**Brief Definitive Report**

**Granzyme A Is an Interleukin 1β-converting Enzyme**

By Martin Irmler,* Silvie Hertig,* H. Robson MacDonald,†
Remy Sadoul,§ J. D. Becherer,‖ Amanda Proudfoot,§
Roberto Solari,‖ and Jürg Tschopp*

From the *Institute of Biochemistry, University of Lausanne, and ♦Ludwig Institute for Cancer Research, Lausanne Branch, CH-1066 Epalinges, Switzerland; Glaxo Institute for Molecular Biology, CH-1028 Plan Les-Ouates, Switzerland; Glaxo Research and Development, Department of Cellular Science, Greenford, Middlesex, United Kingdom; and ♦Glaxo Incorporated, Research Triangle Park, North Carolina 27709

**Summary**

Apoptosis is critically dependent on the presence of the ced-3 gene in *Caenorhabditis elegans*, which encodes a protein homologous to the mammalian interleukin (IL)-1β-converting enzyme (ICE). Overexpression of ICE or ced-3 promotes apoptosis. Cytotoxic T lymphocyte-mediated rapid apoptosis is induced by the proteases granzyme A and B. ICE and granzyme B share the rare substrate site of aspartic acid, after which amino acid cleavage of precursor IL-1β (pIL-1β) occurs. Here we show that granzyme A, but not granzyme B, converts pIL-1β to its 17-kD mature form. Major cleavage occurs at Arg120, four amino acids downstream of the authentic processing site, Asp116. IL-1β generated by granzyme A is biologically active. When pIL-1β processing is monitored in lipopolysaccharide-activated macrophage target cells attacked by cytotoxic T lymphocytes, intracellular conversion precedes lysis. Prior granzyme inactivation blocks this processing. We conclude that the apoptosis-inducing granzyme A and ICE share at least one downstream target substrate, i.e., pIL-1β. This suggests that lymphocytes, by means of their own converting enzyme, could initiate a local inflammatory response independent of the presence of ICE.

IL-1β-converting enzyme (ICE) is a cytoplasmic cysteine protease characterized from cells of monocytic origin based on its ability to process the 31-kD inactive precursor of IL-1β (pIL-1β) to the 17-kD proinflammatory cytokine IL-1β (1, 2). ICE is a member of a novel class of cysteine proteases with a unique (p20/p10)2 homodimeric structure (3, 4), and it displays a rare sequence cleavage preference, i.e., after aspartic acids (5, 6). Activation of human pIL-1β occurs at the Asp116-Ala117 bond.

Besides their role in the activation of pIL-1β, ICE and its structural homologue, Nedd2 (7, 8), appear to be involved in the regulation of programmed cell death, or apoptosis. Overexpression of ICE or Nedd2 in rat fibroblasts or neurons induces cell death (7, 9). The CrmA protein, a viral serpin inhibitor of ICE, has been shown to prevent apoptotic cell death in neurons in vitro (10). Moreover, the expression of ced-3, a *Caenorhabditis elegans* protein belonging to the ICE family, is essential for programmed cell death to occur during worm development (11).

The only other mammalian protease with aspase activity described until now is granzyme B, a serine protease expressed in granules of activated cytotoxic lymphocytes and NK cells (12). Along with granzyme A, a protease with trypsinlike specificity, the pore-forming lytic perforin, and other constituents of the cytoplasmic granules, granzyme B is secreted into the intercellular space between killer and target cell upon specific cell–cell interaction (12). The physiological substrate(s) of granzyme A and B remain unknown, but the analysis of granzyme B (−/−) mice points to a crucial role in the control of apoptosis (13). DNA degradation in target cells lysed by lymphocytes deficient in granzyme B is impaired (13). In addition, granzymes A and B induce apoptotic cell death when added together with sublytic doses of perforin (14, 15).

Since these two aspases occupy key regulatory roles in the induction of the apoptotic cell death program, the question of whether ICE and granzyme B share common substrates arose. We therefore investigated the possibility that the ICE substrate pIL-1β is processed by lymphocyte granzyme B. In spite of common substrate specificities, we show that only ICE produces active IL-1β. However, granzyme A was found to be a potent IL-1β-converting enzyme, suggesting that lymphocytes are able to initiate an inflammatory reaction independent of ICE expression.

**Materials and Methods**

**Reagents and Substrates.** Granzyme A substrate BLT (N-α-benzoyloxycarbonyl-L-lys-thiopeobenzyyl ester) was purchased from Bachem (Bubendorf, Switzerland), and granzyme B substrate...
BAT (Boc-Ala-Ala-Asp-SBzl) was purchased from Enzyme Systems Products (Dublin, CA). The inhibitor for granzyme A (PhNHCO-NHCITeEtOIC, IGA) was a kind gift of Dr. J. Powers (Georgia Institute of Technology, Atlanta, GA), and the ICE inhibitor (Ac-Tyr-Val-Ala-Asp-CHO, YVA) was from the Glaxo Institute for Molecular Biology (Geneva, Switzerland). DCI (3,4-dichloroisocoumarin) was purchased from Boehringer Mannheim (Mannheim, Germany). The polyclonal goat antibody to mouse IL-1ß was a kind gift from G. Mazzei (Glaxo Institute for Molecular Biology, Zürich, Switzerland) and subjected to amino acid sequence determination using a pulsed liquid phase protein sequencer (477 A; Applied Biosystems, Foster City, CA) on-line phenylthiohydantoin-amino-acid analyzer (120 A; Applied Biosystems).

The peptide corresponding to the pIL-1ß sequence Asn140 to Asn152 of human pIL-1ß (Bachem, 10 µg) was incubated with purified granzyme A or B (1 µg each) at 37°C for 16 h, and the cleavage site was determined by laser desorption mass spectroscopic analysis (20).

Lysis by CTLs. Bone marrow-derived macrophages from BALB/c mice (H2b) were stimulated with LPS (10 ng/ml) for 3 h, washed, and then added to the Plasmodium falciparum circumsporozoite protein peptide/H2b specific CTL clone P815D12 (21). The effector to target cell ratio was 4:1. After 4 h, cells were centrifuged and the cell-associated IL-1ß was determined by Western blot analysis using a goat anti-mouse IL-1ß polyclonal antiserum.

Results

As ICE and the lymphocyte granzymes are both crucial mediators of apoptosis, we investigated whether these proteases recognize and cleave common substrates. Potential cleavage of in vitro–translated human pIL-1ß by granzyme A or B was investigated. As expected, the 31-kD precursor form of IL-1ß was efficiently cleaved by ICE after a 10-h incubation into a fragment corresponding to mature IL-1ß (Fig. 1 A). In spite of the shared substrate specificity of granzyme B and ICE, no cleavage of pIL-1ß was observed with the former enzyme. However, incubation with purified granzyme A generated a fragment similar in size to the authentic 17-kD IL-1ß. Cleavage of pIL-1ß by granzyme A was rapid and already observed after 30 min (data not shown). The granzyme A-induced pIL-1ß conversion was completely blocked by serine protease inhibitor DCI, whereas the ICE inhibitor YVA was inactive (Fig. 1 A). In turn, ICE activity was completely inhibited by YVA at identical concentrations (Fig. 1 A).

Efficient cleavage of pIL-1ß by granzyme A was also observed when recombinant human pIL-1ß produced in bacteria was used as a substrate (Fig. 1 B). Recombinant pIL-1ß gave rise to two bands at ∼31 kD by Western blot analysis, which were converted to two species of 16-17 kD upon addition of granzyme A. Both processed species showed an identical NH2 terminus, indicating that the recombinant protein was degraded at its COOH terminus. The conversion was again inhibited with DCI and IGA. Granzyme B had no effect on pIL-1ß.

ICE cleaves between Asp116 and Ala117 of human pIL-1ß to release the active cytokine. Sequence analysis of granzyme A–converted pIL-1ß revealed Ser121 at its NH2 terminus, indicating that pIL-1ß was cleaved after Arg120, in perfect agreement with its thrombinlike activity (Fig. 2 A). Three minor contaminants were found with NH2 termini corresponding to Leu124, Ser121, and Ala124. The Arg120–Ser121 cleavage site was also observed when a synthetic peptide covering the ICE pIL-1ß cleavage site (NEAYVHDAP-VRSLN) was offered to granzyme A (Fig. 2 B). In spite of comparable esterolytic activity on their respective substrates (BLT and BAT), in no case was cleavage detected after the authentic Asp116 by granzyme B.
We next asked whether this granzyme A-generated 17-kD fragment of IL-1β had any significant IL-1β biological activity. We have previously shown that, in an EL-4 T helper clone, EL-4-6.1, IL-2 receptor surface expression was strictly dependent on the presence of picogram quantities of IL-1β (19). Precursor 31 kD IL-1β had no demonstrable activity in this assay (Table 1), as demonstrated by others previously (22). Granzyme A alone was also inactive. FACS® analysis revealed induction of IL-2 receptor surface expression only when pIL-1β incubated with proteolytically active granzyme A was added. Compared to the recombinant human IL-1β, the activity was approximately three- to fourfold lower.

pIL-1β is synthesized as a cytosolic protein without signal peptide. Although ICE is a cytoplasmic protein, processed pIL-1β is not observed to be associated with intact cells but is only generated and released when cells are undergoing apoptosis (23). Only when rapid apoptosis of macrophages is induced by CTLs or ATP is intracellular conversion of pIL-1β observed (23). To evaluate whether this intracellular processing is due to granzyme A, LPS-activated macrophages were used as targets for the cytolytic T cells. The CTL clone used recognizes a peptide derived from the *P. falciparum* protein circumsporozoite in the context of the H2d class I antigen. When the conversion of pIL-1β was analyzed in macrophages during CTL attack, the anti-IL-1β antibodies detected a faint, cell-associated band of 17 kD in addition to the 31-kD precursor, thus confirming results by Hogquist et al. (23) showing that intracellular pIL-1β cleavage was occurring during the cytolytic attack. Processed intracellular pIL-1β was not present in cells not previously sensitized with the peptide (Fig. 3). When CTLs were pretreated for 30 min with IGA, intracellular processing of pIL-1β was abolished, suggesting that granzyme A may be involved in the rapid conversion of pIL-1β (Fig. 3).

**Discussion**

Our results show that, although both granzyme B and ICE exhibit aspase activity and are crucial mediators of apoptotic cell death, they do not act on identical cellular target proteins. pIL-1β, the only known substrate for ICE, is resistant to the proteolytic action of granzyme B. Even when the ICE pIL-1β cleavage site is offered as a 14-mer peptide, no cleavage after the critical Asp residue is observed. These observations indicate that the presence of an Asp residue alone may not be solely sufficient for the efficient processing of pIL-1β at the Asp116-Val117, and other structural constraints may be required. Today no proteinase substrate for granzyme A is known.

**Table 1. Activities of IL-1β**

| Sample                        | Relative activity |
|-------------------------------|-------------------|
| Mature IL-1β (17 kD)          | 100               |
| pIL-1β (31 kD)                | <1                |
| pIL-1β (31 kD) + Granzyme A   | 30                |
| pIL-1β (31 kD) + DCI          | <1                |
| pIL-1β (31 kD) + IGA          | <1                |
| pIL-1β (31 kD) + Granzyme A   | <1                |

IL-1β activities were determined by the EL-4 assay described previously (19). The obtained activities were normalized to the activity of recombinant human IL-1β, which was arbitrarily set to 100%.
zyme B has been found, in spite of its known high esterolytic activity on synthetic thioester substrates. The absence of pIL-1β processing by granzyme B cannot exclude, however, the possibility that other proteases, such as those activated during apoptosis, are substrates shared by ICE and granzyme B.

Unlike granzyme B, the second lymphocyte protease granzyme A expressed in vivo can generate an active IL-1β from the inactive precursor form. Cleavage occurs between Arg120 and Ser121, yielding a cytokine four amino acids smaller in length than the one generated by ICE. Activity of the IL-1β diminishes rapidly as the cleavage site moves away from the genuine Asp116 cleavage site. Mast cell chymase and chymotrypsin generate molecules that are two and three residues longer with no change in activity (24). Trypsin and elastase, in contrast, generate polypeptides that are 13 and 41 amino residues longer than authentic IL-1β but result in almost complete loss of activity (22).

The biological activity of the granzyme A–processed pIL-1β was increased relative to the recombinant one, in agreement with the removal of activity observed when Arg120 is removed or replaced by Asp (25), although structure–activity studies with truncated proteins expressed in COS cells have shown that IL-1β retains full functional activity even when shortened to Met116 (26). By contrast, using in vitro–translated IL-1β, removal of Arg120 resulted in decreased receptor binding (27).

Precursor IL-1β is predominantly found in intact cells. In cells undergoing apoptosis, processing by ICE results in the release of the active cytokine, in contrast to necrosis, in which IL-1β is released almost exclusively in the precursor form (23). Extracellular proteases such as mast cell proteases may then be expected to cleave the 31-kD precursor IL-1β in areas of necrotic damage and thereby to release substantial amounts of IL-1β. Intracellular processing of pIL-1β, however, has been observed in macrophages that are attacked by CTLs (23). CTLs cause rapid apoptosis induced by the action of granzyme B, granzyme A, and Fas ligand, a membrane protein with structural homology to TNF (15, 28, 29). Granzymes are believed to enter the target cell and act on intracellular substrates whose cleavage leads to apoptosis (30). This may account for the rapid intracellular processing of pIL-1β. Indeed, treatment of CTLs with a granzyme A inhibitor abolished intracellular conversion of pIL-1β in the target cell. Although the granzyme A inhibitor displays high specificity for granzyme A, it also blocks other proteases with similar specificity, such as thrombin (our own unpublished data). Whether the absence of intracellular conversion is due to the inactivation of granzyme A or to other factors remains therefore to be shown.

This lymphocyte protease–generated IL-1β is interesting from the perspective that some cells known to synthesize pIL-1β lack the corresponding converting enzyme (31). For example, fibroblasts and keratinocytes have been shown to produce pIL-1β mRNA but lack IL-1β cytokine activity (31, 32). Other secreted proteases may also play a role in processing precursor IL-1β. Inflammatory responses of diverse origins may, in turn, be a consequence of this process. Mast cell chymase generates an active cytokine and is proposed to play a critical role in the initiation of the inflammatory response in the skin (31). Cathepsin G, which also displays pIL-1β-converting activity, is present in synovial fluids and lung lavage from patients with inflammatory polyarthritis (32). A streptomycetes-derived cysteine protease produces biologically active IL-1β, indicating that bacteria are able to modulate the inflammatory response during pathogenesis (33). The granzyme A–generated IL-1β may directly contribute to the inflammatory response in areas of lymphocyte tissue infiltration. IL-1β may recruit T cells to the site of antigenic challenge caused by viruses and parasites and, in turn, activate Th lymphocytes and CTLs whose efficient activation is dependent on the IL-1β costimulatory signal. This would lead to macrophage and neutrophil sequestration to remove cellular membrane debris caused by CTL action. In an effort to support this model, others have attempted to identify the NH2 terminus of IL-1β at inflammatory sites to determine the processing site, but, because of the low concentration of IL-1β present at inflammatory sites or as a result of proteolytic degradation, they have been unsuccessful to date (32). The observation that precursor IL-1β may be processed in vivo by proteases other than ICE may have important implications in the future development of therapeutic approaches. In particular, drugs currently developed to inhibit the pIL-1β processing in monocytes by interfering with ICE activity may be unable to inhibit IL-1β production in vivo.
References

1. Black, R.A., S.R. Kronheim, J.E. Merriam, C.J. March, and T.P. Hopp. 1989. A pre-aspartate-specific protease from human leukocytes that cleaves pro-interleukin-1 beta. J. Biol. Chem. 264:5323–5326.

2. Kostura, M.J., M.J. Tocci, G. Limjucno, J. Chin, P. Cameron, A.G. Hillman, N.A. Charrtain, and J.A. Schmidt. 1989. Identification of a monocye specific pre-interleukin 1 beta convertase activity. Proc. Natl. Acad. Sci. USA. 86:5227–5231.

3. Walker, N.P.C., R.V. Talanian, K.D. Brady, L.C. Dang, N.J. Bump, C.R. Ferenza, S. Franklin, T. Ghayur, M.C. Hackett, L.D. Hammill, et al. 1994. Crystal structure of the cysteine protease interleukin-1-beta-converting enzyme - a (p20/p10) (2) homodimer. Cell. 78:343–352.

4. Wilson, K.P., J.A. Black, J.A. Thomson, E.E. Kim, J.P. Griffith, M.A. Navia, M.A. Murcko, S.P. Chambers, R.A. Al- dape, S.A. Raybuck, et al. 1994. Structure and mechanism of interleukin-1 beta converting enzyme. Nature (Lond). 370: 270–275.

5. Thornberry, N.A., H.G. Bull, J.R. Calaycay, K.T. March, and S.R. Kronheim. 1994. Induction of apoptosis by the mouse nedd2 gene, beta-converting enzyme, a mammalian homolog of the interleukin-1 beta converting enzyme. Genes & Dev. 8:1613–1626.

6. Cerretti, D.P., C.J. Kozlosky, B. Mosley, N. Nelson, N.K. Van, T.A. Greenstreet, C.J. March, S.R. Kronheim, T. Druck, L.A. Cannizzaro, et al. 1992. Molecular cloning of the interleukin-1 beta converting enzyme. Science (Wash. DC). 256:97–100.

7. Ishikawa, S., M. Kinoshita, M. Noda, N.C. Copeland, and N.A. Jenkins. 1994. Induction of apoptosis by the mouse nedd2 gene, which encodes a protein similar to the product of the caenorhabditis elegans cell death gene ced-3 and the mammalian IL-1-beta-converting enzyme. Genes & Dev. 8:1613–1626.

8. Weng, L., M. Miura, L. Bergeron, H. Zhu, and J. Yuan. 1994. Ichi-1, an Ice/ced-3 related gene, encodes both positive and negative regulators of programmed cell death. Cell. 78:739–750.

9. Miura, M., H. Zhu, R. Rotello, E.A. Hartwig, and J. Yuan. 1993. Induction of apoptosis in fibroblasts by IL1 beta converting enzyme, a mammalian homolog of the C. elegans cell death gene ced-3. Cell. 75:653–660.

10. Gagliardini, V., P.-A. Fernandez, R.K.K. Lee, H.C.A. Drexler, R.J. Rotello, M.C. Fishman, and J. Yuan. 1994. Prevention of vertebrate neuronal death by the crmA gene. Science (Wash. DC). 263:826–828.

11. Yuan, J., S. Shaham, S. Ledoux, H.M. Ellis, and H.R. Horvitz. 1993. The C. elegans death gene ced-3 encodes a protein similar to mammalian interleukin-1beta-converting enzyme. Cell. 75:641–652.

12. Jenne, D.E., and J. Tschopp. 1988. Granzymes: a family of serine proteases in granules of cytolytic T lymphocytes. Curr. Top. Microbiol. Immunol. 140:33–47.

13. Heusel, J.W., R.L. Wesselschmidt, S. Shresta, J.H. Russell, and T.J. Ley. 1994. Cytotoxic lymphocytes require granzyme B for the rapid induction of DNA fragmentation and apoptosis in allogeneic target cells. Cell. 76:977–987.

14. Shiver, J.W., L. Su, and P.A. Henkart. 1992. Cytotoxicity with target DNA breakdown by rat basophilic leukemia cells expressing both cytolyisin and granzyme A. Cell. 71:315–322.

15. Shi, L., C.-M. Kam, J.C. Powers, R. Axelrod, and A.H. Greenberg. 1992. Purification of three cytotoxic lymphocyte granule serine proteases that induce apoptosis through distinct substrate and target cell interactions. J. Exp. Med. 176: 1521–1529.

16. Masson, D., and J. Tschopp. 1987. A family of serine esterases in lytic granules of cytolytic T lymphocytes. Cell. 49:679–685.

17. Masson, D., M. Zamaiz, and J. Tschopp. 1986. Identification of granzyme A isolated from cytotoxic T-lymphocyte-granules as one of the proteases encoded by CTL-specific genes. FEBS (Fed. Eur. Biochem. Soc.) Lett. 208:84–88.

18. Odake, S., C.M. Kam, L. Narasimhan, M. Poe, J.T. Blake, O. Krahnenhul, J. Tschopp, and J.C. Powers. 1991. Human and murine cytotoxic T lymphocyte serine proteases: subsite mapping with peptide thioester substrates and inhibition of enzyme activity and cytolyis by isocoumarins. Biochemistry. 30:2217–2227.

19. Le Moal, M.A., M. Stoeck, J.M. CavaiUon, H.R. MacDonald, and B.P. Truffa. 1988. A sensitive, IL-2-independent, assay for IL-1. J. Immunol. Methods. 107:23–30.

20. Chai, BT., and S.B.H. Kent. 1992. Weighing naked proteins: practical, high-accuracy mass measurement of peptides and proteins. Science (Wash. DC). 257:1885–1894.

21. Romero, P., G. Eberl, J.L. Casanova, C. Widmann, I.F. Luescher, G. Corradin, and J.L. Maryanski. 1992. Immunization with synthetic peptides containing a defined malaria epitope induces a highly diverse cytotoxic T lymphocyte response. Evidence that two peptide residues are buried in the MHC molecule. J. Immunol. 148:1871–1878.

22. Black, R.A., S.R. Kronheim, M. Caintrell, M.C. Deely, C.J. March, K.S. Prickett, J. Wignall, P.J. Conlon, D. Cosman, T.P. Hopp, et al. 1988. Generation of biologically active interleukin-1 beta by proteolytic cleavage of the inactive precursor. J. Biol. Chem. 263:9437–9442.

23. Hoggquist, K.A., M.A. Nett, E.R. Unanue, and D.D. Chaplin. 1991. Interleukin 1 is processed and released during apoptosis. Proc. Natl. Acad. Sci. USA. 88:8485–8489.

24. Mizutani, H., N. Schechter, G. Lazarus, R.A. Black, and T.S. Kupper. 1991. Rapid and specific conversion of precursor interleukin 1B (IL-1B) to an active IL-1 species by human mast cell chymase. J. Exp. Med. 174:821–825.

25. Kamogashira, T., M. Sakaguchi, Y. Ohmoto, K. Mizuno, R. Shimizu, K. Nagamura, S. Nakai, Y. Masui, and Y. Hirai. 1988. Site-specific mutagenesis of the human interleukin-1 beta gene: the role of arginine residue at the N-terminal region. J. Biochem. (Tokyo). 104:837–840.

26. Rosenwasser, L.J., A.C. Webb, B.D. Clark, S. Irie, L. Chang, C.A. Dinarello, L. Gehrke, S.M. Wolf, A. Rich, and P.E.
Auron. 1986. Expression of biologically active human interleukin 1 subpeptides by transfected simian COS cells. *Proc. Natl. Acad. Sci. USA.* 83:5243-5246.

27. Mosley, B., S.K. Dower, S. Gillis, and D. Cosman. 1987. Determination of the minimum polypeptide lengths of the functionally active sites of human interleukins 1 alpha and 1 beta. *Proc. Natl. Acad. Sci. USA.* 84:4572-4576.

28. Suda, T., T. Takahashi, P. Golstein, and S. Nagata. 1993. Molecular cloning and expression of the Fas ligand, a novel member of the tumor necrosis factor family. *Cell.* 75:1169-1178.

29. Munger, W.E., G.A. Berrebi, and P.A. Henkart. 1988. Possible involvement of CTL granule proteases in target cell DNA breakdown. *Immunol. Rev.* 103:99-109.

30. Schaerer, E., and J. Tschopp. 1993. Cytolytic T cells keep their secrets. *Curr. Biol.* 3:167-169.

31. Mizutani, H., R. Black, and T.S. Kupper. 1991. Human keratinocytes produce but do not process pro-interleukin-1 (IL-1) beta. Different strategies of IL-1 production and processing in monocytes and keratinocytes. *J. Clin. Invest.* 87:1066-1071.

32. Molineaux, S.M., F.J. Casano, A.M. Rolando, E.P. Peterson, G. Limjucio, J. Chin, P.R. Griffin, J.R. Calaycay, G.J. Ding, T.T. Yamin, et al. 1993. Interleukin 1 beta (IL-1 beta) processing in murine macrophages requires a structurally conserved homologue of human IL-1 beta converting enzyme. *Proc. Natl. Acad. Sci. USA.* 90:1809-1813.

33. Kapur, V., M.W. Majesky, I.L. Li, R.A. Black, and J.M. Musser. 1993. Cleavage of interleukin 1 beta (IL-1 beta) precursor to produce active IL-1 beta by a conserved extracellular cysteine protease from *Streptococcus pyogenes.* *Proc. Natl. Acad. Sci. USA.* 90:7676-7680.