human adherent mononuclear cells or the presence of calcium in the case of lysates of either LPS-activated human adherent mononuclear cells or HTB9 5637 cells. Second, this processing was inhibited either by E-64 (a cysteine protease inhibitor) or by leupeptin (a serine and cysteine protease inhibitor). Although the reason for this partial inhibition is not known, there is a report showing that CANP in its purified form is very sensitive to leupeptin and E-64 but that crude preparations of CANP are far less susceptible to the same inhibitors (15). The possibility that other enzymes can also contribute to the proteolytic cleavage of pre-IL-1α cannot be excluded. Third, this processing occurred most efficiently at neutral pH. Fourth, a specific CANP inhibitor peptide inhibited this proteolysis in the same dose-dependent fashion as it did CANP-mediated proteolysis of pre-IL-1α. Finally, purified CANP generated a 17-kDa peptide from pre-IL-1α whose amino terminus was identical with that reported for human mature IL-1α (14). To our knowledge, this report is the first to identify a processing enzyme for pre-IL-1α.

The processing enzyme for IL-1β is distinct from CANP. As shown in Fig. 1, ionomycin enhanced the processing of pre-IL-1β to 29-kDa intermediate IL-1β but not to mature IL-1β. Furthermore, EGTA failed to block the processing of pre-IL-1β in lysates of LPS-stimulated human monocytes (Y.K., unpublished results). (It should be noted here that the formation of mature IL-1β in LPS-stimulated human monocytes varied with donors.) Recently Black *et al.* (16) and Kostura *et al.* (17) have reported that the processing protease for IL-1β is specifically found in monocytes or monocyte-like cell lines and that the activity is blocked by iodoacetate and *N*-ethylmaleimide but not other class-specific inhibitors and is therefore distinct from CANP.

Besides CANP, there is another well-characterized calcium-dependent protease, the *KEX2* endopeptidase found in the yeast *Saccharomyces cerevisiae* (18). This enzyme is required for proteolytic processing of α mating factor and killer toxin precursors and is a serine protease with an acidic pH optimum. There are several other processing enzymes that have been reported to require calcium and to show an acidic pH optimum, such as the enzyme responsible for the conversion of proinsulin into mature insulin, although it is not known whether these enzymes are serine proteases. Since the enzyme(s) for pre-IL-1α processing in the system described here

is unique in requiring calcium, has a neutral pH optimum, and is a cysteine protease, it is highly probable that the enzyme(s) is CANP. Furthermore, it should be pointed out that IC_{50} of synthetic CANP inhibitor peptide in our proteolytic processing of pre-IL-1α (0.05 μM) is close to that observed in the digestion of casein using purified CANP (0.016 μM) (12).

According to the model proposed by Pontremoli *et al.* (19), autocatalytic activation of pro-CANP occurs at the inner surface of the membrane in the presence of micromolar calcium and phospholipid, and CANP then becomes active as a membrane-bound enzyme. Since the activated form of CANP has never been isolated from tissues, it may be unstable, or only a small portion of proCANP may be activated under ordinary conditions (20). Furthermore, although there has been a report showing the presence of CANP and CANP inhibitor in the synovial fluid of the knee joint of patients, it was not possible to detect any CANP activity before separation with DEAE-cellulose column chromatography (21). Therefore it is likely that intracellular CANP may cleave pre-IL-1α before release, although we cannot exclude the possibility that extracellular CANP also may participate in this proteolysis. It should be noted here that extent of formation of mature IL-1α in cell lysates varies from 60% to 95% and that the digestibility of pre-IL-1α with CANP also varies from experiment to experiment. Although the reason for these observations is not known, it may be the presence of inhibitors to CANP, instability of CANP, or posttranslational modification or pre-IL-1α such as phosphorylation (10) and acylation (22) which may change the susceptibility of pre-IL-1α to CANP.

We have to consider the possibility that pre-IL-1α binds to the membrane by an unknown mechanism in response to a rise in intracellular calcium. In relation to this, we propose that the phosphorylated form of pre-IL-1α binds to acidic phospholipids in the presence of submillimolar calcium. Since CANP is believed to function on inner membranes, it is puzzling that proteolyzed IL-1α has never been detected intracellularly. Although we don't know the correct answer as yet, it is possible that digestion of membrane-associated pre-IL-1α by CANP is coupled with release of mature IL-1α. In support of this idea, human platelet membrane glycoprotein Ib was reported to be cleaved by endogenous CANP and released in response to calcium ionophore A23187 (23). Furthermore, since the submission of this paper for review, calcium ionophore A23187 was reported to enhance both secretion and processing of mouse IL-1α (24). Overall, our results and those of others suggest that, although CANP may not be the only enzyme that can proteolytically cleave pre-IL-1α to the mature IL-1α form, it may be ideally located to be the major enzyme responsible for the proteolytic processing of IL-1α.

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