Communication

Functional Characterization of the Prodomain of Interleukin-1β-converting Enzyme*

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Wim Van Criekinge, Rudi Beyaert, Marc Van de Craen, Peter Vandenabeele, Peter Schotte, Dirk De Valck, and Walter Fiers
From the Laboratory of Molecular Biology, Flanders Interuniversity Institute for Biotechnology and University of Ghent, B-9000 Ghent, Belgium

Interleukin-1β-converting enzyme (ICE) has been identified as the main protease responsible for maturation of the prodomain of interleukin-1β. Recently, it was shown to belong to a larger gene family, members of which play an important role in programmed cell death. A common feature of the ICE family proteases is the presence of a prodomain that has been hypothesized to keep the enzyme in an inactive form. Expression analysis in yeast revealed autocatalytic degradation of p45ICE, but not of p30ICE lacking a prodomain. We further demonstrate that p45ICE, in which the critical cysteine has been mutated, is still able to dimerize in vivo. Dimerization requires the prodomain and occurs prior to autoprocessing. These results provide evidence for a regulatory role of the prodomain of ICE.

Interleukin-1β-converting enzyme (ICE)

Interleukin-1β-converting enzyme (ICE) is a cysteine protease that cleaves the inactive 33-kDa proform of IL-1β into active mature 17-kDa IL-1β (1, 2). IL-1β is a pleiotropic cytokine produced during inflammation, injury, immunological challenge, or infection (3, 4). ICE is constitutively expressed in different cells (5, 6). This broad expression pattern suggests that ICE might be involved, in addition to IL-1β maturation, in other cellular and biological processes. At present, the role of ICE and ICE-related proteases in programmed cell death or apoptosis (7–9) is one of the subjects most studied in immunology and cell biology (10–13).

Several cDNAs encoding ICE-like proteins were recently identified: ICβ-1/NEαD-2 (14, 15), CPP32/Yama/apopain (16, 17), TX/ICβ-2/ICβEl(18), Mch2 (19), ICβEl(20), and Mch3/ICE-LAP3 (21, 22). Interestingly, while transient overexpression of all these cDNAs can induce apoptotic cell death, ICE only is able to cleave the prodomain of IL-1β efficiently. This has been demonstrated clearly with mice deficient in ICE, which do not produce mature IL-1β and are fairly resistant to endotoxic shock, but which show completely normal apoptotic features (23, 24).

Comparison of the cleavage sites in the proform of IL-1β and several other ICE substrates reveals an absolute requirement for an Asp residue at position P1 in order to function as a substrate for ICE (25). ICE is made as a 45-kDa precursor protein (p45), the maturation of which involves removal of an N-terminal prodomain and an internal linker sequence separating a p20 and a p10 subunit. Cleavage of the p45 precursor occurs at four distinct Asp residues, suggesting a role for autocatalytic processing (5).

The crystal structure of ICE as a complex with a tetrapeptide inhibitor revealed that active ICE, lacking the prodomain and linker, is a tetramer of two p20 subunits surrounded by two adjacent p10 subunits that form the dimer-dimer interface (26, 27). The active site of ICE is composed of amino acid residues from both p20 and p10 with Cys-285 and His-237 forming a catalytic dyad (27). Starting from the specific quaternary structure of ICE, two models for the maturation of the ICE proenzyme were proposed. The first model assumes an association of two p45 precursor proteins and subsequent processing. The p20 and p10 subunits forming one active site are derived from two distinct precursor proteins after autoprocessing of interdigitating precursors (27). The second model supposes processing followed by association of mature proteins (27). Elucidation of the molecular events leading to active ICE is important in view of the development of specific ICE inhibitors.

We used the yeast two-hybrid system to study ICE/ICE interaction in vivo and demonstrate that the prodomain of ICE is absolutely required for dimerization and autoactivation of ICE.

MATERIALS AND METHODS

Yeast Two-hybrid System—The yeast two-hybrid system was purchased from Clontech Laboratories (Palo Alto, CA). Polyclonal antibodies recognizing the yeast Gal4 DNA-binding region were purchased from Upstate Biotechnology (Lake Placid, NY). 3-Amino-1,2,4-triazole was purchased from Sigma. XGal was obtained from Saxon Biochemicals (Hannover, FRG). Transformation of the Saccharomyces cerevisiae strain HF7c was achieved with the lithium acetate method (28) according to the manufacturer’s directions. Yeast colonies carrying putative interacting proteins were selected by growth on synthetic minimal media, lacking Trp, Leu, and His, in the presence of 5 mM 3-amino-1,2,4-triazole, and by screening for βGal activity in a filter assay using XGal as a substrate.

Construction of Fusion Proteins and Mutants in Two-hybrid Vectors—Marine ICE cDNA was isolated from an L929r2 cDNA library (2) and an Ndel-BamHI fragment was fused in-frame to Gal4DB in the yeast expression vector pAS2 (Gal4DBp45ICE). Gal4DBp30ICE was obtained by cloning an Eco109-BamHI fragment corresponding to ICE lacking the N-terminal prodomain (p90ICE) in combination with a small linker joining Ndel and Eco109 in pAS2. The above Ndel-BamHI fragment of p45ICE and p30ICE was also ligated to an EcoRI-NdeI adaptor and cloned in-frame of Gal4AC into pGAD424, resulting in Gal4ACp45ICE and Gal4ACp30ICE, respectively. Similarly, Gal4AC-PRO was prepared by cloning the NdeI-BamHI fragment, in which a stop codon was created between the prodomain and p20 by cutting with SacI and religation, in-frame of Gal4AC into pGAD424. Site-directed

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† Research Assistant with the Nationaal Fonds voor Wetenschappelijk Onderzoek.
¶ Postdoctoral Researcher with the Nationaal Fonds voor Wetenschappelijk Onderzoek.
One of the abbreviations used are: ICE, interleukin-1β-converting enzyme; βGal, β-galactosidase; Gal4AC, Gal4 transactivation domain; Gal4DB, Gal4 DNA-binding domain; IL, interleukin; XGal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside.

2 M. Van de Craen, manuscript in preparation.
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mutagenesis was carried out with a kit from Clontech Laboratories in the pMa plasmid containing a chloramphenicol-sensitive gene (29). By using two oligonucleotides containing the mutation of interest and a mutation which inactivates the chloramphenicol-sensitive gene, respectively, one can then select for mutants by growing transformants in the presence of chloramphenicol. Cloning and mutations were verified by DNA sequencing.

**Protein Expression and Western Blotting**—After transformation of the appropriate pAS2 plasmid encoding Gal4DB fusion proteins into *S. cerevisiae* strain HF7c and incubation on plates lacking Trp, single colonies were inoculated into 15 ml of synthetic medium lacking Trp. At least between 1.3 and 1.5, the culture was centrifuged at 2500 rpm for 5 min, the pellet was washed in distilled water and boiled for 3 min in 200 μl of 2 X Laemml loading buffer. 50 μl of this sample was separated by 10% SDS-polyacrylamide gel electrophoresis and blotted onto a nitrocellulose membrane (Schleicher & Schuell, Dassel, FRG). Detection of the expressed fusion proteins was performed with polyclonal anti-yeast Gal4DB antibody and peroxidase-conjugated anti-rabbit antibody using ECL (Amersham Life Science, Amersham, UK).

**RESULTS**

**Role of the Prodomain in Autoproteolysis**—To study the role of the prodomain in dimerization and autoactivation of murine ICE we used the yeast two-hybrid system (30). The coding sequence of p45ICE or ICE lacking the N-terminal prodomain (p30ICE) was fused in-frame to Gal4DB or Gal4AC in the yeast expression vectors pAS2 and pGAD424, respectively. As estimated by multiple sequence alignment with its human counterpart, the prodomain of ICE comprises the first 122 amino acids. A schematic representation of the fusion proteins used is given in Fig. 1.

After transformation into HF7c of pAS2 encoding Gal4DB fusion proteins, single colonies were used to prepare lysates that were separated by SDS-polyacrylamide gel electrophoresis. Subsequent Western blot analysis with antibodies against the N-terminal Gal4DB revealed a band of the expected size (51 kDa) in the case of Gal4DBp30ICE. Remarkably, a slightly faster migrating band (50 kDa) was visible in the case of Gal4DBp45ICE (Fig. 2). Considering its size and recognition by an anti-Gal4DB antibody, the latter band likely resulted from cleavage between p20 and p10 ICE subunits. To determine whether this degradation was due to autocleavage or to a (aspecific) yeast factor, a point mutation was introduced at position 285 to convert Cys in the active site of the conserved QACRG sequence to Ser (C285S). This mutation has previously been shown to result in complete inactivation of the proteolytic activity of ICE (31, 32). Western blot analysis of lysates of yeast cells transformed with the active site mutants Gal4DBp30ICE-C285S or Gal4DBp45ICE-C285S (Fig. 1) only showed bands corresponding to the expected full-length fusion proteins (51 and 62 kDa, respectively; Fig. 2), which means that the previously observed degradation product of wild-type p45ICE was due to autoprocessing at its C-terminal end.

The absence of a smaller degradation product corresponding to a fusion of Gal4DB and the prodomain of ICE (30 kDa) suggests that prior removal of the prodomain is not required for autoproteolysis at a site between the p20 and p10 subunits (Fig. 2). This was also confirmed by mutating two Asp residues to Glu at the end of the prodomain, corresponding to potential cleavage sites. Expression of the obtained mutants Gal4DBp45ICE-C285S (data not shown). These results further indicate that removal of the N-terminal prodomain is not a prerequisite for autoprocessing at the C terminus.

**Role of the Prodomain in Dimerization**—The importance of the prodomain in ICE dimerization was investigated by the yeast two-hybrid system. Several combinations of plasmids, encoding fusions between Gal4DB or Gal4AC and wild-type or mutated p45ICE and p30ICE or the prodomain as such (Fig. 1), were cotransformed in *S. cerevisiae* strain HF7c which contains the his3 and lacZ reporter genes under control of a Gal4 promoter. Cotransformation of the yeast expression vectors pVA3 and pTD1, which encode Gal4 fusion proteins of the strong interacting p53 and SV40-LT oncogene, respectively, served as a positive control. The ability of transformed cells to grow on His-deficient plates in the presence of 5 μM 3-aminotriazole and the expression of βGal activity were used as parameters for interaction. Single plasmid transformation or cotransformations using the appropriate empty plasmids pGAD424 or pAS2 did not result in (auto)activation of the reporter genes. The specificity of the interactions was verified by cotransforming cDNAs coding for interacting irrelevant proteins, viz. p53 or SV40-LT (Table I). None of the transformations with wild-type constructs resulted in His auxotrophy or βGal activity, indicating that there were no interacting ICE proteins present. In contrast, after cotransformation of Gal4DBp45ICE-C285S-encoding and Gal4ACp45ICE-C285S-encoding plasmids, both reporter genes were activated. These results show that there is interaction only between the full-length p45ICE molecules and that autocatalytic processing leads to truncated proteins that are no longer able to interact. Moreover, Gal4DBp45ICE-C285S and Gal4ACp30ICE-C285S were unable to interact, indicating that the prodomain is absolutely required for interaction. Finally, cotransformation of the prodomain of ICE as such (Gal4AC-PRO) with either Gal4DBp30ICE-C285S or Gal4DBp45ICE-C285S did not result in reporter gene activity, suggesting that the prodomain is required, but not sufficient, for dimerization of p45ICE.
DISCUSSION

ICE is synthesized as a precursor protein with an N-terminal prodomain of 11 kDa and a linker sequence which separates a p20 and a p10 subunit (5). Crystal structure analysis of ICE in complex with a tetrapeptide inhibitor revealed that ICE lacking the prodomain and linker between the p20 and p10 subunits, is a tetramer of two p20 subunits surrounding two adjacent p10 subunits forming the dimer-dimer interface. The active site is composed of amino acid residues of both p20 and p10 subunits (27). Structural data of p45ICE have not been presented yet, but two models for maturation of p45ICE have been proposed. In the first model, one assumes an association of two p45 precursor proteins and subsequent processing. The second model proposes processing followed by association of mature proteins. Using the yeast two-hybrid system, we showed that the prodomain of ICE is absolutely required, although not sufficient, for dimerization and autoproteolytic cleavage of ICE. Dimerization could only be observed with functionally inactive mutants, demonstrating that dimerization occurs prior to autoproteolysis. Interestingly, autoproteolysis could only be observed with p45ICE, but not with p30ICE lacking its prodomain. It has been demonstrated previously that separate expression of the p20 and p10 subunits in Sf9 cells results in functional ICE, which suggests a regulatory, rather than a structural, role for the prodomain (16). This is in contrast with numerous other proenzymes, where the prodomain is necessary for proper maturation or folding of the protein (33). Further evidence for a regulatory role of the prodomain is based on the fact that the evolutionary divergence between ICE and the Caenorhabditis elegans homolog CED-3 is larger in the prodomain. Although we demonstrated a role of the prodomain of ICE in dimerization and autoprocessing, other regulatory mechanisms mediated by the prodomain, such as interaction with an inhibitor, post-translational modification, or subcellular localization, cannot be excluded.

The fact that the prodomain of ICE is separated from the p20 subunit by two Asp residues which are potential cleavage sites for ICE has led to the general assumption that the prodomain is removed by autocatalytic processing (5). However, after expression of p45ICE, we could not detect any degradation products resulting from prodomain removal. Similarly, only ICE precursor forms have been detected in mammalian cell lines, although biological ICE activity was present (34). Our results suggest that removal of the prodomain is not an absolute requirement for biological activity of ICE. It is, however, conceivable that p45ICE and processed ICE might have different subcellular specificities and activities (35).

When the presence of different ICE homologs is considered, the situation becomes even more complex. It is not unlikely that the prodomain of ICE can be removed by another ICE homolog. The large family of ICE proteases can, based on phylogenetic comparison, be divided in three subgroups: the ICE group (ICE/TX/ICErel3), the ICH group (ICH), and the CPP32 group (CPP32/MCH-2/MCH-3). The members of the ICE and ICH group all contain large prodomains, while those of the CPP32 group have only a minimal prodomain which needs to be removed for activation. Comparable to the Bcl-2 family, where hetero-oligomerization between the different members is crucial for the biological outcome (36–39), one can imagine a similar mechanism within the ICE family. Indeed, hetero-oligomerization between TX and ICE (40) or CPP32 and Mch-3 (21) has already been demonstrated, although the biological significance is still unknown. Therefore, our observation that the prodomain of ICE is absolutely required for dimerization and subsequent autoprocessing might have important implications for the understanding of a possible interplay between prodomains of different ICE homologs and for the development of specific ICE inhibitors.

REFERENCES

1. Black, R. A. (1988) J. Biol. Chem. 263, 9437–9442
2. Kostura, M. A., Tocci, M. J., Limjuc, G., Chi, J. H., Cameron, P., Hillman, A., Charrtain, N. A., and Schmidt, J. A. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 5227–5231
3. Dinarello, C. A., and Wolff, S. M. (1993) N. Engl. J. Med. 328, 106–113
4. Dinarello, C. A. (1994) Eur. Cytokine Net. 5, 511–521
5. Thornberry, N. A., Bull, H. G., Calaycay, J. R., Chapman, K. T., Howard, A. D., Kostura, M. J., Miller, D. K., Molineux, S. M., Weidner, J. R., Aunins, J., Elliston, K. O., Ayala, J. M., Casano, F. J., Chin, D., Ding, G. J. F., Egger, L. A., Gaffney, E. P., Limjuc, G., Palhya, O. C., Raja, S. M., Rolando, A. M., Sailey, 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 356, 768–774
6. Miller, D. K., Ayala, J. M., Egger, L. A., Raja, S. M., Yamin, T.-T., Ding, G. J. F., Gaffney, E. P., Howard, A. D., Palhya, O. C., Rolando, A. M., Sailey, J. P., Thornberry, N. A., Weidner, J. R., Williams, J. H., Chapman, K. T., Jackson, J., Kostura, M. J., Limjuc, G., Molineux, S. M., Mumford, R. A., and Calaycay, J. R. (1993) J. Biol. Chem. 268, 18062–18069
7. Cohen, J. J. (1993) Immunol. Today 14, 126–130
8. Vaux, D. L., Haecker, G., and Strasser, A. (1995) Cell 76, 777–779
9. Kroemer, G., Petit, P., Zannam, N., Vaysseires, J. L., and Mignotte, B. (1995) FASEB J. 9, 1277–1287
10. Whyte, M. and Evan, G. (1995) Nature 376, 17–18
11. Kumar, S. (1995) Trends Biochem. Sci. 20, 198–202
12. Jacobson, M. D., and Evan, G. I. (1994) Curr. Biol. 4, 337–340
13. Nalin, C. M. (1995) Structure 3, 143–145
14. Kumar, S., Kinoshiita, M., Noda, M., Copeland, N. G., and Jenkins, N. A. (1994) Genes Dev. 8, 1613–1626
15. Wang, L., Liura, M., Bergeron, L., Zhu, H., and Yuan, J. (1994) Cell 78, 75–750
16. Fernandez-Alnemri, T., Litwack, G., and Alnemri, E. S. (1994) J. Biol. Chem. 269, 30761–30764
17. Tewari, M., Quan, L. T., O’Rourke, K., Desnoyers, S., Zeng, Z., Beidler, D. R., Poirier, G. G., Salvesen, G. S., and Dixit, V. M. (1995) Cell 81, 801–809
18. Faucheux, C., Dru, A., Chan, A. W. E., Blanchet, A.-M., Missec, C., Hervé, F., Collard-Dutilleul, V., Gu, Y., Aldape, R. A., Lippke, J. A., Rocher, C., Su, M. S. S., Livington, D. J., Herend, T., and LALANCE, J-L. (1998) EMBO J. 14, 1914–1922
19. Fernandez-Alnemri, T., Litwack, G., and Alnemri, E. S. (1995) Cancer Res. 55, 2737–2742
20. Munday, N. A., Vaillancourt, J. P., Ali, A., Casano, F. J., Miller, D. K., Molineux, S. M., Yamin, T. T., Yu, V. L., and Nicholson, D. W. (1995) J. Biol. Chem. 270, 15870–15876
21. Fernandez-Alnemri, T., Takahashi, A., Armstrong, R., Krebs, J., Fritz, L., Tomaselli, K. J., Wang, L., Yu, Z., Croce, C. M., Salveson, G. S., Kornamish, C. W., Litwack, G., and Alnemri, E. S. (1995) Cancer Res. 55, 6045–6052
22. Duan, H., Chinnaiyan, A. M., Hudson, P. L., Wing, J. P., He, W., and Dixit, V. M. (1996) J. Biol. Chem. 271, 35013–35035
23. Li, P., Allen, H., Banerjee, S., Franklin, S., Herzog, L. John, M., McDowell, J., Paskind, M., Rodman, L., Sallefied, J., Towne, E., Tracey, D., Wardwell, S.,
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Wei, F., Wong, W., Kamen, R., and Seshadri, T. (1995) Cell 80, 491–411

24. Kuida, K., Lippke, J. A., Ku, G., Harding, M. W., Livingston, D. J., Su, M. S., and Flavell, R. A. (1995) Science 267, 2000–2003

25. Howard, A. D., Kostura, M. J., Thornberry, N., Ding, G. J.-F., Limjauco, G., Weidner, J., Salley, J. P., Haggquist, K. A., Chaplin, D. D., Mummord, R. A., Schmidt, J. A., and Tocci, M. J. (1991) J. Immunol. 147, 2964–2969

26. Walker, N. P. C., Talanian, R. V., Brady, K. D., Dang, L. C., Bump, N. J., Ferenz, C. R., Franklin, S., Ghayur, T., Hackett, M. C., Hammill, L. D., Herzog, L., Hugunin, M., Houy, W., Mankovich, J. A., McGuiness, L., Orlewicz, E., Paskind, M., Pratt, C. A., Reis, P., Summani, A., Terranova, M., Welch, J. P., Xiong, L., Miller, A., Tracey, D. E., Kamen, R., and Wong, W. W. (1994) Cell 78, 343–352

27. Wilson, K. P., Black, J. A., Thomson, J. A., Kim, E. E., Griffith, J. P., Navia, M. A., Murcko, M. A., Chambers, S. P., Aldape, R. A., Raybuck, S. A., and Livingston, D. J. (1994) Nature 370, 270–275

28. Giertz, R. D., and Schiestl, R. H. (1991) Yeast 7, 253–263

29. Stanssens, P., Opsomer, C., Mc. Keowny, Y. M., Kramer, W., Zabeau, M., and Fretz, H.-J. (1989) Nucleic Acids Res. 17, 4441–4453

30. Fields, S., and Song, O. K. (1989) Nature 340, 254–256

31. Miura, M., Zhu, H., Rotello, R., Hartweg, E. A., and Yuan, J. (1993) Cell 75, 653–660

32. Howard, A. D., Palyha, O. C., Griffin, P. R., Peterson, E. P., Lenny, A. B., Ding, G. J., Pickup, D. J., Thornberry, N. A., Schmidt, J. A., and Tocci, M. J. (1995) J. Immunol. 154, 2321–2332

33. Baker, D., Shiu, A. K., and Agard, D. A. (1993) Curr. Opin. Cell Biol. 5, 966–970

34. Miossec, C., Decoen, M. C., Durand, L., Fasy, F., and Diu-Hercend, A. (1996) Eur. J. Immunol. 26, 1032–1042

35. Yamin, T. T., Ayala, J. M., and Miller, D. K. (1996) J. Biol. Chem. 271, 13273–13282

36. Yin, X. M., Oltvai, Z. N., and Korsmeyer, S. J. (1995) Curr. Top. Microbiol. Immunol. 184, 311–338

37. Nunez, G., Merino, R., Grillot, D., and Gonzalez Garcia, M. (1994) Immunol. Today 15, 582–588

38. Yang, E., Zha, J., Joels, J., Boise, L. H., Thompson, C. B., and Korsmeyer, S. J. (1995) Cell 80, 285–291

39. Sedlak, T. W., Oltvai, Z. N., Yang, E., Wang, K., Boise, L. H., Thompson, C. B., and Korsmeyer, S. J. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 7834–7838

40. Gu, Y., Wu, J., Faucheu, C., Lalanne, J. L., Dhu, A., Livingston, D. J., and Su, M. S. (1995) EMBO J. 14, 1923–1931