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Immunology

Interleukin 1β (IL-1β) processing in murine macrophages requires a structurally conserved homologue of human IL-1β converting enzyme

(inflammation/autocatalysis/protease)

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ABSTRACT **Murine interleukin 1β (IL-1β) convertase (mICE) was identified in cytosolic extracts of peritoneal exudate cells (PECs) and macrophage cell lines. mICE cleaves both the human and mouse IL-1β precursors (pIL-1β) at sites 1 and 2 but fails to cleave a human pIL-1β (Asp^{116} to Ala) mutant at site 2, indicating that Asp is required to the left of the scissile bond. Ac-Tyr-Val-Ala-Asp-amino-4-methyl coumarin, patterned after site 2 of human pIL-1β, is a fluorogenic substrate for mICE, while the tetrapeptide aldehyde Ac-Tyr-Val-Ala-Asp-CHO is a potent inhibitor (K_i = 3 nM) that prevents generation and release of mature IL-1β by PECs (IC_{50} = 7 μM). Cloning of a full-length 1.4-kb cDNA shows that mICE is encoded as a 402-aa proenzyme (p45) that can be divided into a prodomain (Met^1–Asp^{122}), followed by a p20 subunit (Gly^{123}–Asp^{296}), a connecting peptide (Ser^{297}–Asp^{314}), and a p10 subunit (Gly^{315}–His^{402}). At the amino acid level, p45, p20, and p10 are 62%, 60%, and 81% identical with human IL-1β convertase (hICE). The active site Cys^{284} lies within a completely conserved stretch of 18 residues; however, Ser^{289} in hICE, which aligns with the catalytic region of serine and viral cysteinyl proteases, is absent from mICE. Expression in *Escherichia coli* of a truncated cDNA encoding Asn^{119}–His^{402} generated active enzyme, which was autocatalytically processed at three internal Asp-Xaa bonds to generate a p20 subunit (Asn^{119}–Asp^{296}) complexed with either p11 (Ala^{309}–His^{402}) or p10. Recombinant mICE cleaves murine pIL-1β accurately at the Asp^{117}-Val^{118} bond. The striking similarities of the human and murine enzymes will make it possible to assess the therapeutic potential of hICE inhibitors in murine models of disease.**

Interleukin 1β (IL-1β) is synthesized as a 31-kDa precursor polypeptide (pIL-1β) that must be proteolytically cleaved to generate the active 17.5-kDa cytokine (1, 2). In stimulated human monocytes, processing requires a specific protease, termed IL-1β converting enzyme (ICE), which cleaves pIL-1β at site 1 (Asp^{27}-Gly^{28}) and site 2 (Asp^{116}-Ala^{117}) to yield 28- and 17.5-kDa products, respectively (3). The recent purification and cloning of human ICE (hICE) (4, 5) have revealed that the enzyme is a unique heterodimeric cysteine protease with a highly unusual requirement for Asp to the left of the scissile bond (P1). The active enzyme consists of a 1:1 stoichiometric complex of 19.8- (p20) and 10.2- (p10) kDa subunits, which are derived from a single 45.2-kDa (p45) proenzyme (4). In the proenzyme, Asp-Xaa bonds flank both subunits, as well as an alternatively processed form of p20, termed p24, suggesting that autoproteolysis may be involved in generating the heterodimeric (p20–p10) form of the enzyme (4).

Inhibition of hICE prevents both cleavage of the IL-1β precursor and release of the mature cytokine from monocytes in lipopolysaccharide (LPS)-stimulated whole blood (4) and thus may represent a therapeutic approach for treating inflammatory diseases. To validate ICE as a therapeutic target, it will be necessary to establish the biochemical and functional efficacy of ICE inhibitors in appropriate animal models of human disease.

Several lines of evidence suggest that an IL-1β processing enzyme similar to hICE is present in murine macrophages. First, the minimal substrate requirements for hICE (4) define a peptide sequence that is highly conserved at sites 1 and 2 in the pIL-1βs from all mammalian species thus far examined (3). Second, hICE can cleave murine pIL-1β to yield products congruent with those generated from human pIL-1β (3). Finally, evidence for murine ICE (mICE) activity has been obtained in the murine macrophage cell line J774, where apoptosis results in cleavage of murine pIL-1β at Asp^{117}-Val^{118} (6).

In this report, native mICE has been identified and shown to be similar to hICE with respect to its substrate requirements and its inhibition by a specific, potent tetrapeptide aldehyde. Addition of this inhibitor to cultures of murine peritoneal exudate cells (PECs) blocked the generation of processed, mature IL-1β. Analysis of the mICE cDNA sequence revealed that all of the salient features of hICE are conserved. Expression of the cDNA in *Escherichia coli* showed that active heterodimeric enzyme is generated by autocatalytic processing at Asp-Xaa bonds. Recombinant mICE cleaved recombinant murine pIL-1β at the Asp^{117}-Val^{118} bond to generate authentic mature IL-1β. These findings suggest that the mouse is a useful model in which to examine the role of ICE in regulation of IL-1β-mediated biological functions.

MATERIALS AND METHODS

Isolation of PECs. Female CD-1 mice were injected i.p. with 1 mg of heat-killed *Propionibacterium acnes*. Cells were harvested 6 days after injection by lavage with 5 ml of phosphate-buffered saline (PBS) containing heparin (10 units/ml) (Upjohn), plated (2 × 10^6 cells per ml) for 2 h in RPMI 1640 medium/10% fetal calf serum (Hazelton Labo-

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Abbreviations: IL-1β, interleukin 1β; mICE, murine IL-1β converting enzyme; hICE, human IL-1β converting enzyme; pIL-1β, IL-1β precursor; PEC, peritoneal exudate cell; AMC, amino-4-methyl coumarin; LPS, lipopolysaccharide; DTT, dithiothreitol; LC-ESI-MS, liquid chromatography electrospray ionization mass spectroscopy; ORF, open reading frame.

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ratories, Gaithersburg, MD) at 37°C, and washed. Adherent cells were cultured in fresh medium containing LPS (100 ng/ml) for 18 h before harvest.

Cells and Tissue Culture. J774, IC21, P388D.1, WEHI-3, RAW 8.1, EL4, and NIH 3T3 cells were obtained from the American Type Culture Collection and cultured according to their specifications.

Cell Extracts. Cell-free extracts were prepared by hypotonic lysis (7), dialyzed against 100 mM Hepes/10% sucrose/0.1% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate/2 mM dithiothreitol (DTT), pH 7.5 (4°C), and stored at −80°C.

hICE. hICE was purified to homogeneity from extracts of THP.1 cells by affinity chromatography (4).

pIL-1β Cleavage Assay. ICE activity was monitored by a radiolabeled pIL-1β gel cleavage assay (3, 7).

Peptide Cleavage Assay. The synthetic substrate Ac-Tyr-Val-Ala-Asp-amino-4-methyl coumarin (AMC) was used in a continuous, fluorometric enzyme assay for ICE (4) (1 unit = 1 pmol of AMC per min at 25°C at saturating substrate).

Murine IL-1β Antibodies. The polyclonal antiserum used in the ELISA and immunoblot analysis was generated by injecting New Zealand rabbits i.d. or s.c. with 50 μg of murine IL-1β (R & D Systems, Minneapolis) emulsified in 1× complete Freund's adjuvant or 3× incomplete Freund's adjuvant over an 8-week period. The polyclonal antiserum used to precipitate IL-1β was raised to murine IL-1β adsorbed to alum (8). A monoclonal antibody for murine IL-1β was kindly provided by David Chaplin (Washington University, St. Louis).

Murine IL-1β ELISA. Plates (96-well; Costar) were coated with the monoclonal antibody (5 μg/ml) in PBS, washed, and blocked by addition of bovine serum albumin solution for 30 min. After three washes, 100-μl aliquots of sample were added to the plate and incubated for 2 h at 25°C. The plates were washed four times and incubated for 1 h at 25°C with a 1:500 dilution of the rabbit polyclonal serum. After three washes, plates were incubated with a 1:10,000 dilution of peroxidase-conjugated $F(ab')_2$ fragment of goat anti-rabbit IgG (Accurate Scientific) for 1 h at 25°C. The peroxidase assay (TMB substrate; Kierkegaard and Perry Laboratories, Gaithersburg, MD) was terminated after 1 min by addition of 1 M phosphoric acid, and color development was quantitated by measurement of OD_{450} on a plate spectrophotometer (Molecular Devices).

Characterization of Murine IL-1β Produced by PECs. PECs (2×10^6 cells) were lysed directly in 100 μl of 2× Laemmli buffer, electrophoresed on a 12.5% polyacrylamide gel, and analyzed by immunoblot (9) with polyclonal antiserum against IL-1β. Culture supernatants (2 ml) from PECs were immunoprecipitated by incubation for 18 h at 4°C with 200 μl of a 25% slurry of protein A beads coated with rabbit immune IgG. The immune complexes were washed three times in RIPA buffer (25 mM Tris·HCl, pH 7.5/10 mM NaCl/1 mM EDTA/1% Triton X-100/0.5% sodium deoxycholate/0.2% SDS) and resuspended in 20 μl of 2× Laemmli buffer (10). Electrophoresis and immunoblotting were carried out as described above except that the blots were incubated in SuperBlock (Pierce) for 1 h at 25°C before addition of antibody.

Isolation of mICE cDNA Clones. The hICE cDNA clone was used as a probe to screen a mouse macrophage library (Clontech) under reduced stringency. The filters were prehybridized [30% formamide/5× standard saline citrate (SSC)/5× Denhardt's solution/0.1% SDS/100 μg of salmon sperm DNA per ml] for 1 h at 42°C and then hybridized in the same buffer containing 10% dextran sulfate and ^{32}P radiolabeled probe overnight at 42°C, washed in 2× SSC/0.1% SDS at 42°C, and exposed to x-ray film. A partial mICE cDNA clone isolated in this manner was used as a probe to rescreen a WEHI library (Stratagene) in the same manner except that the hybridization buffer contained 50% formamide.

Isolation of RNA. Total RNA was extracted from 10^8 cells by a guanidine thiocyanate procedure (12), fractionated on 1% agarose gels containing 2.2 M formaldehyde, and blotted to a Duralon membrane. Blots were probed (11) with full-length m- or hICE ^{32}P radiolabeled cDNA.

Recombinant mICE. A construct for expression of recombinant enzyme was generated by PCR with primers that contained *Nde* I sites and placed an initiator methionine in-frame with a truncated form of the mICE proenzyme (Asn^{119}–His^{402}) lacking most of the prodomain. The PCR fragment was cloned into the *Nde* I site of the pET11a expression vector (Novagen), transformed into BL21(pLysS) *E. coli*, and induced with isopropyl β-D-thiogalactoside. After resuspension in 50 mM Tris·HCl, pH 8/0.5 mM EDTA/0.5 mM sucrose/1 mM phenylmethylsulfonyl fluoride/aprotinin (5 μg/ml)/pepstatin (5 μg/ml)/leupeptin (10 μg/ml), cells were homogenized in a Menton–Gaulin press.

Active mICE was purified from concentrated extracts by ligand affinity chromatography as described (4). After elution in reductant-free buffer, the material was directly analyzed by liquid chromatography electrospray ionization mass spectroscopy (LC-ESI-MS) (4).

Recombinant Murine pIL-1β. A full-length murine pIL-1β cDNA (3) was cloned into the pET11a vector as described above. Recombinant pIL-1β was isolated from the inclusion body pellet by washing three times in 1% Triton X-100/50 mM Tris·HCl, pH 8/1 mM phenylmethylsulfonyl fluoride/pepstatin (10 μg/ml)/leupeptin (10 μg/ml), followed by solubilization in 10 vol of 6 M guanidine hydrochloride/100 mM DTT/50 mM Tris·HCl, pH 7.5. The sample was fractionated on a C4 reversed-phase HPLC column, diluted into ICE buffer, and purified to homogeneity by DEAE-5PW HPLC (4).

Cleavage of Murine pIL-1β by mICE. Purified recombinant mICE was activated as described (4). Purified pIL-1β (300

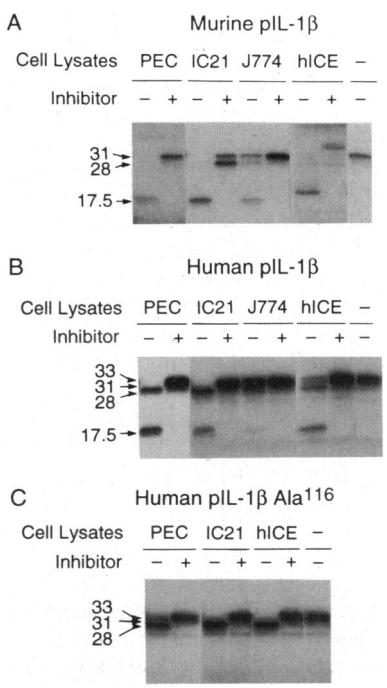

FIG. 1. Cleavage of murine pIL-1β (*A*), human pIL-1β (*B*), or a human pIL-1β (Asp^{116} to Ala) processing site mutant (*C*) by cytosolic extracts of murine PECs and macrophage cell lines. Extracts (4 μl) from *P. acnes*-elicited PECs; IC21 or J774 cells were incubated with 1 μl of radiolabeled pIL-1β for 4 h in the absence (lanes −) or presence (lanes +) of 1 μM inhibitor (Ac-Tyr-Val-Ala-Asp-CHO. Affinity-purified hICE was assayed as a control. ICE activity was monitored by fractionation of cleavage products on SDS/PAGE. The sizes (kDa) of IL-1β proteins are indicated.

μg) and recombinant mICE (200 units) were incubated in 500 μl of ICE buffer (4) for 5 h at 30°C. An aliquot was analyzed by SDS/PAGE and stained with Coomassie brilliant blue G. An additional aliquot was injected onto a narrow-bore C4 reversed-phase HPLC column and the molecular mass values of pIL-1β as well as the 17.5-kDa cleavage product were determined by LC-ESI-MS.

RESULTS AND DISCUSSION

mICE Activity in PECs and Macrophage Cell Lines. To determine whether murine macrophages contained ICE activity, cytosolic extracts prepared from *P. acnes*-elicited PECs were tested in a gel-based assay with *in vitro* synthesized, radiolabeled pIL-1β substrates (Fig. 1). These extracts cleaved murine pIL-1β to a 17.5-kDa product that was the same size as that generated by purified hICE (Fig. 1*A*, lanes 1 and 7). A 28-kDa product was generated following shorter incubations (data not shown). The PEC extracts also cleaved human pIL-1β to 28- and 17.5-kDa products corresponding to cleavage at sites 1 and 2 by hICE (Fig. 1*B*, lanes 1 and 7) (7, 13). Incubation of PEC extracts with a human pIL-1β processing site mutant in which Asp^{116} at site 2 has been changed to Ala (3) yielded only the 28-kDa product, indicating that mICE also requires Asp at P1 (Fig. 1*C*, lanes 1 and 5).

To obtain a convenient source of mICE, several murine monocyte–macrophage cell lines were surveyed for activity. The ICE activity in these cell lines cleaved murine and human pIL-1β at sites 1 and 2, and the Ala^{116} mutant of human pIL-1β only at site 1 (Fig. 1 *A* and *B*, lanes 3 and 5; Fig. 1*C*, lane 3). These studies indicate that elicited murine macrophages and macrophage cell lines constitutively express an ICE activity that exhibits the same macromolecular substrate specificity as hICE.

Time-Dependent Inhibition by a Tetrapeptide Aldehyde. Complete inhibition of cleavage of both murine and human pIL-1β at sites 1 and 2 was obtained upon incubation of the PEC and cell line-derived extracts with a tetrapeptide aldehyde (Ac-Tyr-Val-Ala-Asp-CHO) that is a potent, selective, reversible inhibitor of hICE (K_i = 0.76 nM; Fig. 1 *A* and *B*, lanes 2, 4, 6, and 8; Fig. 1*C*, lanes 2, 4, and 6) (4). The kinetics of inhibition of mICE by the tetrapeptide aldehyde were analyzed by the continuous fluorometric assay developed for the human enzyme (4). The tetrapeptide aldehyde is a slow binding inhibitor as is found with hICE (Fig. 2). The steady-state velocity defines a K_i of 3 nM for the murine enzyme, versus 0.76 nM for hICE. These results along with those described above show that mICE requires Asp in P1 and that the active sites of the native human and murine enzymes are closely related.

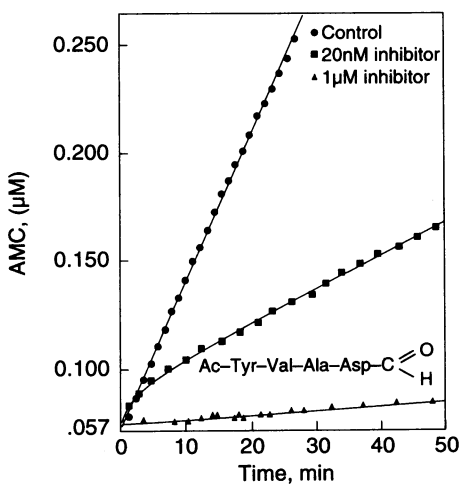

FIG. 2. Inhibition of mICE by the tetrapeptide aldehyde Ac-Tyr-Val-Ala-Asp-CHO. Addition of mICE cytosolic extract to reaction mixtures containing inhibitor (20 nM) and the fluorometric substrate Ac-Tyr-Val-Ala-Asp-AMC (14 μM) under standard assay conditions (4) resulted in first-order decay of the initial rate, v_o, to a final steady-state velocity, v_s, with a rate constant of 6×10^{-3} s^{-1} shown by theoretical lines. The value for K_i (3 nM) was calculated as described (14). The background hydrolysis of substrate was determined from the rate of the reaction with saturating inhibitor (1 μM).

ICE Inhibition Prevents Mature IL-1β Release from Mouse Peritoneal Macrophages. To evaluate the role of mICE in IL-1β production by intact cells, PECs from *P. acnes*-treated mice were isolated by adhesion and cultured for 18 h in the presence of LPS (100 ng/ml) (15). Under these conditions, macrophages secrete significant levels (1–6 ng/ml) of IL-1β as measured by ELISA. Addition of the tetrapeptide aldehyde resulted in a dose-dependent inhibition of IL-1β release with an IC_{50} of 7 μM, as compared to 4 μM in human blood (Fig. 3*A*) (4). Immunoblot analysis of the culture medium and cell lysates showed that the inhibitor prevented generation of all mature extracellular 17.5-kDa IL-1β without affecting the levels of intracellular pIL-1β (Fig. 3*B*). Thus, an inhibitor of mICE completely blocks processing of pIL-1β to mature IL-1β in intact cells.

In human blood, inhibition of mature IL-1β release was paralleled by an inversely proportional increase in extracellular pIL-1β, showing that processing and secretion are not mechanistically coupled (4). While murine pIL-1β does not appear to be released from PECs in the presence or absence of inhibitor (Fig. 3*B*), this may be due to the inability of our antisera, which were raised to the mature form of IL-1β, to immunoprecipitate murine pIL-1β (data not shown) (16).

Isolation of a mICE cDNA Clone. A partial mICE cDNA was isolated by screening a mouse macrophage library under reduced stringency with the full-length hICE cDNA (4). Using this partial cDNA as a probe, six longer clones were isolated by screening a WEHI cDNA library. The longest cDNA had an open reading frame (ORF) encoding a protein of 402 aa with a predicted molecular weight of 45,640 (p45; Fig. 4). This cDNA was 1378 nt long, had 48 and 115 nt of 5′ and 3′ untranslated sequence, respectively, and contained a

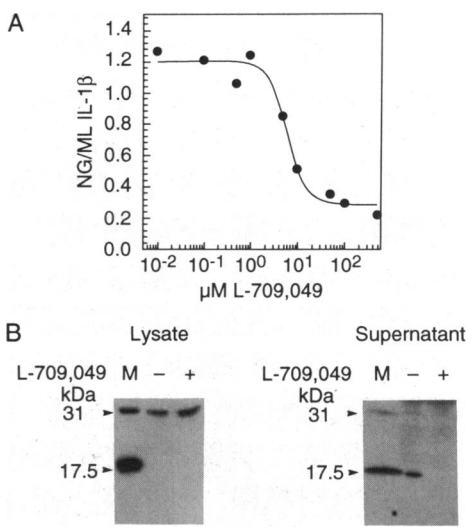

FIG. 3. ICE inhibitor Ac-Tyr-Val-Ala-Asp-CHO (L-709,049) prevents release of mature IL-1β from LPS-activated murine peritoneal macrophages. (*A*) Dose–response of IL-1β release from PECs treated with inhibitor (L-709, 049). IL-1β levels in the culture supernatants were measured by ELISA as the mean of duplicate samples. (*B*) Immunoblot analysis of the forms of IL-1β in lysates and supernatants of activated murine PECs. After incubation in the absence or presence of 100 μM L-709,049, cells and culture media were analyzed for IL-1β as described. The sizes (kDa) of IL-1β protein standards (land M) are indicated.

polyadenylylation signal sequence as well as a poly(A) tail. In the ORF, there is 72% identity in the nucleic acid sequence between the human and murine cDNAs.

mICE and hICE Proteins Are Highly Conserved. Active hICE is a stoichiometric complex made up of two nonidentical subunits, p20 and p10, which are derived from p45 by processing at Asp-Xaa bonds. By analogy with hICE, the ORF of mICE can be divided into a prodomain (Met^{1}–Asp^{122}), followed by p20 (Gly^{123}–Asp^{296}), a short connecting peptide (Ser^{297}–Asp^{314}), and p10 (Gly^{315}–His^{402}) (Fig. 4). Consistent with these assignments, Asp-Xaa bonds are conserved at the C terminus of p20 (Asp^{296}-Ser^{297}) and the N terminus of p10 (Asp^{314}-Gly^{315}). However, the sequence of mICE differs from that of hICE at the N terminus of p20, where Asn^{119} is deleted in the murine protein; nevertheless, a candidate site for ICE cleavage is present within four residues at Asp^{122}-Gly^{123}, suggesting that the N terminus of the murine p20 subunit is Gly^{123}. An alternative form of human p20, termed p24, results from cleavage at the Asp^{103}-Ser^{104} bond (4), which is conserved in mICE. Cleavage at this bond would add 19 residues to the predicted N terminus of murine p20.

mICE and hICE are closely related, with 62% identity at the primary amino acid sequence level. The prodomain and the connecting peptide sequences display 53% and 50% identity with the corresponding domains in hICE, suggesting that they are functionally important. In other proteases, prodomains can act both as folding templates and/or as inhibitors of protease activity (17–19). By analogy with the connecting peptide of insulin, the connecting peptide in p45 may facilitate folding of the subunits into an active complex before excision from the enzyme (20).

The p20 subunit is well-conserved, exhibiting 62% identity with its human counterpart. Cys^{284} in murine p20 aligns with the catalytic Cys^{285} of hICE (4) and lies within a completely conserved stretch of 18 aa. Neither enzyme has extensive homology to known proteins, including other cysteinyl proteases. However, a region adjacent to the active site Cys in hICE (Gly-Asp-Ser^{289}-Pro-Gly) aligns with the consensus sequence for the catalytic Ser/Cys of serine and viral cysteinyl proteases (Gly-Xaa-*Ser/Cys*-Xaa-Gly) (21). The mICE sequence (Gly-Glu-Lys^{288}-Gln-Gly) fails to align, eliminating any relationship between ICE and previously identified protease sequences.

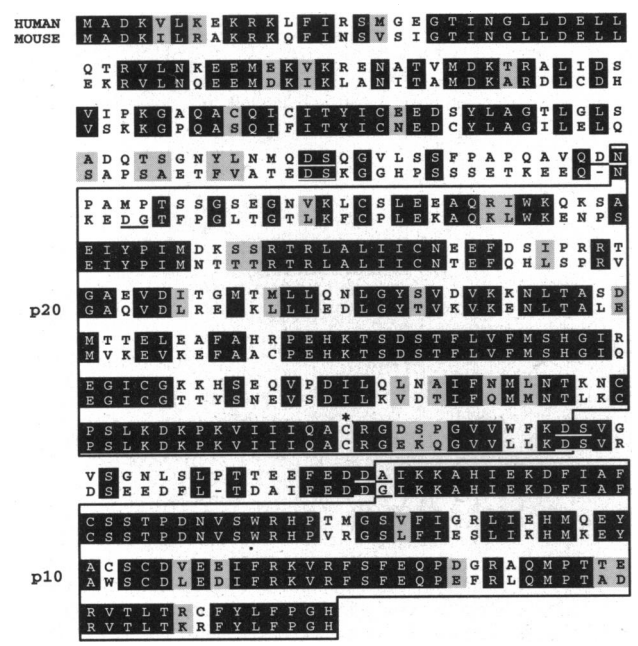

FIG. 4. Comparison of deduced amino acid sequences for the m- and hICE proenzymes. Alignment of the two proteins indicates that the Asp^{119} and Pro^{308} residues in hICE are deleted in mICE. Identical amino acids (black) and conservative substitutions (gray) are indicated. The p20 and p10 subunits are outlined. The active site Cys is denoted with an asterisk. Autoprocessing sites are underlined.

The p10 subunit, which shares 81% amino acid identity with human p10 is the most highly conserved portion of the molecule. The most striking feature is an identical stretch of 26 aa at the N terminus. An earlier observation made with hICE showed that saturating levels of substrate or inhibitor prevent the time-dependent loss of enzymatic activity that occurs upon dilution (4), suggesting that p10 participates directly in substrate binding. The extensive conservation of p10 provides additional evidence that this subunit is important for catalytic activity and may contribute to formation of the active site of ICE. In this regard, many cysteine proteinases contain an essential histidine, which serves a general base function in catalysis (22). It is interesting to note that four of the six conserved histidines are found in p10 (p20, His^{224} and His^{237}; p10, His^{322}, His^{342}, His^{356}, and His^{404}).

ICE mRNA Is Expressed in Monocytes and Other Hematopoietic Cells. By Northern blot analysis, a single 1.6-kb low-abundance ICE mRNA, similar to the major transcript found in human THP.1 cells, is constitutively expressed in IC21, J774, WEHI-3, and RAW8.1 cells, but not in P388D1 cells or 3T3 fibroblasts (Fig. 5). A T-cell line (EL4) also expressed ICE mRNA. The two minor species of ICE mRNA, 2.3 and 0.5 kb in size, that have been detected in human THP.1 cells (4, 5) were not found in this panel of murine cells (Fig. 5). In the monocytic cell lines examined, ICE activity correlated with the presence of ICE mRNA (data not shown). Thus, although LPS or other stimuli are required for IL-1β synthesis and secretion, they are not required for transcription and translation of hICE or mICE (Figs. 1 and 5). Given that preparation of cell extracts may result in activation of the proenzyme, further experiments are needed to determine the structure and subcellular location of the enzyme in intact cells.

Characterization of Recombinant mICE. To obtain sufficient quantities of mICE for detailed characterization, a truncated cDNA encoding Asn^{119}–His^{402} was expressed in *E. coli*. The crude cell extracts contained the primary translation product as well as mICE-derived proteins similar in size to human p20 and p10. Only the proteins corresponding to p20 and p10 (Fig. 6*A*) were isolated following ligand affinity chromatography (4). LC-ESI-MS of the purified enzyme yielded an ion chromatogram with two major peaks (data not shown). Deconvolution of the spectra associated with the second ion peak revealed a major species of M_r 20,171.4, which is within 0.4 atomic mass unit of the mass value predicted for the fully reduced, recombinant, *N*-methionyl form of p20 (Asn^{119}–Asp^{296}) (Fig. 6*C*). Similarly, deconvolution of the spectra associated with the first ion peak revealed two molecular species of M_r 10,478.8 and 11,170.0, which are each within 0.2 atomic mass unit of the mass values

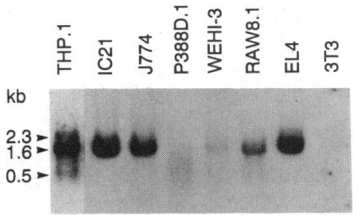

FIG. 5. Northern blot analysis of ICE mRNA expression in murine cells. Total cellular RNA (20 μg) was hybridized to hICE (lane 1) or mICE (lanes 2–8) cDNA probes. Sizes of ICE mRNAs are indicated.

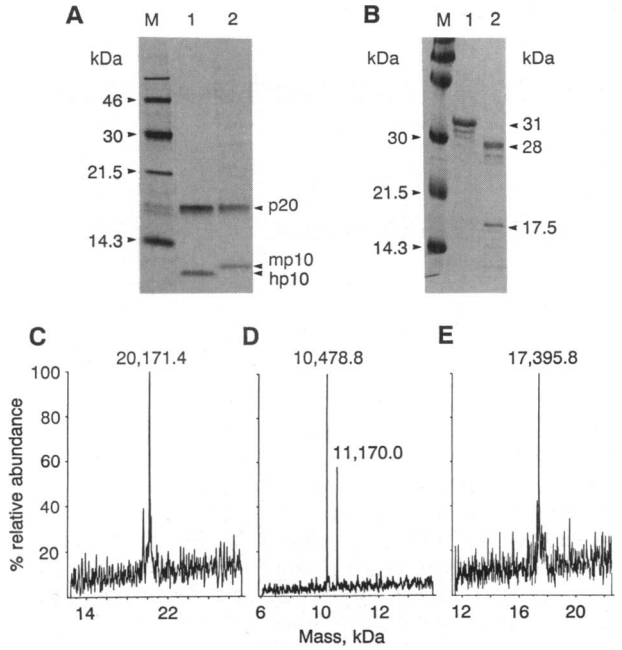

FIG. 6. Structural and functional analysis of affinity-purified recombinant mICE from *E. coli*. (*A*) Silver-stained SDS/PAGE of recombinant mICE (lane 2) from *E. coli* after affinity purification. The same number of units of native, affinity-purified hICE (lane 1) is included as a standard. Positions of human and murine p20, murine p10 (mp10), and human p10 (hp10) are on the right. Molecular mass values of marker proteins (lanes M) are on the left. (*B*) Coomassie-stained SDS/PAGE of recombinant murine pIL-1β before (lane 1) and after (lane 2) a 5-h incubation with recombinant mICE. Molecular mass values (kDa) of marker proteins (lanes M; left) and IL-1β proteins (right) are indicated. (*C–E*) Affinity-purified mICE in *A* and mature 17.5-kDa IL-1β in *B* were analyzed by LC-ESI-MS. The deconvoluted mass spectra of p20 (*C*), p10 (*D*), and mature murine IL-1β are shown.

predicted for fully reduced p10 (Gly^{315}–His^{402}) and p11 (Ala^{309}–His^{402}) (Fig. 6*D*). The latter protein is an alternatively processed version of p10 resulting from cleavage of p45 between Asp^{308} and Ala^{309}, a bond that is not found in hICE. The N termini of p20, p11, and p10 were confirmed by Edman sequencing (data not shown). These results demonstrate that active mICE is a heterodimeric complex consisting of p20 paired with either p10 or p11, the formation of which results from autoprocessing of Asp-Xaa bonds (where Xaa is Ser^{297}, Ala^{309}, and Gly^{315}) in extracts of *E. coli*. Interestingly, the Asp^{122}-Gly^{123} bond in the recombinant form of p20 was not cleaved. All of the Cys residues in the enzyme eluted from the affinity matrix are fully reduced, indicating that enzyme activity is not dependent on formation of disulfide bonds. Moreover, the finding that the prodomain is not required for production of active mICE in *E. coli* is consistent with transfection studies in COS-7 cells, showing that active hICE is generated following expression of a protein lacking the prodomain (5). Additional studies are required to determine whether the prodomain alters the efficiency with which active heterodimer is generated.

To test the ability of recombinant mICE heterodimers to accurately cleave the relevant macromolecular substrate, murine pIL-1β was expressed in *E. coli*, purified, and found by LC-ESI-MS to have a M_r of 30,794.2 (predicted M_r, 30,794.6). Cleavage of murine pIL-1β yielded products of 28 and 17.5 kDa (Fig. 6*B*), consistent with accurate cleavage of sites 1 and 2. The 17.5-kDa cleavage product had a M_r of 17,395.8 (Fig. 6*E*), which is within 0.9 atomic mass unit of the mass predicted for authentic, mature, murine IL-1β (Val^{118}–Ser^{270}) resulting from cleavage at the Asp^{117}-Val^{118} (site 2) amide bond (6).

In summary, comparison of m- and hICE has identified the prototypical features likely to be conserved among all ICE proenzyme sequences: (*i*) a prodomain; (*ii*) a p20 subunit containing the active site Cys residue and flanked by Asp-Xaa bonds; (*iii*) a short linker peptide; and (*iv*) a p10 subunit preceded by an Asp-Xaa bond and terminating with the end of the ORF. Active ICE consists of a stoichiometric complex of p20 and p10, or their alternatively processed forms, and results from autocatalytic cleavage of Asp-Xaa bonds.

A critical biological role for mICE in production of mature 17.5-kDa IL-1β by LPS-stimulated murine peritoneal macrophages has been clearly demonstrated. These data, taken together with the striking conservation of functional, structural, and catalytic properties between m- and hICE, will allow the therapeutic potential of inhibitors to be evaluated in murine models of IL-1β-mediated inflammatory disease.

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