diffusion of secreted substances and promotes paracrine interactions within the islet, where β, α, and δ cells tend to be segregated (*14*).

Our data suggest the notion (*24*, *28*) that the fusion pore of physiological exocytosis is formed by membrane lipids whose lateral diffusion is not prevented by immobile proteins (*23*). If the initial pore is formed from proteinaceous channels (*3*, *10*), such channels would have to be disassembled before expansion of the pore to a diameter of 1.4 nm so as not to prevent lipid diffusion. Thus, the role of fusogenic proteins is more likely to be to control the proximity of two biological membranes and thereby to facilitate formation of the lipidic nanopore. Such proteins may prevent lipid flux along the outer wall of the fusion pore, as described for hemagglutinin-induced cell fusion (*29*); the outer wall of the fusion pore might thus be proteinaceous to some extent. Given that insulin secretion is mediated by an expanding lipidic pore, abnormal lipid metabolism (*14*) in individuals with non–insulin-dependent (type 2) diabetes mellitus may impair secretion at the level of lipidic pore structure. Our approach provides a means of further elucidating the molecular control of fusion pore structures during physiological exocytosis.

**References and Notes**


1. E. Neher, *Neuron* **20**, 389 (1998).
2. L. J. Breckenridge, W. Almers, *Nature* **328**, 814 (1987).
3. J. Zimmerberg, M. Curran, F. S. Cohen, M. Brodwick, *Proc. Natl. Acad. Sci. U.S.A.* **84**, 1585 (1987).
4. G. Alvarez de Toledo, R. Fernadez-Chacon, J. M. Fernandez, *Nature* **363**, 554 (1993).
5. Z. Zhou, S. Misler, R. H. Chow, *Biophys. J.* **70**, 1543 (1996).
6. L. Orci, M. Amherdt, F. Malaisse-Lagae, C. Rouiller, A. E. Renold, *Science* **179**, 82 (1973).
7. H. Plattner, A. R. Artlejo, E. Neher, *J. Cell Biol.* **139**, 1709 (1997).
8. D. E. Chandler, J. E. Heuser, *J. Cell Biol.* **86**, 666 (1980).
9. T. Kanaseki, K. Kawasaki, M. Murata, Y. Ikeuchi, *J. Cell Biol.* **137**, 1041 (1997).
10. J. Zimmerberg, *Trends Cell Biol.* **11**, 233 (2001).
11. W. Denk, J. H. Strickler, W. W. Webb, *Science* **248**, 73 (1990).
12. Materials and methods are available as supporting material on *Science* Online.
13. T. Nemoto *et al.*, *Nature Cell Biol.* **3**, 253 (2001).
14. C. R. Kahn, G. C. Weir, *Joslin's Diabetes Mellitus* (Lea & Febiger, Phildelphia, PA, 1994).
15. N. Takahashi *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **96**, 760 (1999).
16. L. Orci, M. Ravazzola, R. G. Anderson, *Nature* **326**, 77 (1987).
17. W. J. Qian, C. A. Aspinwall, M. A. Battiste, R. T. Kennedy, *Anal. Chem.* **72**, 711 (2000).
18. S. Bonner-Weir, *Diabetes* **37**, 616 (1988).
19. D. G. Pipeleers, *Diabetes* **41**, 777 (1992).
20. N. Takahashi, T. Kadowaki, Y. Yazaki, Y. Miyashita, H. Kasai, *J. Cell Biol.* **138**, 55 (1997).
21. W. J. Betz, F. Mao, G. S. Bewick, *J. Neurosci.* **12**, 363 (1992).
22. N. Takahashi *et al.*, *Diabetes* **51** (suppl. 1), S25 (2002).
23. T. Fujiwara, K. Ritchie, H. Murakoshi, K. Jacobson, A. Kusumi, *J. Cell Biol.* **157**, 1071 (2002).
24. J. R. Monck, G. Alvarez de Toledo, J. M. Fernandez, *Proc. Natl. Acad. Sci. U.S.A.* **87**, 7804 (1990).
25. G. Dodson, D. Steiner, *Curr. Opin. Struct. Biol.* **8**, 189 (1998).
26. S. Barg *et al.*, *Neuron* **33**, 287 (2002).
27. P. A. in't Veld, D. G. Pipeleers, W. Gepts, *Diabetes* **33**, 101 (1984).
28. A. Chanturiya, L. V. Chernomordik, J. Zimmerberg, *Proc. Natl. Acad. Sci. U.S.A.* **94**, 14423 (1997).
29. F. W. Tse, A. Iwata, W. Almers, *J. Cell Biol.* **121**, 543 (1993).



30. We thank H. Gaisano for critical reading of the manuscript; H. Noguchi for helpful discussion; and R. Ijuin, T. Kise, and N. Takahashi for technical assistance. This work was supported by Grants-in-Aid from the Japanese Ministry of Education, Culture, Sports, Science and Technology and the Japan Society for the Promotion of Science; by Research grants from the Human Frontier Science Program Organization, Uehara Memorial Foundation (H.K.), and Japan Diabetes Foundation (N.T.); and by PRESTO of the Japan Science and Technology Corporation (JST) (T.N.).


**Supporting Online Material**

www.sciencemag.org/cgi/content/full/297/5585/1349/DC1
Materials and Methods
Supporting Text
Figs. S1 and S2
Movies S1 and S2

10 May 2002; accepted 28 June 2002

# Requirement for Caspase-2 in Stress-Induced Apoptosis Before Mitochondrial Permeabilization

**Patrice Lassus, Ximena Opitz-Araya, Yuri Lazebnik***

**A current view is that cytotoxic stress, such as DNA damage, induces apoptosis by regulating the permeability of mitochondria. Mitochondria sequester several proteins that, if released, kill by activating caspases, the proteases that disassemble the cell. Cytokines activate caspases in a different way, by assembling receptor complexes that activate caspases directly; in this case, the subsequent mitochondrial permeabilization accelerates cell disassembly by amplifying caspase activity. We found that cytotoxic stress causes activation of caspase-2, and that this caspase is required for the permeabilization of mitochondria. Therefore, we argue that cytokine-induced and stress-induced apoptosis act through conceptually similar pathways in which mitochondria are amplifiers of caspase activity rather than initiators of caspase activation.**

Apoptosis is executed by caspases, a family of proteases that disassemble a cell (*1*, *2*). The pathways leading to caspase activation vary with the cytotoxic stimulus. The stimuli that are collectively referred to as cytotoxic stress, such as DNA damage, activate caspases by initiating signaling pathways that converge on the Bcl-2 family of proteins (*3*). A balance between members of this family is thought to determine whether mitochondria remain intact or become permeabilized and release proteins that promote cell death (*4*). One of these released proteins is cytochrome c, which, in a complex with the cytoplasmic protein Apaf-1, activates caspase-9. Caspase-9, in turn, activates caspase-3, the protease that cleaves the majority of caspase substrates during apoptosis. Two other proteins, Smac (also called Diablo) and Htr2A (Omi), accelerate caspase activation and increase caspase activity by inactivating caspase inhibitors. Mitochondria also release apoptosis- inducing factor (AIF) and endonuclease G, which appear to kill independently of caspases. Therefore, mitochondria are thought to be a central regulatory element in stress-induced apoptosis (*5*).

Another way to activate caspases, used by cytokines, is to assemble receptor complexes that recruit caspase-8 or caspase-10, thereby inducing their autocatalytic processing (*2*). These caspases activate other caspases, including caspase-3, either directly (by proteolytic processing) or indirectly by cleaving Bid, a Bcl-2 family member. A proteolytic fragment of Bid permeabilizes mitochondria, thereby accelerating cell disassembly as described above. Hence, mitochondria in this pathway function as "amplifiers" of the caspase activity rather than as central regulators of apoptosis.

This model of apoptosis was consistent with studies of oncogene-dependent apoptosis, a phenomenon that may provide clues about how to kill cancer cells selectively (*6*). By comparing normal human fibroblasts (IMR90) with fibroblasts transformed with the adenoviral oncogene *E1A* (IMR90E1A), we found that this oncogene sensitizes cells to chemotherapeutic drugs by facilitating the activation of caspase-9 (*7*). *E1A* appears to achieve this effect by promoting the activation of Bax, a proapoptotic Bcl-2 protein that can permeabilize mitochondria, and by repressing a still-unidentified inhibitor of this permeabilization (*8*). These observations were in agreement with a model in which Bcl-2 proteins control caspase activation by regulating mito-

Cold Spring Harbor Laboratory, Cold Spring Harbor, NY 11724, USA.

*To whom correspondence should be addressed. E-mail: lazebnik@cshl.org

chondrial permeability. Contrary to this view, we report that mitochondrial permeability is controlled by a caspase that is activated earlier in the pathway that links DNA damage and cell disassembly.

We used small interfering RNA (siRNA) to silence the expression of various proteins by RNA interference (RNAi) in IMR90E1A cells (*9*). A siRNA to caspase-2, a caspase that has been implicated in apoptosis but whose exact function and regulation remain unknown (*10*–*14*), efficiently and specifically silenced caspase-2 expression (Fig. 1A). Several other siRNAs (*15*) had no effect on the expression of caspase-2. Only the siRNA to Apaf-1 repressed the expression of Apaf-1, and none of the siRNAs used affected the expression of caspase-9 (Fig. 1A). The siRNA to Apaf-1 prevented apoptosis induced by all three DNA-damaging agents [etoposide, cisplatin, and ultraviolet (UV) light] that we used (Fig. 1B). To our surprise, however, inhibition of the expression of caspase-2 and of Apaf-1 prevented apoptosis with equal efficiency. This effect could not be attributed to manipulations required to introduce siRNAs into cells, because the siRNA to caspase-1 had no effect on apoptosis (Fig. 1B). Therefore, we conclude that either caspase-2 is required for apoptosis, or the caspase-2 siRNA interfered with the expression of other proteins.

To test whether apoptosis indeed required caspase-2, we attempted to restore sensitivity to cytotoxic agents by expressing caspase-2 ectopically. To prevent the destruction of the ectopic caspase-2 mRNA by the caspase-2 siRNA, we introduced two silent mutations into the region of the caspase-2 cDNA that is complementary to the siRNA (*16*). Consistent with the reported high specificity of siRNA (*17*), the expression of both the endogenous and ectopic caspase-2 from the wild-type cDNA was efficiently silenced, whereas the expression of caspase-2 from the mutated cDNA (caspase-2si) was not (Fig. 2A). Expression of caspase-2si restored the sensitivity of cells to etoposide (Fig. 2B), indicating that the effect of the siRNA could be explained by repression of caspase-2. Expression of catalytically inactive caspase-2si (in which catalytic cysteine was mutated to serine) (*16*) did not rescue the effect of the siRNA; this result indicates that the proteolytic activity of caspase-2 is required to mediate apoptosis (Fig. 3). The absence of caspase-2 did not affect the rate of apoptosis induced by tumor necrosis factor–α (fig. S1), which indicates that caspase-2 is not required for receptor-mediated apoptosis.

When cells lacking caspase-2 were exposed to etoposide, cytochrome c remained in mitochondria (Fig. 4A) (fig. S3), as did Smac (Fig. 4B) (fig. S3). Both proteins were released in the absence of Apaf-1, which indicates that experimental manipulations required to introduce siRNA into cells do not prevent mitochondria permeabilization. Caspase-2 was proteolytically processed even if processing of caspases 9, 3, and 7 was prevented by the siRNA to Apaf-1; this finding suggests that caspase-2 is activated before or independently of the other three caspases (fig. S2). Hence, caspase-2 is required to permeabilize mitochondria in these cells, a conclusion consistent with an observation that this caspase can permeabilize mitochondria in a cell-free system (*18*).

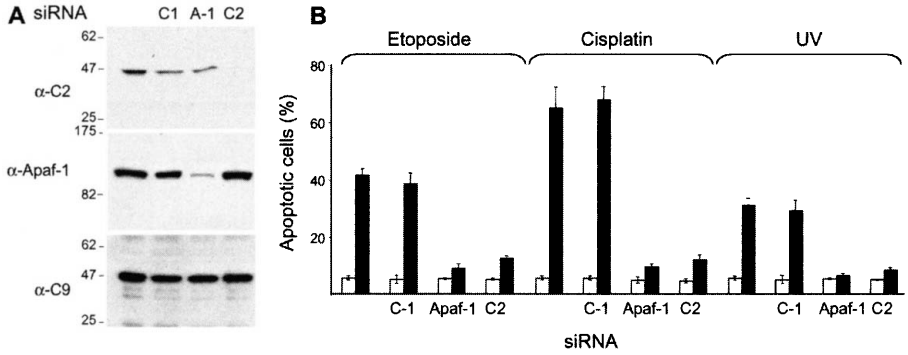


**Fig. 1.** Requirement of caspase-2 for apoptosis induced by several cytotoxic agents. (**A**) IMR90E1A cells were transfected with siRNA to Apaf-1 (A-1), caspase-2 (C2), or caspase-1 (C1) (as a control for the effect of transfection) or were left untransfected (*16*). After culturing cells for 2 days, a portion of the cells was used to determine amounts of caspase-2 and Apaf-1 by immunoblotting (*16*). Molecular mass markers (in kD) are shown. (**B**) The remaining cells were treated with the indicated cytotoxic agents (solid bars) or left untreated (open bars). The final concentration of the drugs in the medium was 50 μM for etoposide and 20 μM for cisplatin. UV was used at 10 mJ/cm$^2$. After 18 hours of treatment, both adherent and floating cells were collected, fixed with 4% paraformaldehyde, and stained with 4′,6′-diamidino-2-phenylindole (DAPI) to reveal chromatin structure. Cells with condensed chromatin were scored as apoptotic.

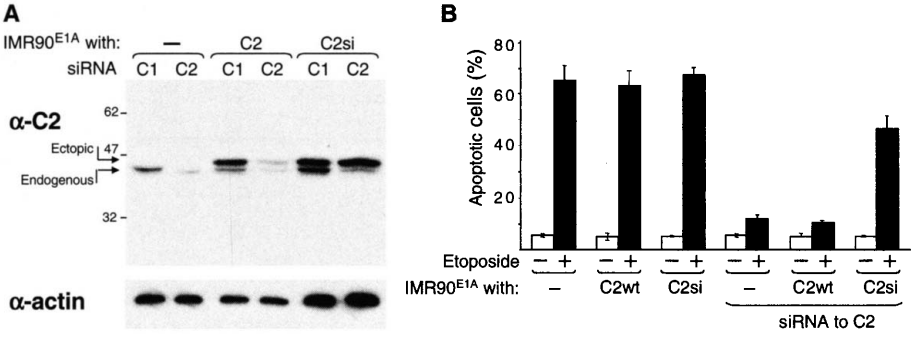


**Fig. 2.** Restored sensitivity of cells to apoptosis after ectopic expression of caspase-2. (**A**) cDNA encoding caspase-2 (C2) or caspase-2 in which two silent mutations prevented interaction between siRNA and the caspase-2 mRNA (C2si) (*16*) were ectopically expressed in IMR90E1A cells by retroviral transduction (*16*). The cell lines were transfected with siRNA to caspase-2 or caspase-1 or left untransfected. Two days later, the amount of caspase-2 in the cell lines was determined by immunoblotting (*16*). The ectopic protein contains a Myc epitope tag that allows distinction of endogenous and ectopic caspase-2 by a difference in electrophoretic mobility. The blot was reprobed with an antibody to β-actin to indicate the relative amount of total protein applied in each lane (*16*). (**B**) The cells transfected with siRNA as described in (A) were treated with 50 μM etoposide or left untreated, and the rate of apoptosis was determined (Fig. 1).

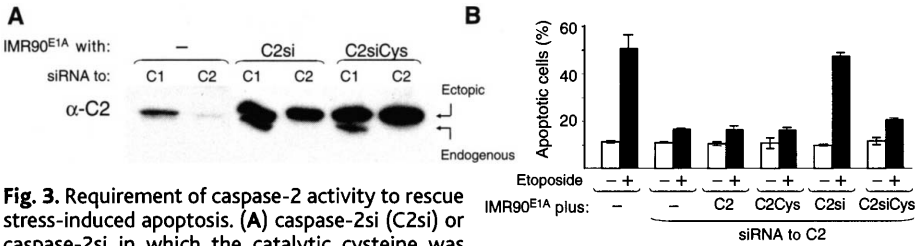


**Fig. 3.** Requirement of caspase-2 activity to rescue stress-induced apoptosis. (**A**) caspase-2si (C2si) or caspase-2si in which the catalytic cysteine was mutated into a serine (C2siCys) (*16*) were expressed in IMR90E1A cells by retroviral transduction (*16*). The cell lines were transfected with siRNA to caspase-2 or to caspase-1 (*16*), and expression of endogenous and ectopic caspase-2 was determined by immunoblotting. (**B**) The cells transfected with siRNA to caspase-2 as described in (A) or left untransfected were treated with 50 μM etoposide for 18 hours or left untreated, and were then scored for apoptosis (Fig. 1).

The earliest detectable change in the apoptotic machinery after DNA damage may be the translocation of the cytoplasmic Bcl-2 family member Bax to mitochondria (*19*). Etoposide induced Bax translocation in cells transfected with the siRNA to Apaf-1. However, Bax remained in the cytoplasm in cells transfected with the siRNA to caspase-2 (Fig. 4C) (fig. S3). Thus, caspase-2 is required to translocate Bax to mitochondria in this experimental system.

Hence, as previously suggested (*20*), our results imply that stress-induced apoptosis can be executed in a pathway that is conceptually similar to that of cytokine-induced apoptosis, in that each pathway begins with the activation of a caspase that uses mitochondria to amplify the total caspase activity in the cell.

We expanded our study to five human tumor cell lines that can be efficiently transfected, are sensitive to cytotoxic agents, and are widely used in cancer research. In the lung adenocarcinoma A549 and the osteosarcoma U2OS, caspase-2 and Apaf-1 were required to produce morphological features of apoptosis (Fig. 5, A and B), and, in both cell lines, caspase-2 was required for cytochrome c release (Fig. 5C). In contrast, breast cancer cell line MCF-7 did not require caspase-2 for release of cytochrome c, perhaps because cytochrome c release is caspase-independent in these cells or because other caspases are involved (*15*). The results obtained with cervix adenocarcinoma HeLa and colorectal carcinoma HCT-116 cells were inconclusive because the viability of these cells is poor when transfected with siRNA.

Our findings have several implications (*16*). Although strong evidence indicates that a failure of apoptosis contributes to cancer progression in experimental systems, the evidence is much weaker for such a relationship in human cancers (*21*). Evidence for the latter has been primarily gathered by correlating tumor properties with deficiencies in the apoptotic machinery. Our findings imply that a critical part of a major apoptotic pathway is yet to be considered by such studies. Indeed, the survival of cancer cells might be enhanced by any changes that prevent caspase-2 activation. How this activation is regulated is unknown, which indicates that even the basic pathways of apoptosis are not yet sufficiently understood to allow the efficient modulation of apoptosis to a therapeutic end. This study also highlights the ability of RNAi to yield results in cancer biology studies that do not depend solely on observations made with genetically modified mice.

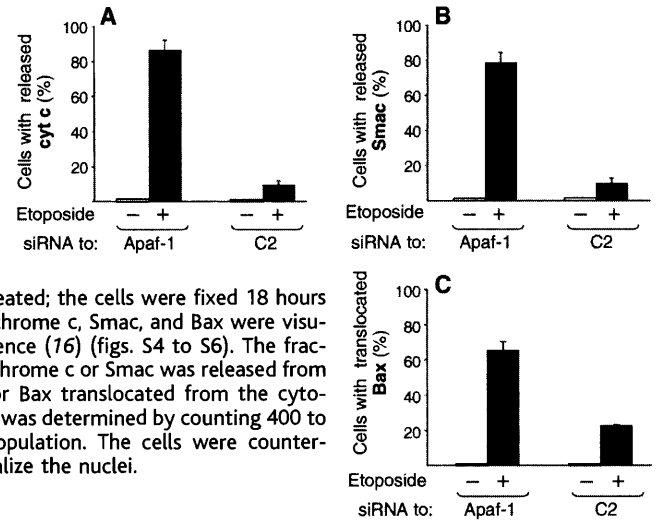


**Fig. 4.** Requirement of caspase-2 for release of cytochrome c and Smac from mitochondria, and for translocation of Bax from the cytoplasm to mitochondria. IMR90E1A cells were transfected with siRNA to either Apaf-1 or caspase-2. After 2 days, the cells were either treated with 50 μM etoposide or left untreated; the cells were fixed 18 hours after treatment, and cytochrome c, Smac, and Bax were visualized by immunofluorescence (*16*) (figs. S4 to S6). The fraction of cells in which cytochrome c or Smac was released from mitochondria (**A** and **B**) or Bax translocated from the cytoplasm to mitochondria (**C**) was determined by counting 400 to 500 cells for each cell population. The cells were counterstained with DAPI to visualize the nuclei.

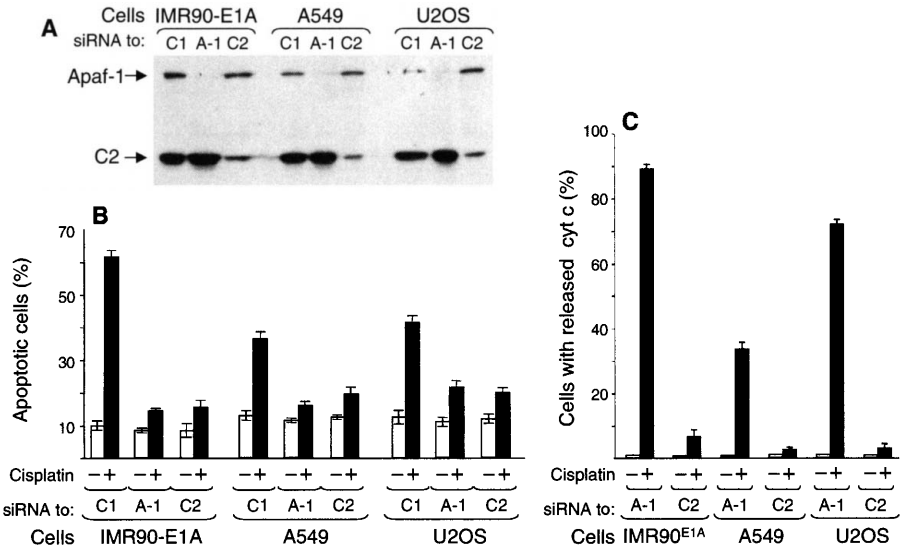


**Fig. 5.** Requirement of caspase-2 for apoptosis and cytochrome c release in human tumor cell lines. (**A**) The indicated cell lines were treated with siRNAs to caspase-1, Apaf-1, or caspase-2 (*16*), and expression of Apaf-1 and caspase-2 was determined by immunoblotting with antibodies to these proteins (*16*). The blot shown was exposed after incubation with both antibodies. (**B**) Cells transfected with siRNA to caspase-1, Apaf-1, or caspase-2 were treated with cisplatin (40 μM) for 20 hours, then collected and scored for apoptosis by observing chromatin condensation (Fig. 1). (**C**) Cytochrome c release in the cells treated as described in (B) was visualized by immunofluorescence, and the fraction of cells with released cytochrome c was determined by counting about 500 cells.

## References and Notes


1. N. A. Thornberry, Y. Lazebnik, *Science* **281**, 1312 (1998).
2. M. O. Hengartner, *Nature* **407**, 770 (2000).
3. A. Gross, J. M. McDonnell, S. J. Korsmeyer, *Genes Dev.* **13**, 1899 (1999).
4. D. C. Huang, A. Strasser, *Cell* **103**, 839 (2000).
5. X. Wang, *Genes Dev.* **15**, 2922 (2001).
6. G. Evan, T. Littlewood, *Science* **281**, 1317 (1998).
7. H. O. Fearnhead *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **95**, 13664 (1998).
8. D. M. Duelli, Y. A. Lazebnik, *Nature Cell Biol.* **2**, 859 (2000).
9. S. M. Elbashir *et al.*, *Nature* **411**, 494 (2001).
10. S. Kumar, M. Kinoshita, M. Noda, N. G. Copeland, N. A. Jenkins, *Genes Dev.* **8**, 1613 (1994).
11. L. Wang, M. Miura, L. Bergeron, H. Zhu, J. Yuan, *Cell* **78**, 739 (1994).
12. L. Bergeron *et al.*, *Genes Dev.* **12**, 1304 (1998).
13. S. Kumar, *FEBS Lett.* **368**, 69 (1995).
14. G. Paroni, C. Henderson, C. Schneider, C. Brancolini, *J. Biol. Chem.* **277**, 15147 (2002).
15. P. Lassus, Y. Lazebnik, unpublished data.
16. See supporting data on *Science* Online.
17. S. M. Elbashir, J. Martinez, A. Patkaniowska, W. Lendeckel, T. Tuschl, *EMBO J.* **20**, 6877 (2001).
18. Y. Guo, S. M. Srinivasula, A. Druilhe, T. Fernandes-Alnemri, E. S. Alnemri, *J. Biol. Chem.* **277**, 13430 (2002).
19. A. Nechushtan, C. L. Smith, Y. T. Hsu, R. J. Youle, *EMBO J.* **18**, 2330 (1999).
20. G. Hausmann *et al.*, *J. Cell. Biol.* **149**, 623 (2000).
21. I. Herr, K. M. Debatin, *Blood* **98**, 2603 (2001).


22. We thank V. Marsden and A. Strasser for enjoyable interactions and for sharing unpublished data, D. Huang for caspase-2 antibody, and the members of the Lazebnik laboratory, S. Kaufmann, G. Hannon, and S. Lowe for helpful discussions. Supported by U.S. Army Prostate Cancer Program grant DAMD 17-01-1-0044, NIH grant CA-13106-31, and a Maxfield Foundation grant (Y.L.).


**Supporting Online Material**

www.sciencemag.org/cgi/content/full/297/5585/1352/DC1
Materials and Methods
Supporting Text
Figs. S1 to S3
References

4 June 2002; accepted 3 July 2002