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Processing and Activation of Pro-Interleukin-16 by Caspase-3*

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Interleukin-16, a proinflammatory cytokine produced in CD8$^+$ lymphocytes, is synthesized as a precursor protein (pro-IL-16). It is postulated that the C-terminal region of pro-IL-16 is cleaved, releasing bioactive IL-16. To characterize IL-16 cleavage, we transfected COS cells with a cDNA encoding a ~50-kDa form of pro-IL-16. Transfected COS cells released a ~20-kDa IL-16 cleavage product shown to consist of the 121 C-terminal residues of pro-IL-16 by immunoblotting and amino acid sequencing. Cleaved IL-16, but not pro-IL-16, exhibited lymphocyte chemoattractant activity. A C-terminal ~20-kDa IL-16 polypeptide was also released when pro-IL-16 was treated with concanavalin A-stimulated CD8$^+$ lymphocyte lysate. Cleavage occurred after an Asp, suggesting involvement of a caspase (interleukin-1β-converting enzyme/CED-3) family protease. Using recombinant caspases and granzyme B, we determined that pro-IL-16 cleavage is mediated only by caspase-3. Relevance to pro-IL-16 processing in primary lymphocytes was supported by identifying the p20 subunit of activated caspase-3 in stimulated CD8$^+$ lymphocytes and by inhibition of CD8$^+$ lymphocyte lysate-mediated cleavage with Ac-DEVD-CHO. Pro-IL-16 is a substrate for caspase-3, and cleavage by this enzyme releases biologically active IL-16 from its inactive precursor.

Interleukin-16 (IL-16)[1] is a pleiotropic proinflammatory cytokine originally identified by Center and Cruikshank in 1982 (1, 2). IL-16 is produced and secreted predominantly by CD8$^+$ T lymphocytes, and it exerts chemoattractant, growth factor, and other activities on lymphocytes, monocytes, and eosinophils by interaction with CD4 (3). Biologically active IL-16 is secreted from CD8$^+$ T cells in response to antigen, mitogen, histamine (4), or serotonin stimulation (5), but the mechanism of IL-16 secretion remains undefined. Natural biologically active secreted IL-16 has a molecular mass of ~56 kDa by Sephadex molecular sieve chromatography, which appears to represent noncovalent association of four identical polypeptide chains (2, 6). Monomers of IL-16 migrate on SDS-PAGE in the range of 17–20 kDa (6). Recent evidence from cDNA cloning indicates that the mature and biologically active secreted IL-16 is derived from the C-terminal region of a larger intracellular precursor protein (pro-IL-16) (7). Like interleukin-1β (IL-1β), the predicted amino acid sequence of IL-16 lacks a signal sequence, suggesting that maturation and release of IL-16 does not proceed by conventional mechanisms.

Reviewing the predicted pro-IL-16 protein sequence, we identified three potential protease cleavage sites that might be targets for processing to release a C-terminal fragment in the size range of secreted IL-16. In this report we present evidence that COS cells transfected with IL-16 cDNA synthesize and naturally process pro-IL-16 and secrete the biologically active mature IL-16. Based on analysis of this processing in COS cells and experiments with primary lymphocytes, we determined that pro-IL-16 is specifically cleaved by a member of the caspase (interleukin-1β-converting enzyme/CED-3 protease) family, caspase-3. We also demonstrated that proteolytic processing of pro-IL-16 was required for the chemoattractant function of mature IL-16. This is the first report of involvement by caspase-3 in the processing of a cytokine precursor protein.

EXPERIMENTAL PROCEDURES

Expression Constructs—A mammalian expression plasmid (pXM-W16) was produced by ligating a 2150-bp IL-16 cDNA (6) fragment to the COS cell expression vector pXM. This cDNA encodes a pro-IL-16 fragment with a predicted molecular mass of ~40 kDa. With pXM-W16 as template, recombination polymerase chain reaction was used to generate single amino acid changes as listed in Fig. 4*B*. All mutations were confirmed by sequence analysis of both DNA strands.

Expression of Pro-IL-16 and Pro-IL-16 Derivatives—COS cells were suspended in serum-free Dulbecco's modified Eagle's medium (Sigma) at 5×10^6 cells/ml. Four $\times 10^6$ cells (0.8 ml) were placed in a cooled electroporation cuvette with a 0.4-cm gap, 10 μg of plasmid DNA was added, and the cells were electroporated at 0.3 kV, 500 microfarads (yielding a time constant of 11–12 ms). Cells were allowed to recover in the cuvette at room temperature for 10 min and were then plated in a 100-mm dish in Dulbecco's modified Eagle's medium with 10% fetal bovine serum. After 24 h, cells were washed twice with PBS and then changed to serum-free Dulbecco's modified Eagle's medium. After an additional 48 h, conditioned medium was collected, cells and debris were removed by centrifugation, and the medium was stored at −20 °C.

Western Blot Analysis—Conditioned medium from transfected COS cells was concentrated 50-fold using a Centricon-10 device (Amicon) before electrophoresis. Transfected COS cells were washed once with PBS, released by scraping, and lysed in 60 mM Tris-HCl (pH 6.8), 1% SDS, 10% glycerol, and 5% β-mercaptoethanol (protein lysis buffer). Both concentrated conditioned medium and cell lysate were boiled in SDS sample buffer and subjected to electrophoresis through a 15% SDS-polyacrylamide gel. Proteins were electrophoretically transferred to nitrocellulose membrane and probed with mouse monoclonal anti-human-recombinant IL-16 antibody (6) designated mAb 14.1. The secondary antibody, an anti-mouse immunoglobulin labeled with horseradish peroxidase (Santa Cruz Biotechnology), was used at a dilution of

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[1] The abbreviations used are: IL-16, interleukin-16; rhIL-16, recombinant human IL-16; Con A, concanavalin A; mAb, monoclonal antibody; PBMC, peripheral blood mononuclear cell(s); PBS, phosphate-buffered saline; IL-1β, interleukin-1β; PAGE, polyacrylamide gel electrophoresis; bp, base pair.

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1:5000. Visualization of the signal was by electrochemiluminescence (Amersham Corp.).

Quantitation of Pro-IL-16 and Mature IL-16—Known amounts of recombinant human Il-16 (rhIL-16) expressed in *Escherichia coli* were included in every immunoblot of IL-16 expressed by COS cells. Immunoblots were then analyzed by laser densitometry, and the mass of each COS cell-produced protein was determined by reference to the rhIL-16 standard. The amount of COS cell supernatant or lysate loaded in each lane generated an immunoblot signal within the linear response range of the laser densitometer.

Cell Preparation—Human PBMC were isolated by Ficoll-Paque (Pharmacia Biotech Inc.) density centrifugation of freshly drawn venous blood of normal human volunteers. After centrifugation, cells were washed three times and suspended at a concentration of 3×10^6 cells/ml in culture medium 199 supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, 22 mM HEPES buffer, and 0.4% bovine serum albumin. Nylon Wool nonadherent human T cells were prepared by a modification of the method of Julius *et al.* (8). Enriched $CD8^+$ T cells were prepared by negative selection by using magnetic beads (BioMag; Advanced Magnetics, Inc.) conjugated with goat anti-mouse IgG. Briefly, 1×10^6 T cells were first incubated with 5 μg of mouse anti-human CD4 monoclonal antibody (Biosource) for 30 min at 4 °C. Labeled T cells were mixed with magnetic beads previously washed three times with cold PBS and then incubated for 30 min at 4 °C. Culture flasks were taped to a strong magnet, and nonadherent cells were collected.

Chemotaxis Assay—Chemotaxis assays were performed with human PBMC in a multiwell chamber fitted with a polycarbonate filter having 5-μm pores as described (9). Each COS cell supernatant or lysate was tested over a range of dilutions.

Lymphocyte Lysates—Unseparated human T cells or $CD8^+$ T cells were cultured overnight at 37 °C in a humidified atmosphere with 5% CO_2 at a cell density of 3×10^6 cells/ml before incubating with Con A (10^{-4} M). After 4 h of stimulation, lymphocytes were harvested by centrifugation, washed once with cold PBS, and lysed by sonication in a buffer containing 0.5% Nonidet P-40, 20 mM HEPES (pH 7.4), 100 mM NaCl. The lysates were cleared by centrifugation and stored in aliquots at −70 °C. The total protein content was determined by Bio-Rad protein assay according to the manufacturer's instructions.

In Vitro Translation of Human Pro-IL-16—Human pro-IL-16 cDNA was cloned into the *Kpn*I-*Eco*RI sites of pCDNAIII vector. Plasmid DNA was translated in the TNT T7 transcription-translation coupled reticulocyte lysate system (Promega). Each coupled transcription-translation reaction contained 2 μg of plasmid DNA in a final volume of 50 μl in a methionine-free amino acid mixture supplemented with ^{35}S-labeled methionine according to the manufacturer's instructions. After incubation at 30 °C for 2 h, the reactions were stored at −20 °C.

In Vitro Cleavage Reactions—*In vitro* cleavage reactions were performed in a buffer containing 0.5% Nonidet P-40, 20 mM HEPES (pH 7.4), 100 mM NaCl, and 20 mM dithiothreitol for 1 h at 37 °C with 3 μl of *in vitro* translated, ^{35}S-labeled pro-IL-16 and the indicated protease preparations or lymphocyte lysates. Reactions were terminated by the addition of SDS gel loading buffer and boiling for 4 min. Products of the cleavage reactions were separated by 15% SDS-polyacrylamide gel electrophoresis, fixed in 20% MeOH/10% acetic acid, vacuum dried, and detected by autoradiography. Recombinant proteases were prepared by transformation of *E. coli* with expression vectors for caspase-1, caspase-2, and caspase-3 as described previously (10). To verify the activity of these protease preparations, 10 μl of bacterial lysate containing recombinant caspase-1 and caspase-3 were determined to cleave >95% of [^{35}S]-pro-IL-1β and [^{35}S]poly(ADP-ribose) polymerase, respectively, when incubated at 37 °C for 1 h.

Immunoprecipitation and Immunoblot—Monoclonal antibody 14.1 was added to either concentrated COS cell conditioned medium or concentrated $CD8^+$ lymphocyte culture medium at a concentration of 2 μg/100 μl and incubated at 4 °C for 1 h under gentle rotation. Protein A Sepharose beads were added, and the mixture was incubated for an additional 1 h. The beads were washed three times with PBS, then resuspended in SDS-PAGE sample buffer, and boiled, and released material was analyzed on a 15% SDS-polyacrylamide gel. After electrophoresis, proteins were transferred to nitrocellulose membrane for immunoblotting analysis.

Protein Sequencing—For protein sequencing, proteins were transferred to polyvinylidene difluoride membrane (Bio-Rad), and Ponceau-stained staining bands were cut out and stored at −20 °C. Blotted proteins were sequenced on a Beckman LF3000 protein sequencer, using the manufacturer's recommended protocol. The released PTH-derivatives were separated by high pressure liquid chromatography and identified by comparison with standards.

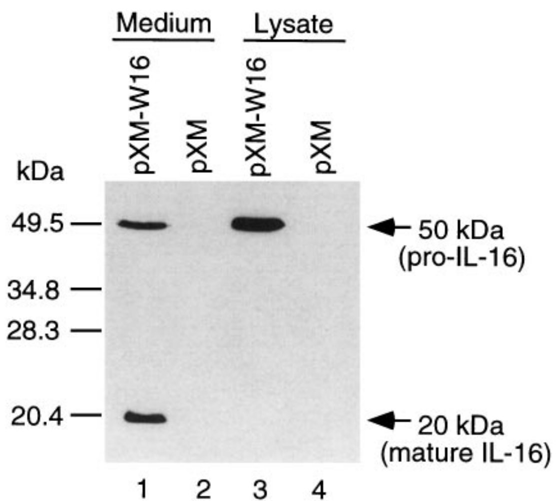

FIG. 1. **COS cell express and process pro-IL-16.** COS cells were transfected with a pro-IL-16 expression plasmid (*pXM-W16*, *lanes 1* and *3*) or with the control vector (*pXM*, *lanes 2* and *4*). Both cell lysates and conditioned medium were prepared and analyzed for the presence of IL-16 protein by immunoblotting with mAb against human recombinant IL-16. Mobilities of pro-IL-16 (~50 kDa) and mature IL-16 (~20 kDa) are indicated on the *right*. There is no detectable IL-16 present in the lysate, and conditioned medium transfected with vector alone (*lanes 2* and *4*).

RESULTS

Processing of pro-IL-16 in COS Cells—To evaluate intracellular processing of pro-IL-16 to IL-16, a 2150-bp cDNA (6, 11) encoding a 374-amino acid form of pro-IL-16 with a predicted molecular mass of ~40 kDa was subcloned into the COS cell expression vector pXM. Following transfection with this plasmid (pXM-W16), both COS cell lysate and serum-free conditioned medium were tested for IL-16 expression by Western analysis. The blot was probed with mAb 14.1 raised against rhIL-16, which binds to an epitope within the C-terminal 130 amino acids of pro-IL-16 and therefore recognizes both the precursor and mature forms. As shown in Fig. 1, a band that migrated at ~50 kDa was present in both COS cell lysate and conditioned medium. This represents the 374-amino acid product expressed by pXM-W16; its molecular mass estimated by SDS-PAGE suggests that there is some post-translational modification. In contrast to the COS cell lysate, only the conditioned medium contained a second band that migrated as ~20 kDa. The appearance of this ~20-kDa band in the conditioned medium suggested that COS cells may possess a protease capable of cleaving pro-IL-16, with this processing event followed by secretion of mature IL-16. The appearance of the ~50 kDa IL-16 in the conditioned medium may have resulted from COS cell lysis due to the effects of overexpression of the exogenous protein or culture in serum-free medium. In the cultures we noted abnormal morphology of transfected COS cells by 48 h post-transfection, and the proportion of cells that appeared abnormal increased by 72 h.

Processing of pro-IL-16 Is Required for Biological Function—To determine whether the 374-amino acid pro-IL-16 fragment exhibited any of the activities of mature IL-16 and to test the effects of cleavage on biological activity, COS cell conditioned medium and lysate of cells transfected with the 2150-bp IL-16 cDNA or control vector were tested for chemoattractant activity. As shown in Fig. 2*A*, conditioned medium of COS cells transfected with pXM-W16-stimulated lymphocyte migration with half-maximal activity at a dilution of 1:27. No activity was detected in supernatant of COS cells transfected with the con-

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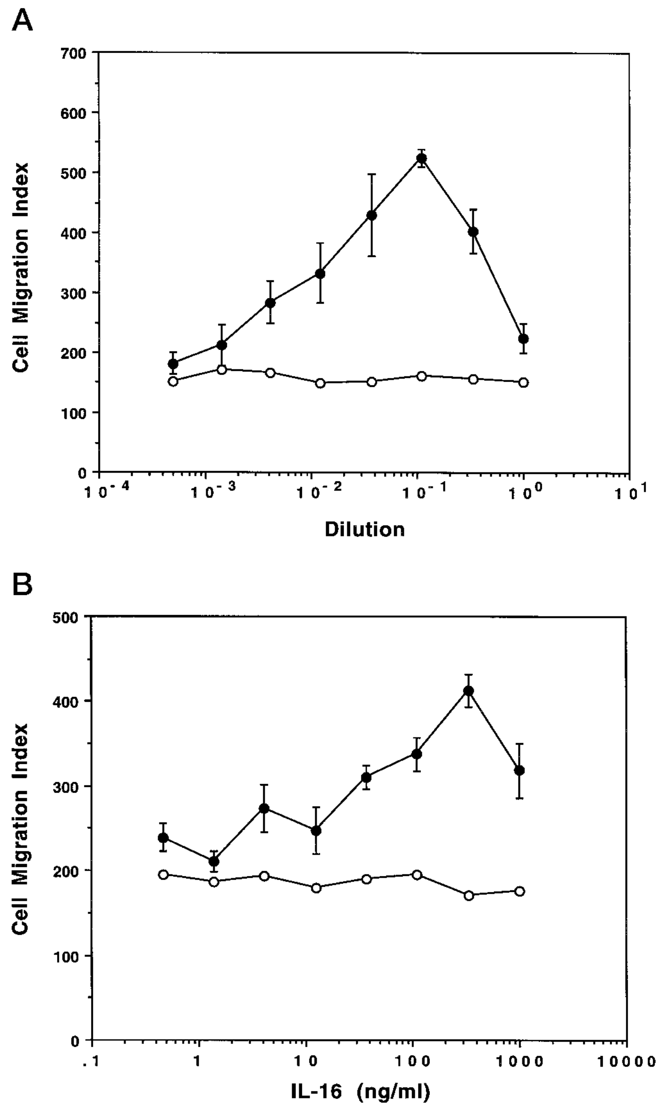

FIG. 2. *A*, T lymphocyte chemoattractant activity in medium conditioned by transfected COS cells. Dilutions of medium from cells transfected with a human pro-IL-16 expression plasmid (*closed circles*) or a control plasmid (*open circles*) were tested for chemoattractant activity as described under "Experimental Procedures." Each point represents the average value for duplicate wells, and *error bars* represent the range. These results are typical of two similar experiments. *B*, chemoattractant activity of mature IL-16 from pXM-W16-transfected COS cell conditioned medium (*closed circles*) and pro-IL-16 from transfected COS cell lysate (*open circles*). Quantitation of IL-16 is described under "Experimental Procedures." Each point represents the average value for duplicate wells, and *error bars* represent the range. These results are typical of two similar experiments.

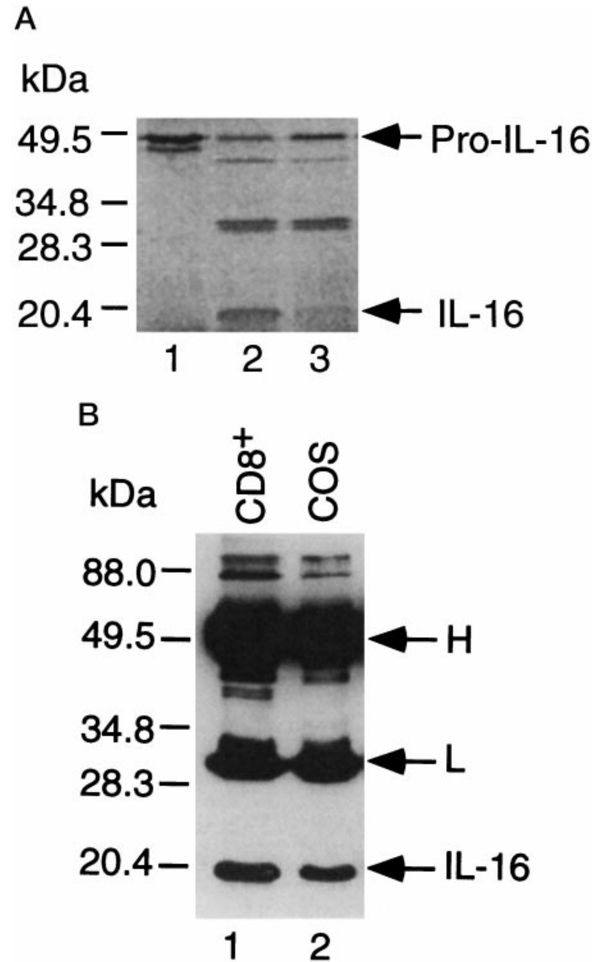

FIG. 3. *A*, generation of mature IL-16 by stimulated $CD8^+$ T cell lysates. Lysates of $CD8^+$ T cells were incubated with *in vitro* translated and ^{35}S-labeled pro-IL-16 (~50 kDa) at 37 °C. The processed forms of the protein were then detected by autoradiography. Lysate of unstimulated cells converted little pro-IL-16 to the ~20-kDa mature form (*lane 3*) after 2 h of incubation, but a lysate of cells that had been stimulated with 3 μg/ml of Con A for 4 h clearly generated a fragment of about 20 kDa (*lane 2*). When pro-IL-16 was incubated with PBS alone, no mature form of IL-16 could be detected after 2 h of incubation at 37 °C, followed by overnight exposure (*lane 1*). *B*, co-migration of natural IL-16 secreted by Con A-stimulated $CD8^+$ lymphocytes and COS cell-processed IL-16 is indicated by the *arrow*. Conditioned medium of pXM-W16-transfected COS cells and culture medium of $CD8^+$ lymphocytes stimulated with Con A for 24 h were collected and concentrated, respectively. Following immunoprecipitation with anti-rhIL-16 mAb, both samples were probed with the same antibody and detected with horseradish peroxidase-conjugated goat anti-mouse IgG secondary antibody visualized by ECL. The secondary anti-mouse IgG also detects both heavy and light chains of the mouse primary antibody used for immunoprecipitation. In the figure, *H* indicates heavy chain and *L* denotes light chain of IgG.

trol vector pXM. As shown in Fig. 1, conditioned medium from pXM-W16-transfected COS cells contains both uncleaved (~50 kDa) and cleaved (~20 kDa) forms of IL-16, whereas only the uncleaved form is present in the lysate. To determine if the uncleaved form of IL-16 could contribute to the observed chemoattractant activity, we compared the relative chemoattractant activities of conditioned medium containing ~20- and ~50-kDa forms of IL-16 *versus* COS cell lysate, which contained only the uncleaved ~50-kDa form. No chemoattractant activity was detected in the lysate of COS cells transfected either with pXM-W16 (Fig. 2*B*) or the control vector pXM (data not shown). These results indicate that pro-IL-16 is processed resulting in the release of biologically active mature IL-16 from COS cells. Cleavage of pro-IL-16 is required for the chemoattractant function of IL-16.

Co-Induction of Processing Activity and Mature IL-16 from Con A Stimulated $CD8^+$ Lymphocytes—Previous studies have found that IL-16 release from $CD8^+$ lymphocytes can be stimulated by Con A (2). To determine if the cleavage of pro-IL-16 in COS cells correlated with the *in vivo* processing mechanisms of human lymphocytes, the 2150-bp IL-16 cDNA was subcloned into pCDNAIII for *in vitro* transcription and translation in the presence of [^{35}S]methionine. When the *in vitro* translated pro-IL-16 was incubated with a crude lysate from Con A-stimulated $CD8^+$ lymphocytes, the ~50-kDa pro-IL-16 was cleaved into two major peptides of ~20 and ~30 kDa after 2 h. Incubation of *in vitro* translated pro-IL-16 with control lysate prepared from unstimulated lymphocytes resulted in minimum production of cleaved IL-16 (Fig. 3*A*). This suggests that $CD8^+$ lym-

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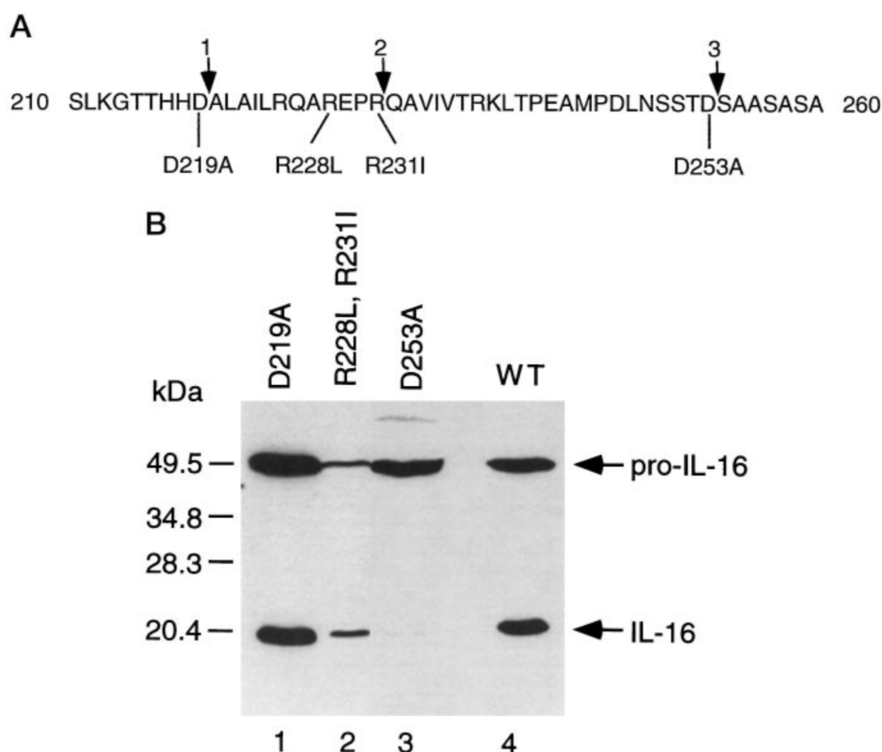

FIG. 4. *A*, amino acid sequence of human pro-IL-16 cDNA from amino acid 210 to amino acid 260. *Vertical arrows* denote the possible sites of cleavage. Site 1 has sequence HHDA, which is similar to the caspase-1 cleavage site VHDA of pro-IL-1β. Site 2 has sequence REPR, which is putative proteolytic cleavage site of dibasic processing endoproteases. Site 3 has sequence STDS, which is a potential cleavage site of caspase-family cysteine proteases. The *underlined residues* denote the mutated amino acids. *B*, immunoblot of COS cell conditioned medium transfected with wild type (*WT*) and mutated pro-IL-16 expression vectors.

phocytes have some basal IL-16 processing activity that can be further activated by Con A stimulation, consistent with the previous observation that $CD8^+$ T cells contain trace amounts of preformed biologically active IL-16 in an intracellular compartment (4). In addition to Con A, the secretagogues serotonin and histamine and antigenic stimulation have also been shown to induce IL-16 release from primary lymphocytes (3). To provide additional evidence that the processing and secretion of IL-16 by COS cells resembles the mechanism employed by lymphocytes, we compared natural IL-16 released from Con A-stimulated lymphocytes with IL-16 released from pXM-W16-transfected COS cells by immunoblot. As shown in Fig. 3*B*, transfected COS cells secrete a 20-kDa polypeptide that comigrates with natural IL-16 released from primary lymphocytes, and the COS cell processed IL-16 has the same immunological reactivity to the anti-rhIL-16 mAb. These data suggest that the ~20-kDa polypeptide released by transfected COS cells is the same C-terminal region and similar in size to that cleaved from pro-IL-16 by primary lymphocytes.

The Processing Site and N-terminal Sequence of Mature IL-16—Although it is recognized that IL-16 is derived from the C-terminal region of pro-IL-16, the exact cleavage site has not been established, and the N-terminal sequence of the mature natural protein has not been reported. Having demonstrated that processing is required for the function of IL-16, we next sought to identify the specific cleavage site of pro-IL-16 and the N-terminal amino acid sequence of mature IL-16. Within the amino acid sequence predicted by the IL-16 cDNA, we identified three potential proteolytic cleavage sites based on reported protease recognition sequences that could possibly yield a C-terminal fragment of the expected size from pro-IL-16 (Fig. 4*A*). These three potential cleavage sites are clustered in a 36-amino acid region (from His^{218} to Ser^{254}). As shown in Fig. 4*A*, site 1 and site 3 have putative aspartase cleavage characteristics (12), whereas site 2 could be a cleavage site for a dibasic processing endoprotease (13). To determine the exact cleavage site of IL-16 processed by COS cells, we made three mutant IL-16 constructs using Ala to replace Asp^{219} (site 1) and Asp^{253} (site 3) and using Leu to replace Arg^{228} and Ile to replace Arg^{231} (site 2). Immunoblotting of conditioned medium of COS cells transfected with these three mutant constructs (Fig. 4*B*) indicated that site 3 is exclusively used for cleavage of pro-IL-16 in these cells. To further confirm the use of this cleavage site, conditioned medium of COS cells transfected with the native pXM-W16 were immunoprecipitated with mAb 14.1, and the secreted ~20-kDa polypeptide was sequenced. The N-terminal sequence, Ser-Ala-Ala-Ser-Ala-Ser-Ala-Ala-Ser, confirmed that cleavage occurred after Asp^{253}, indicating that an aspartase-specific protease was likely to be involved.

Two families of proteases with substrate specificity for aspartate have been identified. One is the caspase (interleukin-1β-converting enzyme/CED-3) family of cysteine proteases, and the other is a serine protease, granzyme B, which is involved in cytotoxic T lymphocyte-mediated cell killing (14) and activation of caspase-like cysteine proteases (15). Therefore, we next determined whether pro-IL-16 could serve as a substrate for any of aspartate-specific proteases.

In Vitro Cleavage of Pro-IL-16 with Aspartate-specific Proteases—Cleavage of pro-IL-16 by recombinant caspase-1 (interleukin-1β-converting enzyme), caspase-3, and granzyme B was tested using ^{35}S-labeled *in vitro* translated pro-IL-16 as a substrate. Only caspase-3 cleaved the ~50-kDa pro-IL-16 into two polypeptides of ~20 and ~30 kDa, whereas no cleavage was observed using caspase-1 or granzyme B (Fig. 5*A*). In a similar experiment, recombinant caspase-2 (ICH-1) did not cleave pro-IL-16 (data not shown). The 20-kDa band released by caspase-3 was immunoprecipitated by mAb 14.1 (Fig. 5*B*, *lane 2*), and cleavage was blocked by the caspase-3 inhibitor Ac-DEVD-CHO (Fig. 5*B*, *lane 4*), indicating that caspase-3 digestion released the C-terminal region of pro-IL-16. To confirm the caspase-3 cleavage site, mutant pro-IL-16 with Ala substituted for Asp^{253} was *in vitro* translated and digested with recombinant caspase-3. As shown in Fig. 5*C*, the generation of 20-kDa band was abolished (Fig. 5*C*, *lane 2*). This indicates that caspase-3 cleaves pro-IL-16 after the same Asp residue as COS cells. Taken together, these results suggest that caspase-3 medi-

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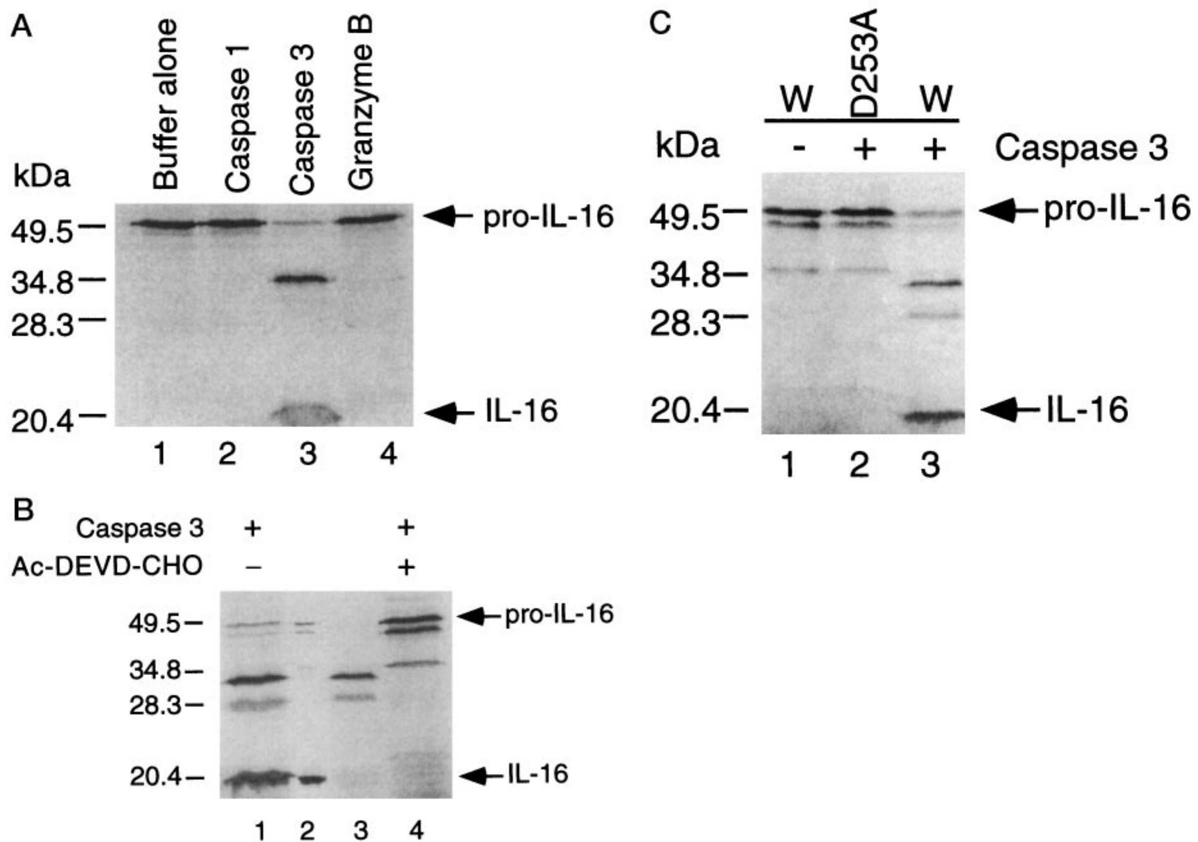

FIG. 5. **Cleavage of *in vitro* translated pro-IL-16 with aspartate-specific proteases.** *A*, *in vitro* synthesized, ^{35}S-labeled pro-IL-16 was incubated in the absence (*lane 1*) or the presence of 10 μl of lysate from *E. coli* transformed with expression vectors for caspase-1 (*lane 2*), caspase-3 (*lane 3*), or granzyme B (*lane 4*). The enzyme activity was defined as described under "Experimental Procedures." After incubation at 37 °C for 1 h, the samples were subjected to SDS-PAGE. The gel was exposed to film for 18 h at room temperature. *B*, *in vitro* translated pro-IL-16 was cleaved by caspase-3 yielding a ~20-kDa fragment (*lane 1*). The products of this digestion were immunoprecipitated by anti-rhIL-16 mAb and applied to the same gel (*lane 2*). A ~30-kDa fragment remained in the supernatant after immunoprecipitation (*lane 3*). The cleavage of pro-IL-16 by recombinant caspase-3 was completely abolished by the specific inhibitor Ac-DEVD-CHO (*lane 4*). *C*, the *in vitro* translation product of a mutated pro-IL-16 (Asp^{253} to Ala) was resistant to cleavage by recombinant caspase-3 (*lane 2*).

ates pro-IL-16 cleavage in COS cells, resulting in the release of mature, biologically active IL-16, and that this enzyme might also be responsible for processing IL-16 in primary lymphocytes.

Detection of Caspase-3 in Activated CD8$^+$ Lymphocyte Lysate—An inhibitor sensitivity study was conducted to determine whether the pro-IL-16 is cleaved by caspase-3 in Con A-stimulated CD8$^+$ lymphocyte lysate. Only the caspase-3 inhibitor Ac-DEVD-CHO significantly inhibited cleavage of *in vitro* translated pro-IL-16. Minimal inhibition was observed with the caspase-1 inhibitor Ac-YVAD-CMK (Fig. 6*A*). The expression of activated caspase-3 in Con A-stimulated CD8$^+$ lymphocytes was confirmed by Western blot analysis with an antibody against the p20 subunit of caspase-3 (Fig. 6*B*). These data indicate that caspase-3 is the primary if not the only protease in stimulated CD8$^+$ lymphocytes that cleaves pro-IL-16 to release the mature polypeptide.

DISCUSSION

The IL-16 cDNA was originally cloned from a COS cell expression library, and it was believed to be encode a 393-bp open reading frame. The IL-16 protein isolated in this screening displayed the unique constellation of biological activities of natural IL-16 and the two proteins migrated similarly on SDS-PAGE (6). However, the predicted sequence contained no signal peptide commonly present in secreted proteins. This inconsistency of function-related structure and our identification of higher molecular weight bands in Western blots of activated lymphocyte lysate probed with anti-rhIL-16 antibodies (16) led us to reexamine the cDNA sequence. Independently, Bazan and Schall (17) and Bannert *et al.* (11) reported findings suggesting that the IL-16 open reading frame in this cDNA extended further in the 5′ direction and could encode a larger precursor protein with a predicted molecular mass of ~40 kDa. We confirmed a longer open reading frame by repeat DNA sequence analysis of the original cDNA (data not shown). More recently, Baier *et al.* (7) described additional 5′ cDNA sequence potentially encoding a ~67-kDa pro-IL-16, and they identified ~80- and ~60-kDa bands in activated PBMC lysate by Western analysis with anti-IL16 antibody. The N-terminal sequences of the natural pro-IL-16 and mature IL-16 produced in primary lymphocytes have not been determined.

In this report, we used a 2150-bp IL-16 cDNA (6, 7, 11) to study pro-IL-16 processing. Expression of this cDNA in COS cells generates a ~50-kDa intracellular protein, whereas both this form and a ~20-kDa band are released from these cells into supernatant. It is unclear whether the ~50-kDa form is actively exported; more likely its appearance in conditioned medium is the result of COS cell lysis. The ~20-kDa form was not detected by immunoblotting lysate of transfected COS cells or Con A-stimulated CD8$^+$ lymphocytes, suggesting that the process of cleavage is linked to secretion.

By chemotaxis assay and immunoblotting, the ~20-kDa form was shown to be the mature and biologically active IL-16, which is cleaved from the C-terminal region of pro-IL-16. In retrospect, the constitutive capacity of COS cells to process and release pro-IL-16 was critical for the success of our original effort to clone the IL-16 cDNA from a COS cell expression

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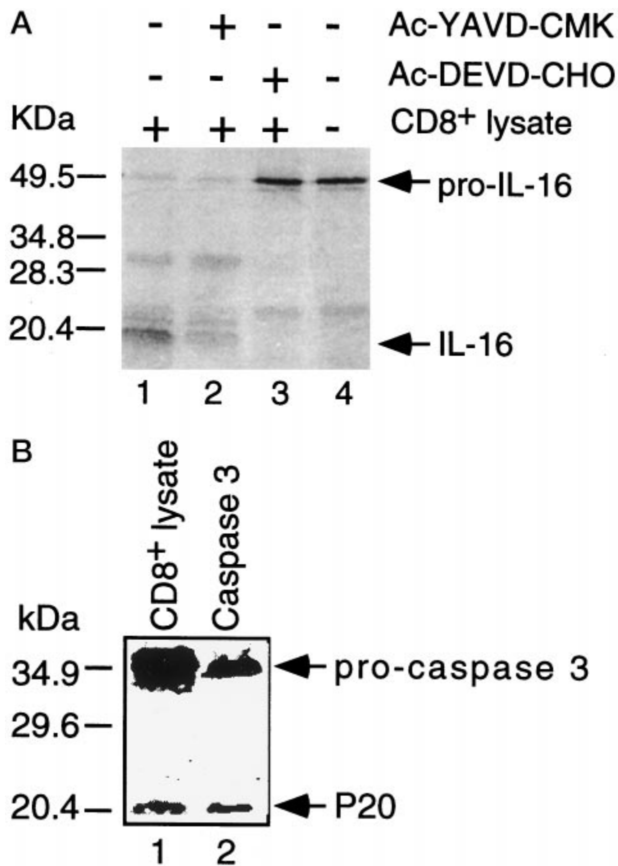

FIG. 6. *A*, effects of protease inhibitors on the cleavage of pro-IL-16 by Con A-stimulated CD8$^+$ T cell lysate. *In vitro* translated ^{35}S-labeled pro-IL-16 was incubated with Con A-stimulated CD8$^+$ T cell lysate for 2 h at 37 °C in the absence of inhibitor (*lane 1*) or in the presence of 1 μM Ac-YVAD-CMK (*lane 2*) or 0.5 μM Ac-DEVD-CHO (*lane 3*). Cleavage products were analyzed by SDS-PAGE and fluorography. *B*, activation of caspase-3 in Con A-stimulated CD8$^+$ T cells. Con-A-stimulated CD8$^+$ T cell lysate (*lane 1*) and recombinant caspase-3 (*lane 2*) were resolved by 15% SDS-PAGE followed by blotting onto polyvinylidene difluoride membrane. Caspase-3 was detected using an antibody against its P20 subunit followed by chemiluminescence.

library screened for bioactivity in transfected cell supernatants (6). In the current studies, we demonstrated that cleavage of pro-IL-16 in COS cells is mediated by caspase-3. Based on N-terminal sequencing we showed that cleavage occurs at Asp^{253}, at a site that is in a compatible context for caspase-3 recognition and cleavage. Mutation of this site but not of two other nearby potential protease recognition sequences blocked cleavage in transfected COS cells. Furthermore, recombinant caspase-3 but not caspase-1, caspase-2, or granzyme B cleaved *in vitro* translated pro-IL-16. The relevance of caspase-3 to IL-16 processing in primary cells was confirmed by incubating *in vitro* translated pro-IL-16 with lysate of Con A-stimulated CD8$^+$ lymphocytes. Cleavage of ~50-kDa pro-IL-16 to bands of ~20 and ~30 kDa was observed, and this cleavage was specifically and strongly inhibited by Ac-DEVD-CHO. The presence of active caspase-3 in Con A-stimulated CD8$^+$ lymphocytes was confirmed by immunoblot. It remains possible that a caspase-3-like protease, not caspase-3 itself, is the true IL-16 processing enzyme.

Interleukin-16 was originally identified as a lymphocyte chemoattractant factor secreted by CD8$^+$ T cells following stimulation with histamine, serotonin, mitogen, or antigen (3). Our findings reported here indicate that stimulation of resting lymphocytes activates an IL-16 processing mechanism that involves cleavage of pro-IL-16 by caspase-3, linked to secretion of biologically active IL-16. The mechanism of secretion remains undefined but our data suggest similarities with the processing and secretion of IL-1β. However, there is no significant sequence similarity between pro-IL-16 and pro-IL-1β, and spectra of biological activities are different.

Although there is evidence that caspase family proteases are mediators of apoptosis, the definitive roles of each isozyme present in different tissues and the substrates for each enzyme have yet to be clarified. The previously reported substrates for caspase-3, including poly(ADP-ribose) polymerase (18), DNA-dependent protein kinase (19), D4-GDI (20), fodrin (21), sterol regulatory element binding proteins (22), and actin all appear to be related to the involvement of this enzyme in apoptosis (23). It has been reported that mature biologically active IL-1β is released from murine peritoneal macrophages undergoing apoptosis by lipopolysaccharide stimulation (24). Although IL-1β itself is not a mediator of apoptosis, its release from apoptotic macrophages could be used as signal that this event has occurred, leading to an appropriate inflammatory response. It is unknown at this time if the processing of pro-IL-16 by caspase-3 in primary lymphocytes is related to apoptosis. The ability of the secretagogues histamine and serotonin to induce IL-16 release suggests that apoptosis is not a requirement for IL-16 release, implying that caspase-3 might be utilized in a nonapoptotic pathway. However, it is possible that the release of IL-16 following mitogen or antigen stimulation could occur in the context of apoptosis. In this regard, caspase-3 could mediate a pro-inflammatory function by processing IL-16, which acts to recruit and activate CD4$^+$ lymphocytes, monocytes, and eosinophils, amplifying the inflammatory response.

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