**Purification and Characterization of Active Human Interleukin-1β-converting Enzyme from THP.1 Monocytic Cells***

(Received for publication, February 22, 1993, and in revised form, May 6, 1993)

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Interleukin-1β-converting enzyme (ICE) was purified from dialyzed cytoplasmic extracts of THP.1 human monocytic cells by a combination of DEAE-5PW and SP-5PW ion exchange and C₄ reverse phase high performance liquid chromatography. Sequence information from tryptic and Asp.N peptides on the isolated 20-kDa (p20) and a 10-kDa (p10) proteins enabled the subsequent cloning of ICE (Thornberry, N. A., Bull, H. G., Calaycay, J. R., Chapman, K. T., Howard, A. D., Kostura, M. J., Miller, D. K., Molineaux, S. M., Weidner, J. R., Aunins, J., Elliston, K. O., Ayala, J. M., Casano, F. J., Chin, J., Ding, G. J.-F., Egger, L. A., Gaffney, E. P., Limjucuo, G., Palyha, O. C., Raju, S. M., Rolando, A. M., Salley, J. P., Yamin, T.-T., Lee, T. D., Shively, J. E., MacCross, M., Mumford, R. A., Schmidt, J. A., and Tocci, M. J. (1992) *Nature* 358, 768-774) and localized the active site Cys. Immunoblots with ICE specific antibodies and NH₂-terminal sequencing indicated that ICE active column fractions contained in addition to p20 and p10 an alternatively processed form of the p20 protein (p22) containing an extra 16 amino acids NH₂-terminal to the p20. Furthermore, immunoblot analysis of the ion exchange column effluent showed that p20 and p22 were found together in three separate fractions distinguished by differences in p10: an intact p10 with complete ICE activity, a COOH-terminally truncated form of p10 with decreased ICE activity, and an absence of p10 with no ICE activity. These results indicate that the p10 protein is essential for ICE activity and that the ICE holoenzyme contains an intact p10 subunit paired with a p20 or p22 catalytic subunit.

Interleukins-1α and -1β are proinflammatory cytokines that stimulate leukocyte accumulation, prostaglandin synthesis, joint swelling, and cartilage destruction, and are implicated in chronic inflammatory diseases such as rheumatoid arthritis (1-8). They are synthesized largely by monocytes and mononuclear cell lines as 31-kDa cytoplasmic precursor proteins (pIL-1) that lack functional leader sequences and are released from the cells in vivo by means not involving the classical endoplasmic reticulum/Golgi network (9-15). pIL-1α is fully active on IL-1 receptors (16), and it remains largely cell-associated; its presence extracellularly in relatively small amounts may result from its release from damaged cells (14). In contrast, pIL-1β is inactive on IL-1 receptors until it is cleaved into a mature 17.5-kDa species (mIL-1β) (15, 16) at which time it is rapidly and quantitatively secreted from monocytes (14). mIL-1β is the major form of IL-1 found in biological fluids during diseased states (17). Since mIL-1β is found only extracellularly whereas pIL-1β is the exclusive intracellular form, the active secretion of mIL-1β following cell stimulation must be closely coupled to intracellular cleavage of pIL-1β.

A unique cytoplasmic cysteine protease, termed IL-1β-converting enzyme (ICE), has been identified which cleaves the Asp¹⁶-Ala¹⁷ bond of pIL-1β to generate the mature form of IL-1β (18-23). This novel cleavage by ICE has been identified only in monocytic cells (19, 22, 24). Furthermore, cells lacking ICE activity do not secrete mIL-1β even if transfected with pIL-1β (25, 26). Studies with macromolecular and peptide substrates have established that aspartate is required in P1 for efficient catalysis. The minimal peptide substrate for ICE contains four amino acids to the left of the cleavage site (optimally Ac-Tyr-Val-Ala-Asp) and a small hydrophobic amino acid in P1' (23).

We have reported that ICE is composed of 20- and 10-kDa polypeptides encoded by a common 45-kDa precursor protein (23). In the present paper we expand our earlier work by reporting a more extensive biochemical and immunological analysis of the ICE activity obtained from THP.1 human monocytic cells. We detail the initial purification of ICE by conventional HPLC techniques as well as the Edman sequencing of these p20 and p10 proteins which enabled the cloning of the cDNA and the identification of the active site cysteine. Antibodies raised against either active affinity-purified ICE or specific peptide regions have now been used to identify and characterize alternatively processed forms of both the p20 and p10 polypeptides and establish that the p10 is required for catalytic activity.

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MATERIALS AND METHODS

Preparation of THP.1 Cell Extracts—THP.1 cells were grown in suspension in 46-300-liter suspension flasks and fermentors in Iscove's modified Dulbecco's medium supplemented with 9% horse serum and 0.1-0.3% F68 pluronide (added to reduce shear force; JRH Biosciences, Lenexa, KS). The cells were isolated in a Prostak concentration system (50 square feet (4.6 square meters) of 0.65-μm pore size, 14 ml in a hypotonic buffer containing 25 mM HEPES, pH 7.5, 5 mM MgCl₂, and 1 mM EGTA; Protein inhibitors were added (1 mM phenylmethylsulfonyl fluoride and 10 μg/ml each of pepstatin and leupeptin), and the cells were broken with 15-25 strokes of a tight fitting Dounce homogenizer (100- or 300-ml capacity; Kontes Glass, Vineland, NJ) to yield 90-95% breakage. Nuclei and unbroken cells, removed by centrifugation at 2,000 × g for 20 min, were resuspended in buffer containing phenylmethylsulfonyl fluoride, 10 μg/ml each of pepstatin and leupeptin, or 5 mM EDTA. Protease inhibitors were added (1 mM leupeptin), and the cells were broken with 15-25 strokes of a tight fitting Dounce homogenizer (100- or 300-ml capacity; Kontes Glass, Vineland, NJ) to yield 90-95% breakage. Nuclei and unbroken cells, removed by centrifugation at 2,000 × g for 20 min, were resuspended in about one-fourth the original volume of the hypotonic buffer, rehomogenized for 10 strokes, and recentrifuged. The postnuclear supernatants were combined, 6 mM EDTA was added, and membranes were removed by centrifugation at 24,000 × g for 20 min (16,000 rpm, SW 40 rotor; Du Pont), followed by centrifugation for an additional 60 min at 200,000 × g (50,000 rpm, 50.2-Ti rotor; Beckman). Following the addition of 2 mM dithiothreitol, the supernatant was stored at −80 °C until further purification.

Measurement of ICE Activity—Three assays employing different substrates have been used to measure ICE activity in monocyte cell extracts: (i) cleavage of macromolecular 31-kDa [³⁵S]-pIL-1β to its 17-kDa mature form followed by SDS-PAGE and fluorography; (ii) cleavage of a peptide substrate that spans the cleavage site of pIL-1β, NEAYVHDAPVRSLN, followed by HPLC and measurement of absorbance at 280 nm of the P' side cleavage product NEAYVH; or (iii) cleavage of the fluorometric substrate AcYVAD-aminoethylcoumarin (AMC; 7-amino-4-methylcoumarin) followed by HPLC separation and measurement of the P' side product AMC by fluorescence (18, 19, 23). The [³⁵S]-methionine pIL-1β gel assay (20 μl) was performed as previously described (18). For the AcYVAD-AMC assay (20 μl), samples were incubated for 1 h at 30 °C (20% of the final volume) or by the [³⁵S]-methionine-labeled pIL-1β gel assay (20 μl) was performed as previously described (18). For the AcYVAD-AMC assay (20 μl), samples were incubated for 1 h at 30 °C (20% of the final volume) or by the [³⁵S]-methionine-labeled pIL-1β assay (inset, positions of the 31-kDa precursor [³⁵S]-methionine-labeled pIL-1β and 17.5-kDa mIL-1β are indicated) by the 150-ml bed volume (150 mm × 10 cm) DEAE column (see below for measurement) were pooled, diluted with an equal volume of the HSCD buffer, adjusted to pH 7.0 with HCl, and applied to a 150-ml bed volume (150 × 21.5-mm, inner diameter) TosoHaas SP-5PW HPLC column. Elution was performed at 4 ml/min with a gradient of 0.5 M KCl in HSCD buffer.

DEAE and SP HPLC—For DEAE-5PW HPLC separation about 1 liter of the cytosolic supernatant (3–5 g of total protein, 1.4 × 10⁹ cells) was dialyzed overnight at 5 °C versus HSCD buffer. The extracts were clarified by a Microcon 0.22-μm hollow fiber filtration system (1-square foot (0.09 square meters) filtration cartridge; Laguna Hills, CA) and, where indicated in the text, concentrated 10–20-fold with an Amicon YM3 spiral cartridge (Beverly, MA). The clarified THP.1 extract was applied to a 240-ml bed volume Tosohaas DEAE-5PW HPLC column (200 × 86-mm, inner diameter; Montgomeryville, PA) with a Waters 650E liquid chromatography system (Milford, MA). Proteins were eluted at 5 °C, 25 ml/min, with a linear gradient of 0.4 M NaCl and 240 mM Tris-HCl, pH 7.8, in 10% sucrose, 0.1% CHAPS, and 2 mM dithiothreitol (B buffer) and increasing proportions of 0.4 M NaCl and 240 mM Tris-HCl, pH 7.8, in 10% sucrose, 0.1% CHAPS, and 2 mM dithiothreitol (B buffer). The ICE active fractions from the DEAE column (see below for measurement) were pooled, diluted with an equal volume of the HSCD buffer, adjusted to pH 7.0 with HCl, and applied to a 150-ml bed volume (150 × 21.5-mm, inner diameter) TosoHaas SP-5PW HPLC column. Elution was performed at 4 ml/min with a gradient of 0.5 M KCl in HSCD buffer.

### FIG. 1. Purification of ICE by preparative DEAE-5PW HPLC (upper panel) and SP-5PW HPLC (lower panel) ion exchange chromatography. Upper panel, THP.1 cytosolic extracts that cleaved [³⁵S]methionine-labeled pIL-1β were chromatographed over a DEAE-5PW HPLC column eluted with a gradient of NaCl and Tris-HCl, pH 7.8, and monitored for protein (A₂₈₀, solid line). Aliquots of the eluting fractions were monitored for ICE activity by the [³⁵S]methionine-labeled pIL-1β assay (inset, positions of the 31-kDa precursor [³⁵S]methionine-labeled pIL-1β and 17.5-kDa mIL-1β are indicated) or the cleavage of either AcYVAD-AMC or NEAYVHDAPVRSLN peptide substrates measuring free AMC (●) or NEAYVH (O), respectively. Lower panel, SP-5PW HPLC chromatography of a pool of ICE active fractions from the DEAE column (upper panel) measuring ICE activity with the [³⁵S]methionine-labeled pIL-1β assay (inset) or with the AcYVAD-AMC (●) or NEAYVHDAPVRSLN (O) substrates. Bars indicate fractions containing immunoreactive ICE protein (see Fig. 8).
Enzymatic Cleavage and Edman Sequencing of ICE—Trypsin (Boehringer Mannheim) was incubated with individual proteins at an enzyme:substrate ratio of 1:100 (w/w) in 50 μl of 50 mm ammonium bicarbonate, pH 9.0, for 16 h at 37 °C and neutralized with 5 μl 10% trifluoroacetic acid prior to HPLC separation. The peptides were fractionated on a Vyda C18, 2.1 × 150-mm column eluted at 280 μl/min at 50 °C with detector units of 214 nm/0.25 absorbance units in full scale. A linear gradient of 2-75% solvent B (89.945% acetonitrile, 10% water, 0.055% trifluoroacetic acid) in water was run in 60 min followed by 10 min of 75-95% B. Asp(N) (Boehringer Mannheim) was incubated with the proteins with an enzyme:substrate ratio of 1:20 for 90 min at room temperature. All other conditions were identical to that of the trypsin. Conventional Edman degradation microsequencing was performed on a modified Applied Biosystems 477A gas phase sequenator equipped with a continuous flow reactor as described by Shively et al. (29).

Affinity Purification of ICE—A column of Sepharose CL-4B coupled to Ac-YVKD-CHO was prepared and activated as described previously (23). From about 4 × 10^10 units present in a DEAE-5PW ICE pool, about 100 μg of active purified ICE was generated in this manner.

ICE Antibody Preparation—Peptides were synthesized as described previously on an Applied Biosystems 430A peptide synthesizer (23) using solid phase chemistry with a norleucine and a cysteine at one end. The peptides were coupled via the cysteine to thyroglobulin using the Sulfo-MBS technique (Pierce; Ref. 30). Antibodies were raised in rabbits or guinea pigs by subcutaneous and/or intranodal injection of either the peptide conjugates or affinity-purified ICE emulsified in complete Freund’s adjuvant. All antipeptide sera were shown to bind ICE specifically by immunoblotting of THP-1 cytosolic extracts. Prebleeds of the sera did not label ICE proteins, and preincubation of the immune sera with a 5 mM concentration of the immunizing peptide prevented ICE labeling (not shown).

Immunoblotting Procedure—SDS gels were transferred to polyvinylidene difluoride membranes (Millipore), blocked with nonfat dry milk, incubated with antibody at a 1:25:1:600 dilution, and visualized with 125I-protein A as described previously (31).

RESULTS

Purification of ICE by Sequential Ion Exchange HPLC Chromatography—The protocol used to purify ICE from THP.1 cytoplasmic extracts utilized sequential DEAE-5PW and SP-5PW HPLC chromatography, yielding more than a 10,000-fold purification. One liter of dialyzed extract (3-5 g from about 1.4 × 10^11 cells) was clarified by filtration and fractionated over a DEAE-5PW HPLC column. The resultant column fractions were assayed for ICE activity using three different substrates as described under “Materials and Methods.” All three assays identified a single peak of ICE activity, DEAE peak 1 (Fig. 1, upper panel). The active fractions were pooled and chromatographed on SP-5PW. Again a single peak of activity, SP peak I, was found with the three assays (Fig. 1, lower panel). Quantitative assessment of the specific activity of ICE at each stage of purification is complicated by the concentration dependence of ICE activity. Previous studies have suggested that the active ICE holoenzyme is assembled from inactive monomers: dilution dissociates ICE to inactive subunits, whereas reconstitution restores complete activity (see Ref. 23). Recovery of enzyme activity was thus enhanced by a concentration of the DEAE peak I by ultrafiltration: a 20-fold concentration often produced up to a 10-fold increase in total ICE activity (not shown). The yield of enzyme after DEAE HPLC fractionation and concentration typically was greater than 20,000 units from about 10^11 cells (50 μg of total protein in the DEAE pool). Because of the higher initial ICE concentration, the concentration of SP peak I produced only a 2-fold increase in total activity (about 50,000 units, 250 μg of total protein). Silver-stained SDS-PAGE of SP peak I from a number of separate purifications (Fig. 2A) showed that two protein bands with an M, of 22,000 and 10,000 were consistently present in all of the preparations.

Purification of ICE Proteins by Reverse Phase HPLC—

**Fig. 2. SDS gradient gels (17–27%) of preparative SP-5PW-purified ICE fractions. Arrowheads mark the location of molecular weight standards (left) and positions of p20 and p10 (right). The arrow in panel C indicates the location of a smaller proteolyzed form of p10. Panel A, silver-stained SDS gels of the ICE active regions of successive DEAE-5PW/SP-5PW preparations with approximately 10 units of ICE activity loaded in each lane. Asterisks refer to preparations separated by C1, narrow-bore chromatography (Fig. 3) and where the p20 and p10 proteins were individually sequenced. The preparation lane resulted from the HPLC profile of Fig. 1. The first lane 9 R refers to a fraction of preparation 9 which had not been retained by the SP-5PW column because of a high salt concentration, resulting in its elution at the end of the flow-through peak. This ICE pool was dialyzed and rechromatographed on an analytical SP-5PW column resulting in a cleaner ICE fraction used subsequently as a molecular weight standards (left) and positions of p20 and p10 (right). The arrow in panel C indicates the location of a smaller proteolyzed form of p10. Panel A, silver-stained SDS gels of the ICE active regions of successive DEAE-5PW/SP-5PW preparations with approximately 10 units of ICE activity loaded in each lane. Asterisks refer to preparations separated by C1, narrow-bore chromatography (Fig. 3) and where the p20 and p10 proteins were individually sequenced. The preparation lane resulted from the HPLC profile of Fig. 1. The first lane 9 R refers to a fraction of preparation 9 which had not been retained by the SP-5PW column because of a high salt concentration, resulting in its elution at the end of the flow-through peak. This ICE pool was dialyzed and rechromatographed on an analytical SP-5PW column resulting in a cleaner ICE fraction used subsequently as a standard for successive gels. Panel B, immunoblot of various SP-5PW preparations electrophoresed and blotted with the anti-ICE peptide antibody 1150–158 (1:100 dilution, see Fig. 7). 10–II refers to a second ICE-active peak eluted at higher salt from an analytical SP-5PW column following concentration and rechromatography of preparation 10 (panel A) which was concentrated and rerun on an analytical SP-5PW column (see Fig. 9). AP refers to affinity purified ICE (see Fig. 4). Panel C, immunoblot of SP-5PW preparations blotted with the anti-ICE peptide antibody 1317–330 (1:25 dilution, see Fig. 7).

Specificity of pIL-1β Cleavage by Purified ICE—To establish that the purified enzyme (SP peak I) correctly processes the pIL-1β, in vitro translated [3H]valine- and [3H]leucine-radiolabeled pIL-1β were separately incubated with purified ICE. The products were immunoprecipitated with a human IL-1β-specific antibody, electrophoresed on SDS gels, blotted onto polyvinylidene difluoride membranes, and sequenced. The results indicated that accurate processing of the pIL-1β between Asp^116 and Ala^117 had occurred (not shown).

C1, Reverse Phase HPLC Chromatography.—The SP-5PW ICE active fractions were fractionated (20–100 μl/injection) on an Applied Biosystems C1, narrow-bore HPLC column (2.1 × 100 mm; Foster City, CA) connected to an Applied Biosystems model 130A HPLC. Elution (200 μl/min) was performed with a linear acetonitrile gradient in 0.1% trifluoroacetic acid. Samples were dried in a Savant Speed Vac concentrator (Farmingdale, NY) prior to sequencing or electrophoresis.
When individual SP-5PW fractions from a single run were chromatographed over C₄ narrow-bore HPLC columns (Fig. 3), two peaks eluting at about 50% acetonitrile were obtained, containing the 22-kDa protein in the earlier eluting peak and the 10-kDa protein in the later eluting peak (as determined by SDS-PAGE; Figs. 3A and 4). These proteins comigrate with the two proteins purified by affinity chromatography using a highly selective tetrapeptide aldehyde ligand (Fig. 4) (23). Catalytic activity of ICE was lost after the reverse phase HPLC and was not regained upon recombination of the two proteins as tested by the 36S-pIL-1/3 cleavage assay (not shown). The integrated area of each peak was directly proportional to the amount of ICE activity applied (Fig. 3B). Regression analysis showed that the peak areas were highly correlated ($r = 0.99$) with a ratio of p10 to p20 of 1.273, assuming equal molar absorbance at 214 nm (Fig. 3C). These two proteins were shown by mass spectrometry to have molecular weights of 19,866 and 10,248, and are hereafter referred to as p20 and p10, respectively (see Ref. 23). The total amount of ICE obtained from the complete HPLC purification (700 pmol/liter of cytosol) was slightly less than by the affinity technique (830 pmol/liter), in which an amount equivalent to about 5,000 copies of active enzyme per cell was obtained.

A total of four separate ICE preparations were purified by the HPLC method and used for sequencing studies (asterisks, Fig. 2A). In two of these purifications (SP pools 6 and 7; Fig. 2), the earlier eluting C₄ HPLC peak contained in addition to p20 a protein 2 kDa larger, termed p22. ICE affinity purified from certain DEAE-5PW preparations also yielded mixtures of p22 along with p20 and p10 (Fig. 4A, sample 1) whereas other preparations yielded only p20 and p10 (Fig. 4A, sample 2). The p22 protein was separated from p20 by reduction and alkylation and rechromatography on the C₄ narrow-bore column and obtained in pure form (not shown).

Sequence Analysis of ICE Polypeptides—Peptide maps of the p20 and p10 proteins were generated by digesting 40-50 pmol of the C₄-purified proteins with trypsin or Asp.N and chromatographing the products on a C₄ narrow-bore HPLC column. Each protein had a distinct NH₂-terminal sequence (Fig. 4B) and produced a distinct spectrum of cleavage products (Fig. 5, A-D), indicating that the proteins were unique. The NH₂-terminal sequence of the p20 and p10 isolated by either HPLC or affinity chromatographic methods was identical (not shown). Edman sequencing of a number of the isolated peptides provided sequences (Fig. 5E) that were later used for polymerase chain reaction and cloning of the entire ICE sequence (23). Cloning indicated that both polypeptides were contained within a single 45-kDa precursor protein (p45) with three domains: a prodomain with unknown function, the p22/p20, and the p10 (see Fig. 7).

Edman NH₂-terminal sequence analysis of p22 revealed that it represents a 16-amino acid extended form of the p20 in which its NH₂ terminus begins with residue 104 of the deduced sequence rather than 120; beginning with cycle 18 the p22 sequence was identical with the NH₂ terminus of the p20 protein (Fig. 4B; see Ref. 23). The NH₂-terminal residue of p20 sequenced as an Asp instead of the Asn residue that is found both within the p22 as well as in the cDNA of the
cloned ICE protein. The mechanism for the deamidation of the Asn to Asp may be an efficient NH2-terminal deamidase activity present in the cellular extracts (32). Interestingly, both the NH2 termini of the p20 and the p22 begin following an Asp-X ICE-like cleavage site in the ICE precursor protein. The calculated molecular mass of the NH2-terminal extension on p22 is 1.6 kDa (see Fig. 7), consistent with the 2-kDa larger size seen on SDS-PAGE.

To identify the location of the catalytic Cys, ICE from SP peak I was labeled with [14C]iodoacetic acid and isolated by SDS-PAGE, a process that resulted in labeling of the p20 protein. Labeling of the p20 was prevented by including saturating levels of the substrate AcYVAD-AMC (see Ref. 32). Interestingly, both the NH2 termini of the p20 and the p22 begin following an Asp-X ICE-like cleavage site in the ICE precursor protein.

The antibody to intact, active ICE (JD3) labeled the p22 protein as well as the p20 (Fig. 8). p22 was also found in each DEAE peak, and within the fractions of each peak, p22 eluted slightly in advance of p20. Immunoblotting with the three antipeptide antibodies directed toward various regions of the p20 (1150–158, 1216–228, and 1285–297) gave the same pattern of p22 and p20 proteins as did JD3. The observation that I285–297 labeled p22 and p20 with relative intensity similar to the 1150–158 and 1216–228 antibodies indicates that these proteins have overlapping sequences and include the COOH-terminal region of p20. No p45 protein was found in any of these DEAE-5PW fractions, and the antibody to the prodomain 134–47 did not label any bands in common with the other ICE antibodies.

The presence of p10 in these DEAE fractions was assessed with the I335–346 antipeptide antibody as well as the I376–388 antibody (not shown). In DEAE peak I (Fig. 1, upper panel), ICE activity was proportional to the amount of immunoreactive p10; maximal activity as well as p10 and p20 were present in fraction 102 with less of each in adjoining fractions (Fig. 8). In contrast, DEAE peak II contained no p10 protein and no ICE activity. The p22/p20 proteins in DEAE peak II did not appear to be proteolytically altered themselves; they were identical to the p22/p20 proteins in the DEAE peak I as judged by order of elution, immunoblotting with all three p20 peptide antibodies, and mobility on SDS-PAGE. These data strongly suggest that ICE activity requires the presence of p10 and that the differences in activity do not reflect changes in the p20.

Immunoblot Analysis of SP-5PW Fractions—As noted earlier, when DEAE peak I column fractions were pooled, concentrated, and applied to an SP HPLC column, typically a single peak of activity was observed. Immunoblot analysis of the fractions indicated that p20 and p10 are found only in the active ICE fractions. Some SP peak I preparations also contained p22 in addition to p20 (Fig. 2B), but the relative amount of p22 was much less than that seen following DEAE chromatography (see Fig. 8). Immunoblotting of a number of SP peak I fractions containing an antibody specific for the NH2 terminus of p10 (I285–297, Fig. 7) indicated the presence of a single p10 band (Fig. 2C).

An alternatively processed form of ICE protein could be generated when the SP peak I pools were highly concentrated, dialyzed, and rechromatographed on an analytical SP-5PW column (Fig. 9). In addition to the peak normally eluting at 70–90 mM salt (Fig. 9A, SP peak I), a second, smaller peak of activity was generated which eluted at a higher salt concentration (Fig. 9A, SP peak II). A silver-stained SDS gel of SP peak I eluting from the analytical column confirmed that SP peak I contained p20 and p10 (Fig. 9B, lane 10–1). Silver-stained gels of peak II, however, showed that it consisted of an almost pure preparation of p20 (as confirmed by the I150–158 antibody, Fig. 2B) together with lesser amounts of a protein with M, less than that of p10 (Fig. 9B, lane 10–II). Immunoblots using the NH2-terminal antipeptide antibody I317–330 indicated that the lower M, band was a form of p10.
IL-1β-converting Enzyme Purification

**FIG. 5.** Tryptic (panels A and C) and Asp.N maps (panels B and D) of the p20 (left) and p10 (right) together with peptide sequences (panel E) from various of the peptides. Tryptic peptides (T) were generated from 50 pmol of protein, whereas Asp.N peptides (N) were generated from 40 pmol of protein as described under "Materials and Methods."

**FIG. 6.** C4 HPLC tryptic map of the [14C]iodoacetic acid-labeled p20. The UV tracing (bottom) is plotted versus the corresponding radioactivity of each indicated fraction (top). To identify the catalytic Cys, 17,000 units of SP-5PW purified ICE (preparations 11 and 13, Fig. 2A) were labeled with 100 μM [14C]iodoacetic acid as described previously (23). The p20 was purified by C4 HPLC (100 pmol collected), cleaved with trypsin (see "Materials and Methods"), the peptide mixture was chromatographed and collected in fractions, and 1% of the fractions were counted. Fraction 20 was repurified by C4 HPLC and sequenced resulting in the indicated VIIIQAC sequence. All of the cpm were found associated with the Cys.

(Fig. 2C, lane 10-II) and suggested that the p10 was most likely cleaved at the COOH-terminal end to generate the altered form. SP peak II contained over 3-fold lower activity than would be predicted from its p20 content, suggesting that the form of the enzyme containing a truncated p10 has a lower specific activity. The K_m values of both activity peaks, SP peaks I and II, against the AcYVAD-AMC substrate are equal. This suggests that the reduced activity of SP peak II is caused by a reduced k_cat.

**FIG. 7.** Schematic drawing of the ICE protein showing the location of the p22, p20, and p10 proteins relative to the 46-kDa precursor protein (p45) as cloned (23). The p20 and p10 proteins present in the active enzyme are shaded black. Indicated below are short regions to which antipeptide antibodies have been developed. The antibodies are labeled by their sequence number relative to the p45 protein and the actual sequence to which a cysteine and/or norleucine (N) were added for conjugation to thyroglobulin. Tags on the blocked peptides indicate on which end the tether was made to the thyroglobulin.

**DISCUSSION**

In this report we have characterized the forms of ICE proteins found in active cytoplasmic extracts of THP.1 cells by HPLC purification and immunoblot analysis using specific ICE antibodies. The presence of two proteins, p20 and p10, correlated strongly with ICE activity. Tryptic mapping and site-specific labeling showed that these two proteins were distinct and that a single Cys (285) near the COOH terminus of p20 contained the active site thiol. Sequence analysis of the tryptic and Asp.N peptides enabled the subsequent cloning of an ICE cDNA from a THP.1 cDNA library encoding a protein of about 45 kDa, which includes the sequences for the p20 and p10 proteins (23). We have found that the p20 migrates on SDS-PAGE with an M_r of 22,000. Two other groups have identified this same protein as an ICE protein, referring to it as a 22-kDa protein (33-36). Only in this study and in our earlier work, however, was the p10 identified, isolated in pure form, and shown to be essential for ICE activity.

Antibodies were developed against purified active ICE as well as against various peptide regions of both the p20 and p10 proteins. Immunoblot analysis of the ion exchange-purified ICE resulted in the identification of several different ICE forms that differed in their catalytic activity. In the first case, crude extracts of active THP.1 cytosol contain comparable amounts of p20 and p22, an alternatively processed form of

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2 N. A. Thornberry, unpublished observations.
p20. p22 coelutes with p20 in all of the HPLC fractionations used to purify p20. Because this form has an $M_r$ of 24,000 on SDS gels it was previously referred to as p24 prior to any detailed analysis (23). We now know that immunoblotting with the I285–297 COOH-terminal antiserum indicates that the p22 contains the same COOH terminus and active site as p20 and that protein sequencing reveals that this larger ICE form contains an additional 16 residues at its NH$_2$ terminus, an amount sufficient to account for the 2,000 larger size on gels. Sequence comparison with the cDNA indicates that the NH$_2$ terminus of the p22 results from a cleavage at an Asp residue in the ICE precursor, a highly unusual proteolytic cleavage site. Given the specificity of ICE for Asp in P1, it is likely that p22 is generated by autoproteolysis, as has been suggested for p20 and p10. P22 is found in large amounts in the DEAE-5PW ICE active fractions, yet it is found in reduced and variable amounts in the active SP-5PW pools (Fig. 2) and in purified ICE (Fig. 4A). It is possible that the p22 could be proteolytically processed to the p20 during purification by an autocatalytic mechanism.

It is unclear as to the extent of p22 activity. Because p22 contains the catalytic site Cys and binds to an inhibitor bound affinity column (Fig. 4), p22 is catalytically active itself or it is associated as part of an active ICE complex. On the other hand, comparison of the ICE activity with the amount of p20 or p22 protein found in fractions of ICE (Fig. 8) indicates that the ICE activity tracks most closely with the levels of p20 but not p22. Furthermore, retrospective immunoblot analysis has shown that p22 was present in the SP-5PW-purified ICE preparation used previously for the active site labeling with [$^{14}$C]iodoacetic acid (Fig. 2B, preparation 17). In that experiment, however, only the p20 and not the p22 was labeled (Fig. 3b in Ref. 23). Both results suggest that the catalytic activity of the p22/p10 is low relative to the p20/p10. Recent cloning of the mouse and the rat forms of ICE, however, has indicated the absence of the Asp necessary for the processing of p45 to generate p20, whereas the Asp that generates p22 is conserved in all three species (37, 38). This suggests that p22 has the potential to take the place of p20 for ICE activity completely. Mutagenesis of each of these cleavage sites may be necessary to obtain definitive evidence, and such experiments are in progress.

In addition to identifying the existence of p22, immunoblot analysis has also shown that the p22/p20 elutes on ion exchange columns in three distinct peaks associated both with varying amounts of activity and varying amounts of p10. The first, most active peak contained equal amounts of p20 and p10 (DEAE and SP peak I; Fig. 8, fractions 100–103). A second peak with little activity contained a decreased amount of a COOH-terminally truncated p10 (SP peak II), and a third contained neither activity nor detectable levels of p10 (DEAE peak II; Fig. 8, fractions 142–145). In some DEAE column purifications immunoblotting identified small amounts of the second of these peaks eluting midway between DEAE peaks I and II (not shown). No apparent differences between the p20 and p22 were found within each peak as judged by the following three criteria: (i) the same order of elution of p22 and p20 from the ion exchange column; (ii) the $M_r$ values on SDS-PAGE of p22 and p20 as observed in each of the three peaks; (iii) the pattern and intensity of the protein domains as measured by immunoblot analysis. These observations suggest that the differences among the activities of the peaks are not because of major alterations in the structure of p22 and p20.

In contrast, what is most distinctive about these ICE protein peaks is the change in the amount and form of the p10 associated with them: the less the p10 found in the peak, the lower its activity. This suggests that the p10 is absolutely essential for activity even though it does not have the catalytic Cys. The importance of the p10 is underscored by the observation that it is more highly conserved among mouse, rat, and human ICE than is the p20 (34, 37, 38). Just as other ICE proteins are cleaved following Asp, the p10 that had been truncated at its COOH terminus (SP peak II) may have also been formed by an autocatalysis of ICE since it is generated most prominently after ICE concentration. Recent expression of ICE in an Escherichia coli expression system has identified a 7-kDa form of p10 cleaved after an Asp in the COOH-terminal region (Asp$^{381}$ of the cDNA sequence) comparable to the 7–8-kDa piece currently seen in Fig. 9.

There is no evidence that the p20 and p10 polypeptides coincide with the enzymatic subunits of ICE where active
oligomers freely dissociate to inactive monomers (23). Reconstitution experiments have not been successful because of the inability to purify the individual subunits by conventional chromatography. Furthermore, active ICE could not be produced by mixing together p20 and p10 individually expressed in E. coli or by coexpressing p20 and p10 together in E. coli (39).

The observation that proteolysis of ICE occurs in THP.1 cells to give different sized fragments is not surprising since the ICE clone encodes a 45-kDa protein (23, 33) that is the precursor of the active ICE enzyme described in this report. Although a 45-kDa protein corresponding to the primary translation product is identified in the THP.1 lysates by ICE antibodies (not shown), it is not found in any active ICE fractions and has little activity in cleaving pIL-1/B (40). The relationship of the presently described cleaved forms of ICE to the, as yet, ill defined naturally occurring form that cleaves pIL-1/B in intact, stimulated monocytic cells is currently being investigated.

Acknowledgments—We thank Drs. John Schmidt, Michael Tocci, Philip Davies, and Alan Williamson for support during the progress of this research.

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