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Identification and Characterization of Ich-3, a Member of the Interleukin-1β Converting Enzyme (ICE)/Ced-3 Family and an Upstream Regulator of ICE*

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Suyue Wang§§, Masayuki Miura††, Yong-keun J un§§, Hong Zhu§§, Valeria Gagliardini, Lianfa Shi***, Arnold H. Greenberg****, and Junying Yuan§§§

From the Cardiovascular Research Center, Massachusetts General Hospital-East, Charlestown, Massachusetts 02129, the Department of Medicine, Harvard Medical School, Boston, Massachusetts 02115, the Department of Molecular Neurobiology, Center for Tsukuba Advanced Research Alliance and the Institute of Basic Medical Sciences, University of Tsukuba, I-1-1 Tennoudai, Tsukuba, Ibaraki 305, Japan, and the Manitoba Institute of Cell Biology, Manitoba Cancer Treatment and Research Foundation, University of Manitoba, Winnipeg, Manitoba R3E 0V9, Canada

We report here the isolation and characterization of a new member of the ice/ced-3 family of cell death genes, named ich-3. The predicted amino acid sequence of Ich-3 protein shares 54% identity with murine interleukin-1β converting enzyme (ICE). Overexpression of ich-3 in Rat-1 and HeLa cells induces apoptosis, which can be inhibited by CrmA and Bcl-2. The mRNA and proteins of ich-3 are dramatically induced in vivo upon stimulation with lipopolysaccharide, an inducer of septic shock. The ich-3 gene product can be cleaved by cytotoxic T cells granule serine protease granzyme B, suggesting that Ich-3 may mediate apoptosis induced by granzyme B. Ich-3 does not process pro-IL-1β directly but does promote pro-L-1β processing by ICE. These results suggest that Ich-3 may play a very important role in apoptosis and inflammatory responses and may be an upstream regulator of ICE.

Interleukin-1β converting enzyme (ICE)† family is a growing family of cysteine proteases involved in cytokine maturation and apoptosis (1). ICE is a cytoplasmic cysteine protease responsible for proteolytically processing pro-interleukin-1β (31 kDa) into active form (17 kDa) (2). The amino acid sequence of ICE shares 29% identity with Caenorhabditis elegans cell death gene product Ced-3 (3). Expression of ice in a number of mammalian cell lines induces apoptosis (4, 5). Microinjection of an expression vector of crmA, a cowpox virus gene encoding a serpin that is a specific inhibitor of ICE, prevents death of neurons of dorsal root ganglia and ciliary ganglia induced by trophic factor deprivation (6, 7). Expression of crmA can also suppress apoptosis induced by TNFα and Fas (8–11). These experiments suggest that the members of the ICE family play important roles in controlling mammalian apoptosis.

Cytotoxic T lymphocytes (CTL) are important players in host cell-mediated immunity (12). Granzyme B (GraB) is a serine protease that plays a major role in apoptosis induced by CTLs because mice that are deficient for GraB generated by gene targeting technique are severely defective in CTL-induced apoptosis (13). GraB can induce apoptosis of many if not all cell types in the presence of pore forming protein perforin (14, 15). A recent report showed that CPP32, a member of the ICE family, is activated by cytotoxic T-cell-derived GraB, suggesting that CPP32 is important for CTL killing (16). CPP32, however, cannot be the only ICE family member activated by CTL because CrmA is a very poor inhibitor of CPP32 (17). Tewari et al. (18) showed that expression of crmA completely blocks the Ca2+-independent component of CTL killing (i.e. Fas-mediated); if CPP32 were the only ICE family member responsible for CTL cytotoxicity, expression of crmA should not suppress CTL killing. We predict that there are additional members of the ICE family that play an important role in CTL-induced apoptosis. The amino acid sequence of GraB is not homologous with ICE; however, GraB and ICE share many enzymatic similarities. Like ICE, GraB requires Asp at P1 position for cleavage. Inhibitors of ICE or the ICE family, CrmA, ICE-1γ, and a mutant ICE are effective inhibitors of GraB/perforin-induced apoptosis. Embryonic fibroblasts that are deficient in ICE from ice/— mice are resistant to GraB/perforin-induced apoptosis, suggesting that ICE is critical for GraB/perforin-induced apoptosis in at least certain cell types. ICE itself cannot be directly cleaved by GraB (20), and thus, although ICE is required for GraB/perforin-induced apoptosis in certain cells, GraB does not activate ICE directly. One possibility is that GraB activates another ICE family member that may then directly or indirectly activate ICE, and the activator of ICE can be inhibited by CrmA.

The mammalian ICE family now includes ICE, Nedd-2/ich-1, CPP32/YAMA, MCH-2, TX/ICH-2/ICErelII, and ICErelIII (2, 5, 21–26). Overexpression of nedd-2/ich-1 induces cell death very effectively (5, 21). Expression of CPP32/YAMA in full-length cDNA induces apoptosis of insect Sf9 cells but not
that of mammalian cells (22). Recombinant CPP32/YAMA is inactive, and cleavage of CPP32/YAMA by ICE in vitro activates the precursor (24), suggesting that in vivo CPP32/YAMA may be activated by another protease to induce apoptosis. Expression of MCH2a also induces apoptosis of insect Sf9 cells but not that of mammalian cells (27). Thus, the members of the ICE family can be classified into two classes: those that when overexpressed in mammalian cells can induce apoptosis (e.g. ice and ich-1) and those that when overexpressed in mammalian cells cannot induce apoptosis (e.g. CPP32 and Mch-2). These experimental evidence suggest that in vivo members of the ICE family may be arranged in proteases cascades, and certain members of the ICE family may activate other members of the ICE family.

We report here the isolation and characterization of a new member of the ICE family named ich-3. The predicted amino acid sequence of Ich-3 exhibits 46% identity with murine ICE, 45% identity with human ICE, 60 and 54% identities with human ICE-like proteases TX (TX, ICE-rp-II, and Ich-2 are the same protein) and ICE-rp-II, respectively. It shares 26–32% of sequence identity with Ced-3, human ICH-1L, and CPP32/YAMA. Overexpression of ich-3 in Rat-1 and HeLa cells induces apoptosis, which can be inhibited by CrmA and Bcl-2. Expression of ich-3 is dramatically elevated in vivo after stimulation of LPS, an endotoxin secreted by Gram-negative bacteria that induces sepsis. In addition, Ich-3 can be cleaved by granule serine protease granzyme B in vitro. Ich-3 does not process proIL-1β directly but promotes processing of proIL-1β by ICE. Our results suggest that ich-3 may play an important role in apoptosis and inflammatory responses and may be an upstream regulator of ICE.

MATERIALS AND METHODS

Cloning and Construction of Plasmids—Mouse thymus cDNA library (Stratagene) containing 10^9 plaque-forming units was screened by human ICE cDNA as a probe. Hybridization was performed at 40 °C overnight in 5 × SSPE, 20% formamide, 10 × Denhardt’s solution, 1% SDS. Filters were washed in 1 × SSPE, 0.5% SDS twice at room temperature and then twice at 42 °C. Two ich-3 cDNA clones were originally obtained and subcloned into pbLueScript II (named m29 and mno). Additional ich-3 cDNA were also obtained from the same cDNA library by direct screening with a ich-3 probe and were subcloned in pbLueScript II (named BSN01, BSN03, BSN09, and BSN12). These cDNA clones contain inserts with overlapping segments of the ich-3 gene. mno contained the longest insert (2 kilobase pairs) including the ATG initiation codon; however, this insert was longer than the size of ich-3 mRNA determined by Northern blot. mno contains an unexpected duplication of ich-3 cDNA sequence at its 5′ end. To confirm the 35-base pair upstream sequence from the ATG codon, we performed the reverse transcriptase-PCR analysis by using the primer set mNOF (5'-CTTCCCCAGTCGGAAAAGAAC-3') and mNO9 (5'-GGTCGACCTATCATATATATATATATGTG-3') as primers. The PCR was performed in the condition: 1 × Taq polymerase buffer (Promega), 0.3 mM dNTPs, 2.5 mM MgCl₂, 0.5 μM each primer, 1 unit of Taq DNA polymerase (Promega) in a total volume of 25 μL. DNA was denatured at 94 °C for 1 min, annealed at 55 °C for 1 min, and elongated at 72 °C for 1 min with 28 cycles. The PCR product was digested overnight at 62 °C in 1% BSA, 0.5 mM sodium phosphate, pH 7.2, and 17.5% SDS. The blots were washed in 40 mM sodium phosphate, pH 7.2, 1 mM EDTA, 1% SDS and 70 mM NaCl at 65 °C. For RT-PCR, first strand cDNA was synthesized by using total RNA of larvae and random primers with the Moloney murine leukemia virus reverse transcriptase (Life Technologies, Inc.) as described previously (5). The primers used for PCR to amplify ich-3 were mNOF (5'-CTTCCCCAGTCGGAAAAGAAC-3') and mNO9 (5'-GGTCGACCTATCATATATATATATATGTG-3'). Hybridization was performed at 40 °C overnight in 1% BSA, 0.5 mM sodium phosphate, pH 7.2, and 17.5% SDS.

Expression and Purification of Ich-3 from Escherichia coli—An EcoRI fragment of mouse proIL-1β cDNA was cloned into pcDNA3 and placed under control of CMV promoter. The construct was named as pCMV511. To test whether ich-3 can process proIL-1β, pCMV511 was cotransfected with ich-3 cDNA construct (pCMVM22) into COS cells. proIL-1β (pCMV511) was also cotransfected with ioisilacZ fusion construct and vector pIacGal (Promega). Vector pIacGal was added to each transfection to equalize the total amount of transfected DNA. 24 h after transfection, supernatant was collected and stored at −80 °C or used immediately for ELISA according to the manufacturer’s protocol (Genzyme, Cambridge, MA). In some experiments the cells were stained by X-Gal as described previously (4) to test the efficiency of the transfection.

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Ich-3, an Upstream Regulator of ICE

Ich-3 is a Member of the ICE Family—To identify additional members of the ICE family, we screened a mouse thymus cDNA library at low stringency condition using human ice mRNA as a probe. Two positive clones were identified that encode a protein similar but not identical to murine ICE. The protein encoded by these two clones was named Ich-3. A full-length cDNA of Ich-3 was isolated through additional cDNA library screening (see "Materials and Methods").

The cDNA sequence of Ich-3 (Fig. 1) contains an open reading frame of 373 amino acids. The first ATG translational start codon is at the nucleotide 35–37. An opal stop codon is at the residue.

Generation of Ich-3 Antibodies and Western Blotting Analysis—A 15-amino acid peptide (HTEFKHLSLRYGAKFD)8-multiple antigen peptide-linked within the p20 region of Ich-3 was used for the generation of polyclonal antibodies. The peptide and rabbit polyclonal antibodies against p20 region of Ich-3 was made by Research Genetics (Huntsville, AL) and purified using 4% sucrose, and 10 mM dithiothreitol in a total volume of 10 ml. The mixture was incubated at 30°C for 1 h, and the cleavage was detected by Western blotting with a peptide antibody against the p20 portion of Ich-3.

**RESULTS**

Ich-3 is a Member of the ICE Family—To identify additional members of the ICE family, we screened a mouse thymus cDNA library at low stringency condition using human ice mRNA as a probe. Two positive clones were identified that encode a protein similar but not identical to murine ICE. The protein encoded by these two clones was named Ich-3. A full-length cDNA of Ich-3 was isolated through additional cDNA library screening (see "Materials and Methods").

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According to the x-ray crystal structural analysis of ICE (28, 29), His237, Gly238, and Cys285 of ICE are involved in catalysis, and all three are conserved in Ich-3 (His206, Gly207, and Cys254) (Fig. 2A). The residues whose amino acid side chains form the P1 pocket are indicated by a caret above the residue. And those for binding P2-P4 residues are indicated by a \{. Known and predicted Asp-Xaa cleavage sites that result in the p20/p10 subunits are indicated by arrows below the residue. The potential processing residues are underlined. Residues conserved in more than three ICE members are highlighted. The numbers at the end of each lane are the numbers of amino acid of the protein. B, the structure motifs of hCE, mICE, Ich-3, and hTX. The predicted Asp residues of Ich-3 cleavage sites are indicated. The position of the absolutely conserved pentapeptide sequence QACRG, which includes the catalytic cysteine residue, is indicated above the bars. The black bars and hatched bars represent p20 and p10 domains, respectively.

### Ich-3, an Upstream Regulator of ICE

| A | C-terminal of p20, which would produce a subunit of 20 kDa. |
| B | hCE p45 |
|   | mICE p45 |
|   | Ich-3 p42 |
|   | hTX p44 |

Fig. 2. Structural features of Ich-3. A, sequence and structural comparison of the mouse Ich-3 with other closely related members of cysteine protease family. TX, ICE, and Ich-2 are the same protein. Dotted lines are spaces in the sequence to allow optimal alignment. The catalytic Gly238, Cys285, and His237 residues are marked by asterisks above the residues as indicated by x-ray crystallography analysis (28, 29). The residues whose amino acid side chains form the P1 pocket are indicated by a caret above the residue. And those for binding P2-P4 residues are indicated by a \{. Known and predicted Asp-Xaa cleavage sites that result in the p20/p10 subunits are indicated by arrows below the residue. The potential processing residues are underlined. Residues conserved in more than three ICE members are highlighted. The numbers at the end of each lane are the numbers of amino acid of the protein. B, the structure motifs of hCE, mICE, Ich-3, and hTX. The predicted Asp residues of Ich-3 cleavage sites are indicated. The position of the absolutely conserved pentapeptide sequence QACRG, which includes the catalytic cysteine residue, is indicated above the bars. The black bars and hatched bars represent p20 and p10 domains, respectively.
Ich-3, an Upstream Regulator of ICE

The expression levels of ich-3 in wild type mice in control conditions are very low. Endotoxins (LPS) are strong inducers of proIL-1β synthesis and mature IL-1β secretion. To examine if the expression of ich-3 can be induced by LPS, we prepared RNA from mice either before or 5 h after injection of a lethal dose LPS (40 mg/kg of body weight). Northern blot analysis showed that ich-3 RNA expression is dramatically induced at least 30-fold after LPS stimulation in thymus, lung, spleen, and kidney but not in brain where ich-3 expression is low in control mice (Fig. 3). Iec transcription is not induced in spleen, kidney, lung, heart, and brain after LPS stimulation. Ice mRNA is only induced significantly in thymus (Fig. 3). We also examined the levels of ich-3 protein before and after LPS stimulation. Proteins were isolated from tissues of 7–10-week-old mice before and 4 and 20 h after LPS stimulation (40 mg/kg) and analyzed on Western blot using a monoclonal antibody that recognizes Ich-3 specifically. Ich-3 protein level is very low in Western blot of tissues isolated from control mice. In LPS stimulated mice, Ich-3 is detected as two proteins of 43 and 38 kDa. 43 kDa is very close to the predicted protein size of full-length ich-3 cDNA that we have. We know that these proteins are from ich-3 locus because the ich-3−/− mice that we have generated using gene targeting technique are specifically missing these two proteins. LPS stimulation results in at least 20–30-fold increase in levels of Ich-3 proteins. In spleen two additional bands of 30 and 26 kDa were detected that may be cleavage products of 43 or 38 kDa. Elevated levels of Ich-3 proteins are found at both 4 and 20 h after LPS stimulation, suggesting that LPS induces an immediate and sustained increase in levels of ich-3 proteins. In contrast, Western blot analysis of the same tissue samples using a polyclonal anti-ICE antibody detected no difference in expression before and after LPS stimulation (data not shown). These results suggest that ich-3 may be an important regulator of endotoxic shock in mice.

Overexpression of ich-3 Induces Apoptosis—To examine whether expression of ich-3 may be able to induce apoptosis, we used the same transient expression system used for ice and ich-1 (4, 5). The mouse ich-3 cDNA was fused with the E. coli lacZ gene and placed the fused gene under the control of either chicken β-actin promoter (pπactM24Z) or CMV promoter (pCMVM26Z). A mutant ich-3 was generated by site-directed mutagenesis in which the Cys residue in the conserved pen-tapeptide QACRG domain was converted to a Gly residue. This mutant was also fused to the lacZ gene and placed under the control of chicken β-actin promoter and named pπactS56Z. These expression constructs were transfected into different culture cells, and their ability to induce apoptosis was tested by counting round dead blue cells to flat blue cell after X-Gal staining. As shown in Fig. 4 and Table I, induction of Rat-1 cell apoptosis by ich-3 is as efficient as ice, both at about 97%. The percentage of cell death induced by ich-3-lacZ under the control of chicken β-actin promoter (pπactM24Z) is similar to that of CMV promoter (pCMVM26Z). ich-3 is less effective in inducing HeLa cell apoptosis (43%) than that of ice (94%). Because Rat-1 cells are not transformed, whereas HeLa cells are of tumor origin, this result suggests that ich-3-induced apoptosis may be more sensitive to apoptosis suppressors than that of ice. Consistent with this hypothesis, bcl-2 is somewhat more effective in suppressing ich-3-induced cell death than that of ice (Table II).

The cowpox virus gene crmA encoding a serpin that is a specific inhibitor of ICE (30). CrmA is much more effective in inhibiting ICE-induced apoptosis than ICH-1−, induced apoptosis (5). CrmA is 100-fold more potent in inhibiting ICE than CPP32 (17). These results suggest that CrmA can discriminate among different members of the ICE family. Because expres-

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### Table I

| Tissue          | LPS (h) | Ich-3 induction (fold) |
|-----------------|---------|-----------------------|
| Brain           | 4       | 30                    |
| Thymus          | 4       | 30                    |
| Lung            | 20      | 30                    |
| Spleen          | 4       | 30                    |
mission of crmA can suppress trophic factor deprivation-induced neuronal cell death (6), CTL, Fas, and tumor necrosis factor \( \alpha \)-induced apoptosis (8, 9, 11, 18, 31), it became critical to examine whether cell death induced by a particular ICE family member can be suppressed by CrmA. Expression constructs of ice and ich-3 were transiently transfected into Rat-1 cells stably expressing crmA (4), and the percentage of round dead blue cells among total blue cells was counted. As showed in Table II, ich-3 induced only 55% cell death in rat-1/crmA cells compared with 97% cell death in Rat-1 cells. Similar inhibition of cell death was observed in ice-induced cell death, which is reduced from 97 to 57%. Such experiments showed that CrmA is effective in suppressing ice-3-induced cell death as that of ice.

Ich-3 Can Be Cleaved by Granzyme B in Vitro—Recent studies suggest that ice may be involved in GrB/perforin-mediated CTL-induced apoptosis.\(^2\) CTLs induce apoptosis via granzymes in the presence of the pore forming protein perforin (14, 15). It has been shown that ICE cannot be cleaved directly by GrB; nevertheless, ICE is important for GrB-induced apoptosis in at least certain cell types.\(^2\) Other ICE family members may be processed by GrB, which in turn may directly or indirectly activate ICE. To examine whether GrB can cleave Ich-3, we expressed a His-tagged Ich-3 protein in E. coli. His-tagged Ich-3 protein purified from bacteria was mixed with or without active GrB and incubated at 30 °C for 1 h. The cleavage products were identified by Western blot with a peptide antibodies against the p20 or a monoclonal antibody against p10 portion of Ich-3. As shown in Fig. 5 (right panel), the full-length Ich-3 band disappeared after incubation with GrB; a new 20-kDa band appeared that is detected by an anti-Ich-3 p20 antibody and a new 10-kDa band that is recognized by a monoclonal antibody against p10 of Ich-3. The Ich-3 protein purified from bacteria is processed into p30 (perhaps by autocleavage) but not p20 and p10, whereas GrB can cleave Ich-3 into p20 and p10. Fragments around 30 kDa are the predicted sizes of the cleavages at Asp\(^{59}\) and Asp\(^{80}\). An additional cleavage at Asp\(^{201}\) will generate a 20- and a 10-kDa subunit. To confirm that p10 and p20 are generated from predicted p30 region, we expressed a T7-tagged p30 ich-3 in E. coli. Cleavage of this T7-tagged p30 generated predicted p20 and p10 subunits recognized by p20- and p10-specific antibody (Fig. 5, left panel). The cleavage of Ich-3 by GrB suggests a possible role played by Ich-3 in granzyme B/perforin-induced apoptosis.

Ich-3 Does Not Process proIL-1\( \beta \) Directly but Can Potentiate ICE for Cleavage of proIL-1\( \beta \)—Mice with a homozygously disrupted ice gene are severely defective in generating mature IL-1\( \beta \) (19); hence, ICE plays a critical role in processing proIL-1\( \beta \) to mature IL-1\( \beta \). Because both mature IL-1\( \beta \) and ich-3 mRNA can be dramatically induced by LPS in vivo, we hypothesize that Ich-3 may directly or indirectly contribute to proIL-1\( \beta \) processing. A transient transfection assay combined with ELISA was used to test the ability of Ich-3 in cleaving proIL-1\( \beta \). A mouse proIL-1\( \beta \) expression construct pCMVS11 was cotransfected into COS cells together with either ice (pjaeM102) or ich-3 (pCMVM 262) expression constructs. 24 h after transfection, secreted of mature IL-1\( \beta \) was observed, indicating that Ich-3 could not process proIL-1\( \beta \) by itself. Cotransfection of expression vectors of both ice and ich-3 with that of mouse proIL-1\( \beta \) into COS cells resulted in 50% increase in the amount of mature IL-1\( \beta \) observed, indicating thatIch-3 could promote processing of proIL-1\( \beta \) by ICE. There is no increase of mature IL-1\( \beta \) production when ice was cotransfected with vector (pjaeGalo) or mutant ich-3 (pjaeS6Z) (Fig. 6), suggesting that Ich-3 enzyme activity is required for promoting ICE function in generating mature IL-1\( \beta \) in vivo.

**DISCUSSION**

We described here the molecular cloning and characterization of murine ich-3, a new member of the ice/ced-3 family. The predicted Ich-3 protein is 373 amino acids long and contains the 100% conserved ICE family signature peptide QACRG. Five additional members of the ICE family have been identified (5, 25, 26). These ICE homologs can be classified into two different groups by their sequence homology: one group (ICE) is more homologous to ICE than to ced-3 (TX/ICErelII/ICH2 and ICErelIII) and the other (Ced-3) is more homologous to Ced-3 (ICErelI).
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defective in processing pro-IL-1β by ICE. COS cells were cotransfected with mouse pro-IL-1β (pCMVS11) and with either ice (pIactM102) or ich-3 (pCMVM26Z) or both. Vector DNA was added to each transfection to equalize the total amount of transfected DNA so that total amount of DNA is the same in each group of transfection. Each set of data was from at least three independent transfection results. 24 h after transfection, supernatant was collected and subjected to ELISA test. The height of the bars represent the concentration of detected mature IL-1β in pg/ml.

Expression of ich-3 mRNA is low in normal healthy tissues. The levels of ich-3 proteins are generally undetectable on Western blots of tissues from healthy mice. LPS stimulation dramatically induces ich-3 mRNA and proteins, which persists at least 20 h after LPS stimulation. In contrast, expression of ice is not elevated in most tissues after LPS stimulation with the exception of thymus where its level is moderately elevated. Ich-3 protein is undetectable in normal conditions in mice. Upon stimulation by LPS, two proteins of 43 and 38 kDa are detected. Both proteins are products of ich-3 gene because a null mutation in ich-3 locus eliminates both proteins.4 43 kDa is very close to the predicted protein size (42 kDa) generated from full-length ich-3 cDNA. The 38-kDa protein may be an alternatively spliced product of ich-3. These results suggest that Ich-3 may play a very important role in inflammatory responses. Consistent with its role in inflammatory responses, mice with a homozygous null mutation in ich-3 gene are resistant to LPS-induced septic shock.4 Ich-3 proteins, however, are not likely to be directly involved in processing of pro-IL-1β for the following two reasons. First, there is no in vivo evidence of existence of another protease playing a significant role in pro-IL-1β processing because ICE knock-out mice are at least 90% defective in processing pro-IL-1β (10, 19). Second, expression of ich-3 in COS cells does not lead to pro-IL-1β processing directly; rather it promotes processing of pro-IL-1β by ICE. This result suggests that Ich-3 is an upstream regulator of ICE. It is not clear, however, how Ich-3 activates ICE. The simplest possibility that Ich-3 directly cleaves ICE to activate, but it may not be true because we have consistently failed to observe cleavage of pro-ICE by Ich-3 either in enzymatic assay using GrakB activated Ich-3 or in cells by double transfection. We hypothesize that there may be one or more intermediate step between Ich-3 and ICE. Expression of ich-3 in COS cells activates this intermediate step(s) which in turn activates ICE. This may also explain why we only observe 50% increase in mature IL-1β production when we co-express both ice and ich-3: because there is an intermediate step(s) that is in limited quantity in COS cells. This intermediate step may be another member of the ICE family. Alternatively, Ich-3 may activate ICE indirectly by inactivating an ICE inhibitor.

A question was raised whether the role of ICE is primarily in inflammation or apoptosis (19). It is clear now that ICE has functions in both processes because ice-/– cells are defective in both production of mature IL-1β and Fas- and Grab-induced apoptosis (10, 19).2 The same question can be asked for ich-3: expressing ich-3 can induce apoptosis, which indicates that ich-3 has the ability to induce apoptosis, which does not prove that it has a role in inducing cell death in vivo. Ich-3-/– thymocytes are partially resistant to Fas-induced apoptosis, and ich-3-/– EF cells are resistant to Grab-induced apoptosis. These in vivo data are consistent with in vitro data presented here, which all suggest that Ich-3 is an upstream regulator of ICE.

Like ice-/– mice, a lethal dose of LPS fails to induce production of IL1 in the sera of ich-3-/– mice. The critical difference, however, is that ich-3-deficient macrophages and monocytes in vitro can produce mature IL-1β as well as wild-type cells when stimulated with LPS and ATP (for macrophages) or LPS alone (for monocytes); thus, ich-3 mutant cells still have the normal ICE function, whereas ice-deficient macrophages and monocytes do not produce mature IL-1 when stimulated in vitro (19). These results suggest that Ich-3 may be an upstream regulator of ICE in vivo. When mice are stimulated with LPS, Ich-3 may be induced first and activated, which in turn indirectly activates ICE. This hypothesis is entirely consistent with the data presented here; Ich-3 does not process pro-IL-1β directly but does promote pro-IL-1β processing when ICE is present. The requirement for Ich-3 is bypassed in vitro, however, when cells are stimulated with a strong signal.

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