Cleavage of 14-3-3 Protein by Caspase-3 Facilitates Bad Interaction with Bcl-x(L) during Apoptosis*

Jungyeon Won‡, Doo Yeon Kim‡, Muhnho La§, Doyeun Kim‡, Gary G. Meadows‡, and Cheol O. Joe¶∥

From the ‡Department of Biological Sciences, Korea Advanced Institute of Science and Technology, Taejon 305-701, South Korea and the ¶Department of Pharmaceutical Sciences, Cancer Prevention and Research Center, Washington State University, Pullman, Washington 99164-6510

The 14-3-3e protein was identified as one of the caspase-3 substrates by the modified yeast two-hybrid system. The cellular 14-3-3e protein was also cleaved in response to the treatment of apoptosis inducers in cultured mammalian cells. Asp238 of the 14-3-3e protein was determined as the site of cleavage by caspase-3. The affinity of the cleaved 14-3-3 mutant protein (D238) to Bad, a death-promoting Bcl-2 family protein, was lower than that of wild type or the uncleavable mutant 14-3-3 protein (D238A). However, Bad associated with the cellular Bcl-x(L) more effectively in human 293T cells co-expressing Bad with wild type or the uncleavable mutant 14-3-3 protein (D238A). The present study suggests that the cleavage of 14-3-3 protein during apoptosis promotes cell death by releasing the associated Bad from the 14-3-3 protein and facilitates Bad translocation to the mitochondria and its interaction with Bcl-x(L).

During the early events of apoptosis, mitochondria have been thought to act as central coordinators of cell death (1, 2). Some apoptotic signal cascades induce mitochondrial membrane permeabilization under the control of Bcl-2-related proteins in the mitochondria. The loss of transmembrane potential in the mitochondria then induces the release of apoptotic activators such as cytochrome c, Smac/DIABLO, HtrA2/Omi, AIF, Endo G, and caspases from the mitochondria (3). Released cytochrome c activates caspase-9, binds to Apaf-1, and induces Apaf-1 oligomerization to form apoptosome (4). The apoptosome recruits and activates procaspase-9, which, in turn, activates inactive procaspase-3 into the active caspase-3, the executor molecule of apoptosis. Cytochrome c release was reported to be blocked by anti-apoptotic Bcl-2 or Bcl-x(L) and accelerated by pro-apoptotic Bax (5, 6). Bcl-2 family proteins are key regulatory proteins that play critical roles in mediating the signal transduction path that leads to apoptosis. The multi-BH domain members of the Bcl-2 protein family either suppress (Bcl-2 or Bcl-x(L)) or promote (Bax or BAK) apoptosis, whereas “BH3-only members” (Bad or Bid) exclusively promote apoptosis (7, 8).

Bad is a death-promoting BH3-only member of Bcl-2 family proteins and heterodimerizes with anti-apoptotic proteins such as Bcl-2 and Bcl-x(L). Survival kinases including Akt, protein kinase A (PKA), and ribosomal S6 kinase 1 (RSK1) phosphorylate serine residues of Bad (9–12). Bad phosphorylation has been shown to be necessary for the release of Bad from its association with Bcl-x(L).

14-3-3 proteins are conserved dimeric regulators in eukaryotic cells. There are seven isotypes of 14-3-3 proteins in mammals and two in yeast. 14-3-3 proteins are known for their ability to bind multiple cellular protein ligands. As many as 100 cellular proteins have been found to interact with 14-3-3 proteins to date. 14-3-3 proteins require phosphorylation of their target proteins for the interaction (13, 14). The large number and diversity of ligands for 14-3-3 proteins implicate a suggestion that 14-3-3 proteins are involved in many different cellular events, including signal transduction, cell cycle regulation, apoptosis, stress response, cytoskeleton organization, and malignant transformation (15). Despite the fact that the function of 14-3-3 proteins still remains unknown, there exist some general themes about the anti-apoptotic function of 14-3-3 proteins. 14-3-3 proteins have been implicated in signaling for apoptosis through interaction with pro-apoptotic molecules such as Bad (11, 16, 17), FKHRL1 (18), ASK1 (19), and Nur77 (20). Phosphorylated Bad by survival kinases interacts with 14-3-3 proteins resulting in the retention of Bad in the cytoplasm and the subsequent prevention of cytotoxic interaction with Bcl-x(L) at the mitochondrial membrane.

Caspases play important roles in the execution of apoptosis and inflammatory responses. A large set of cellular proteins has been surveyed for their ability to be cleaved by caspases, and various signaling proteins were found to be caspase substrates (21, 22). In this study, we identified the 14-3-3e protein as one of the caspase-3 substrates using a modified yeast two-hybrid genetic system. We explored the possibility that the cleavage of 14-3-3 proteins by caspase-3 during apoptosis might contribute to cell death by preventing the association of 14-3-3 proteins with Bad and facilitating Bad translocation to the mitochondria in which Bad associates with Bcl-x(L).

EXPERIMENTAL PROCEDURES

Yeast Strains—The expression of the fusion protein was selected in the yeast EGY48 strain (Mat a, his3, trp1, ura3, LexAop-Leu2). The YM4271 strain (Mat a, ura3–52, his 3–200, lys2–801, ade2–101, ade 5, trp1–901, leu 2–3, 112, tyr1–501, gal4, gal80, ade6::hisG) was the mating partner for the EGY48 strain and was used to introduce plasmids expressing human caspases.

Plasmids and cDNA Library—The B42 transcriptional activation domain of pJG4-5 (Clontech) was cloned into the XhoI and XbaI sites of
14-3-3 Cleavage during Apoptosis

pEG202 (Clontech) to generate pSub. Human PARP1 and its mutant, PARP234A, were cloned into the XhoI and PvuII sites of pSub to generate pSub-PARP and pSub-PARP234A. The BALB/c mouse brain cDNA library was constructed using Universal RiboClone cDNA synthesis system (Promega) and was inserted into pSub to generate pSub-cDNAs (23). The B42 transcriptional activation domain of pJG4-5 was deleted to generate the caspase expression vector pExp. Human caspase-1 or 3 was cloned into the Km1 and XhoI sites of pExp for the expression in yeast YM4271 cells. Human Bad was cloned into a mammalian GST fusion vector, pEBG, to produce GST-fused Bad. Human 14-3-3ε and its mutants, D238 and D238A, were cloned into the EcoRI and XhoI sites of cloning vector pcDNA3 (Invitrogen). Hemagglutinin was inserted into the BamHI and HindIII sites of pcDNA3 to generate the N-terminal HA-tagged 14-3-3ε and its mutants.

Yeast Transformation and Matting—Yeast EGY48 cells were transformed with ppsilacZ (Clontech) bearing the LexA-responsive lacZ gene. EGY48 cells transformed with pSub-cDNA and YM4271 cells transformed with a human caspase expression vector pExp-C3 were mated on a single plate containing YPD-rich media (24). After incubation for 6 h at 30 °C, the plate was replica-plated to the plates containing the selective media consisting of 40 mg/liter X-gal either with 2% dextrose or 2% galactose plus 1% raffinose.

Caspase Activity Assay—The activities of caspase-1 or 3 were measured using a fluorogenic peptide substrate, zVAD-AMC (Bachem) as described (25). Yeast YM4271 cells transformed with human caspase expression vector encoding caspase-1 or 3 were cultured in the media containing 1% raffinose for 12 h. The expression of caspases was induced by the addition of 2% galactose to the media. After incubation for 6 h, cell pellets were placed in liquid nitrogen and lysed using glass beads (Sigma) in a disruption buffer containing 20 mM Tris, pH 7.9, 10 mM MgCl₂, 1 mM EDTA, 5% glycerol, and protease inhibitor mix (1 μg/ml leupeptin, 1 μg/ml pepstatin, and 50 μg/ml phenylmethylsulfonyl fluoride). Cell lysate proteins (25 μg) were incubated with 50 μM zVAD-AMC in a standard interleukin-β-converting enzyme (ICE) reaction buffer (25). Cleavage of zVAD-AMC was monitored by a fluorescence spectrophotometer at an excitation/emission wavelength pair of 355 nm/460 nm.

In Vitro Cleavage of 14-3-3ε Protein by Caspase-3—Human 14-3-3ε and its mutant, D238A, were cloned into pcDNA3 (Invitrogen). Site-directed mutagenesis was performed using the procedures of QuickChange site-directed mutagenesis kit (Stratagene). 35S-labeled 14-3-3ε protein and D238A were produced using the TNT T7 coupled reticulocyte lysate system (Promega). In vitro translated 35S-14-3-3ε protein or 35S-D238A was incubated with Escherichia coli BL21(DE3) cell lysate in the presence of recombinant human caspase-3 at 30 °C for 8 h. Proteolytic cleavage of 35S-labeled 14-3-3ε protein and D238A was analyzed by 12% SDS-PAGE and autoradiography.

Immunoblotting and Co-immunoprecipitation—Cos cells were harvested 10 h after staurosporine treatment or 24 h after UVC irradiation. Cells were lysed in buffer A containing 50 mM Tris, pH 7.5, 120 mM NaCl, 0.5% Nonidet P-40, 10 mM EDTA, 5% glycerol, and protease inhibitor mix (1 μg/ml leupeptin, 1 μg/ml pepstatin, and 50 μg/ml phenylmethylsulfonyl fluoride). Cell lysate proteins (25 μg) were incubated with 50 μM zVAD-AMC in a standard interleukin-β-converting enzyme (ICE) reaction buffer (25). Cleavage of zVAD-AMC was monitored by a fluorescence spectrophotometer at an excitation/emission wavelength pair of 355 nm/460 nm.

RESULTS

Identification of 14-3-3ε Protein as Caspase Substrate by the Modified Yeast Two-hybrid System—Fig. 1A illustrates the strategy for identifying caspase substrates in the yeast genetic system. The cDNAs encoding caspase substrates were fused between the LexA DNA-binding domain and the B42 activation domain. Yeast EGY48 cells expressing the fusion products in which the LexA DNA-binding domain and the B42 transcriptional activation domain were linked by the mouse brain cDNA library. The bacterial lacZ gene was used as a LexA-dependent transcriptional reporter. Yeast cells expressing the fusion products grew as the blue β-galactosidase positive colonies on the plate containing the indicator X-gal, whereas the transformants that failed to express lacZ grew as the white β-galactosidase negative colonies. B, a plasmid carrying a fusion product in which PAP or parapim resistant PARP234A links the LexA DNA-binding domain and the B42 transcriptional activation domain was transformed into yeast cells. The expression of human caspases was induced by adding 2% galactose to the culture media. Note that yeast cells expressing PAP grew as the white β-galactosidase negative colonies after the induction of caspase-3, whereas the transformants expressing caspase-resistant PARP234A grew as the blue β-galactosidase positive colonies after the induction of caspase-3. C, the expression of human caspase-1 or 3 under the GAL1 inducible promoter was induced in yeast cells by adding 2% galactose to the culture media. Each yeast cell lysate was incubated with a fluorogenic peptide substrate, zVAD-AMC (0.5 μM), and the activities of caspases in YM4271 cells were fluorometrically measured (excitation/emission of 355 nm/460 nm). Ac-TyVAD-CHO (0.5 μM) and Ac-DEVED-CHO (0.5 μM) were used as specific inhibitors of caspase-1 and 3, respectively.

The abbreviations used are: PARP, poly(ADP)ribose polymerase; HA, hemagglutinin; GST, glutathione S-transferase; FACS, fluorescence-activated cell sorter; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; Ac-DEVED-CHO, acetyl-Asp-Val-Ala-Asp-aldehyde.
expressing the fusion product containing caspase-resistant PARP (PARD234A) in which Asp214 at the P1 position was substituted with Ala (D238A) was not cleavable by caspase-3 (Fig. 2C). Cleavage of PARP implicates the activation of caspase-3 in COS cells after UVC irradiation or staurosporine treatment. Indeed, the activities of caspase-3 were elevated in parallel with the cleavage of cellular 14-3-3e in COS cells after UVC irradiation (data not shown). Amino acid sequence analysis of the cleavage product revealed that caspase-3 cleaves the 14-3-3e protein at Asp258 of MQGAG. A mutant 14-3-3e protein in which Asp 238 was substituted with Ala (D238A) was not cleavable by caspase-3 (Fig. 2C).

Cleavage of 14-3-3e Protein Impairs Its Binding Ability with Bad and Facilitates Bad-mediated Apoptosis—Bad and 14-3-3e constructs were over-expressed in cultured 293T cells. The cleaved 14-3-3e protein fragment, D238, interacted with Bad with lower affinity than its wild type or the uncleavable counterpart, D238A. Bad interacted with the endogenous Bcl-x(L) in cells co-expressing Bad either with the wild type 14-3-3e protein or the uncleavable D238A. Interestingly, co-expression of the truncated 14-3-3e protein D238 with Bad significantly increased Bad association with cellular Bcl-x(L) (Fig. 3), and the increased Bad association with Bcl-x(L) appeared to contribute to Bad-mediated apoptosis. Bad-mediated apoptosis was not affected by the ectopic expression of the wild type 14-3-3e protein of the uncleavable D238A (Fig. 4A). The expression of the truncated D238 also enhanced staurosporine-induced apoptosis, whereas the expression of the uncleavable D238A did not increase apoptosis in 293T cells after staurosporine treatment (data not shown). Cleavage of the 14-3-3e protein by caspase-3 did not seem to change the pattern of 14-3-3e protein dimerization (Fig. 4B).

Fig. 2. Cleavage of 14-3-3e protein at Asp258 by caspase-3. A, the 35S-labeled 14-3-3e protein and PARP were produced by in vitro transcription/translation procedures. 35S-labeled 14-3-3e protein or 35S-labeled PARP was cleaved by the recombinant human caspase-3, and the cleavages were analyzed by 12% SDS-PAGE and subsequent autoradiography. Ac-DEVD-CHO was used as a specific caspase-3 inhibitor. B, COS cells (10⁷ cells) were treated with staurosporine or UVC and incubated for 10 h or 24 h respectively. Cell lysate proteins (50 μg) were separated on 12% SDS-PAGE and subsequent autoradiography. Ac-DEVD-CHO was used as a specific caspase-3 inhibitor. C, the 35S-labeled 14-3-3e protein and the uncleavable D238A were produced by in vitro transcription/translation procedures. The cleavage of the 35S-labeled proteins by the recombinant human caspase-3 was analyzed by 12% SDS-PAGE and subsequent autoradiography. Ac-DEVD-CHO was used as a specific caspase-3 inhibitor.
in 293T cells. After transfection for 24 h, cells were harvested and fixed with cold ethanol. Cells were stained with propidium iodide (PI) for 4 h after fixation. The percentage of apoptosis was quantified and fixed with cold ethanol. Cells were stained with propidium iodide expressed in 293T cells. After transfection for 24 h, cells were harvested equally well with the GST-14-3-3 protein dimer very tightly but at very slow rate (13, 29). Data showed that the truncated 14-3-3 protein disrupted the anti-apoptotic function of the wild type 14-3-3 protein and increased apoptosis in response to UV irradiation. In the present study, we suggest that the 14-3-3e protein is cleaved by the activated caspase-3 to promote cell death during exposure to apoptotic signals. The cleaved 14-3-3e protein impaired its binding affinity to Bad and released Bad to translocate to the mitochondrial outer membrane where Bad heterodimerizes with Bcl-x(L). 14-3-3 proteins have been shown to inhibit Bad-induced apoptosis through interaction at the phosphorylation sites of Bad (11, 16). A number of studies have described the dimeric array of 14-3-3 proteins that interact with 14-3-3 binding proteins at two 14-3-3 consensus binding motifs. The dominant-positive lethal effect of the truncated 14-3-3e protein (D238) on Bad-induced apoptosis in 293T cells (Fig. 4A) cannot be explained by the failure of the intermolecular association of D238 with Bad (Fig. 3), because the endogenous cellular 14-3-3e protein in 293T cells expressing D238 should also have interacted with Bad. The results in Fig. 4B offered an explanation that the truncated D238 heterodimerized with the endogenous 14-3-3e protein and lowered the intracellular level of self-associated wild type 14-3-3e dimers that could sequester Bad in co-transfected cells. 14-3-3 proteins may form heterodimers as well as homodimers; therefore the truncated D238 may also dimerize with isoforms of 14-3-3 proteins in 293T cells. It is unlikely that cleavage of the 14-3-3e protein by caspase-3 changes the pattern of dimerization during Bad-induced apoptosis, because the residues of 14-3-3 isoforms involved in the dimer formation are largely conserved among mammalian isoforms (34). The fact that D238 forms heterodimers with isoforms of 14-3-3 proteins may not affect the interaction of D238 with Bad, because the different isoforms of the 14-3-3 protein were reported to interact equally well with Bad (14). The binding ability of HA-D238 with Bad was compared with that of HA-14-3-3e or the unbeatable HA-D238A in an experiment in which HA-14-3-3e or HA-D238A were co-expressed with or without wild type 14-3-3e protein in 293T cells. Over-expression of the 14-3-3e protein did not interfere with the binding affinity of HA-D238 with Bad, suggesting that cleavage of the 14-3-3e protein by caspase-3 lowers its binding affinity with Bad regardless of the state of the intermolecular association of the cleaved 14-3-3e protein (data not shown). The released death promoting Bad then heterodimerizes with Bcl-x(L) located in the outer membrane of the mitochondria. The binding of Bad to Bcl-x(L) may cause Bcl-x(L) to interact with pro-apoptotic Bax to release cytochrome c or regulate other Bcl-x(L) activities (3), leading to the progression of cell death.

REFERENCES

1. Green, D. R., and Reed, J. C. (1998) Science 281, 1309–1312
2. Reed, J. C., and Green, D. R. (2002) Mol. Cell 9, 1–3
3. Ravagnan, L., Roumier, T., and Kroemer, G. (2002) J. Cell. Physiol. 192, 131–137
4. Cam, K., Brutton, S. B., Langlois, C., Walker, G., Brown, D. G., Sun, X. M., and Cohen, G. M. (2000) J. Biol. Chem. 275, 6067–6070
5. Kluck, R. M., Bossy-Wetzel, E., Green, D. R., and Newmeyer, D. D. (1997) Science 275, 1132–1136
14-3-3 Cleavage during Apoptosis

6. Yang, J., Liu, X., Bhalia, K., Kim, C. N., Ibraheem, A. M., Cai, J., Peng, T. I., Jones, D. P., and Wang, X. (1997) Science 275, 1129–1132
7. Huang, D. C., and Strasser, A. (2000) Cell 104, 839–842
8. Conradt, B., and Horvitz, H. R. (1998) Cell 93, 519–529
9. Lizzano, J. M., Morrice, N., and Cohen, P. (2000) Biochem. J. 349, 547–557
10. Tan, Y., Demeter, M. R., Ruan, H., and Comb, M. J. (2000) J. Biol. Chem. 275, 25865–25869
11. Yang, S., Tsumitso, H., and Soderling, T. R. (1995) Science 268, 584–587
12. Harada, H., Becknell, B., Wilm, M., Mann, M., Huang, L. J., Taylor, S. S., Scott, J. D., and Korsmeyer, S. J. (1999) Mol. Cell 3, 413–422
13. Rittinger, K., Budman, J., Xu, J., Volinia, S., Cantley, L. C., Smerdon, S. J., and Yaffe, M. B. (1999) Mol. Cell 4, 153–166
14. van Hemert, M. J., Steensma, H. Y., and van Heusden, G. P. (2001) Bioessays 23, 936–946
15. Wang, H. G., Pathan, N., Ethell, I. M., Krajewski, S., Yamaguchi, Y., Shi-basaki, F., McKeon, F., Bode, T., Franke, T. F., and Reed, J. C. (1999) Science 284, 339–342
16. Kennedy, S. G., Kandel, E. S., Cross, T. K., and Hay, N. (1999) Mol. Cell Biol. 19, 5890–5910
17. Brunet, A., Bonni, A., Zigmond, M. J., Lin, M. Z., Luo, P., Hu, L. S., Anderson, M. J., Arden, K. C., Iblis, J., and Greenberg, M. E. (1999) Cell 96, 687–698
18. Zhang, L., Chen, J., and Pa, H. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 8511–8515
19. Masuyama, N., Oishi, K., Mori, Y., Ueno, T., Takahama, Y., and Getoh, Y. (2001) J. Biol. Chem. 276, 32799–32805
20. Miller, D. K. (1997) Semin. Immunol. 9, 35–49
21. Villa, P., Kaufmann, S. H., and Earnshaw, W. C. (1997) Trends Biochem. Sci. 22, 388–393
22. Agan, K., Kusano, T., Suzuki, N., and Kitagawa, Y. (1990) Nucleic Acids Res. 18, 1071
23. Bendixen, C., Gangloff, S., and Rothstein, R. (1994) Nucleic Acids Res. 22, 1778–1779
24. Sambrook, J., and Russell, D. W. (2001) Molecular Cloning: A Laboratory Manual, 3rd Ed., pp. 16.19–16.20, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
25. Kim, J. W., Won, J., Sohn, S., and Joe, C. O. (2000) J. Cell Sci. 113, 955–961
26. Widmann, C., Gibson, S., and Johnson, G. L. (1998) J. Biol. Chem. 273, 7141–7147
27. Muslin, A. J., and Xing, H. (2000) Cell Signal. 12, 703–709
28. Zha, J., Harada, H., Yang, E., Jockel, J., and Korsmeyer, S. J. (1996) Cell 87, 619–628
29. Zha, J., Harada, H., Yang, E., Jockel, J., and Korsmeyer, S. J. (1996) Cell 87, 619–628
30. Zha, J., Harada, H., Yang, E., Jockel, J., and Korsmeyer, S. J. (1996) Cell 87, 619–628
31. Zha, J., Harada, H., Yang, E., Jockel, J., and Korsmeyer, S. J. (1996) Cell 87, 619–628
32. Zha, J., Harada, H., Yang, E., Jockel, J., and Korsmeyer, S. J. (1996) Cell 87, 619–628
33. Zha, J., Harada, H., Yang, E., Jockel, J., and Korsmeyer, S. J. (1996) Cell 87, 619–628
34. Zha, J., Harada, H., Yang, E., Jockel, J., and Korsmeyer, S. J. (1996) Cell 87, 619–628
Cleavage of 14-3-3 Protein by Caspase-3 Facilitates Bad Interaction with Bcl-x(L) during Apoptosis

Jungyeon Won, Doo Yeon Kim, Muhnho La, Doyeun Kim, Gary G. Meadows and Cheol O. Joe

J. Biol. Chem. 2003, 278:19347-19351.
doi: 10.1074/jbc.M213098200 originally published online March 25, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M213098200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 33 references, 14 of which can be accessed free at http://www.jbc.org/content/278/21/19347.full.html#ref-list-1