Cytotoxic T Lymphocyte-assisted Suicide

CASPASE 3 ACTIVATION IS PRIMARILY THE RESULT OF THE DIRECT ACTION OF GRANZYM E B*

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Cytotoxic T lymphocyte-induced apoptosis can occur either through the directed exocytosis of granzyme B and perforin or via ligation of Fas. Both pathways involve the activation of a family of cysteine proteinases, the caspases, that cleave substrates at aspartic acid and are themselves activated by cleavage at internal aspartate residues. Fas recruits caspase 8, which initiates the death program through the subsequent activation of caspase 3. Granzyme B can process both caspase 8 and 3 in vitro, suggesting that both Fas and granzyme B access the apoptotic program in the same way. Here we demonstrate that although the two mechanisms are similar, the events that lead to activation of caspase 3 can be distinguished in vivo on the basis of their sensitivities to both pharmacological and virus-encoded caspase inhibitors. In cytotoxic T lymphocytes-mediated death the initial cleavage event on caspase 3 is insensitive to benzoyloxycarbonyl-Val-Ala-Asp fluoromethyl ketone (zVAD-fmk) inhibition in both mouse and human systems. During Fas-mediated death, however, activation of caspase 3 is completely inhibited to zVAD-fmk. In addition, the viral serpin SPI-2, a homologue of cytokine response modifier A (crmA), is an effective inhibitor of the Fas but not the granzyme pathway. Our results demonstrate that whereas Fas-mediated activation of caspase 3 requires an upstream caspase activity that is zVAD-fmk-sensitive, the initial cleavage of caspase 3 during granule-mediated cell death is insensitive to zVAD-fmk, suggesting that caspase 3 is cleaved directly by granzyme B in vivo.

Cytotoxic T lymphocytes (CTL), the major effectors of cellular immunity, have evolved two distinct lytic mechanisms to kill target cells by hijacking their built-in latent apoptotic program (1, 2). Both the calcium-dependent, perforin/granzyme-mediated, and the calcium-independent Fas ligand/Fas (Apo-1/CD95)-mediated CTL lytic pathways cause target cell destruction by activating members of the caspase family and initiating an apoptotic protease cascade. Fas ligand engagement of Fas on the target cell results in the direct recruitment of caspase 8 (FLICE/MACH/Mch5) to the activated Fas complex and results in the proteolytic activation of this enzyme (3, 4). Activated caspase 8 has been shown to be capable of processing a number of the procaspases in vitro, which likely accounts for its mode of action in vivo (5). In the granule exocytosis CTL pathway, the pore-forming protein perforin facilitates the entry of a family of granule-localized serine proteases, the granzymes, into the target cell. One of these serine proteases, granzyme B, is critical for initiating DNA fragmentation that occurs in the target cells (6–8) and is the only eukaryotic non-caspase protease known that exhibits specificity for aspartate residues (9–11). It is now widely believed that granzyme B utilizes its aspase activity to initiate the apoptotic protease cascade since it has been demonstrated that granzyme B can cleave and activate a number of the caspases in vitro, including caspases 3, 7, 8, 9, and 10 (12–18).

Both the Fas and the granzyme B lytic pathways cause a caspase activation sequence that converges at caspase 3 (CPP32/Yama/apopain). Inhibition of caspase 3 activity blocks DNA fragmentation in response to Fas (19–21), granzyme B (22), and a variety of other apoptotic stimuli, placing this caspase at a central control point. Since caspase 3 was the first identified substrate for granzyme B, it was initially proposed that direct cleavage and activation of this caspase explained its mode of action. However, with the numbers of granzyme B caspase substrates increasing steadily, it is possible that both direct and indirect (that is, via other granzyme B-activated caspases) activation of caspase 3 is involved in granzyme B-initiated apoptosis. Specifically, since the apoptotic pathways initiated by both granzyme B and Fas converge at caspase 3, it is of great interest to determine whether they share a common pathway proximal to this point. In fact, it is conceivable that since caspase 8, the caspase that is recruited to an activated Fas complex, activates caspase 3 (23) and also functions as a substrate for granzyme B in vitro (3, 4), the granule exocytosis pathway may initially cause activation of the same caspase as Fas and the two CTL lytic mechanisms may share a common course starting from the most proximal point possible.

To investigate the similarities between Fas- and granzyme B-mediated apoptosis of susceptible target cells, we examined the effects of two different peptide-based inhibitors of caspases and a virus-encoded caspase inhibitor on DNA fragmentation and caspase 3 cleavage. Benzoyloxycarbonyl-Val-Ala-Asp fluoromethyl ketone (zVAD-fmk) is a general pan-specific caspase
inhibitor and benzoxycarbonyl-Asp-Glu-Val-Asp fluoromethyl ketone (zDEVD-fmk) is a specific inhibitor of caspase 3-like enzymes. Granzyme B activity is not affected by either of these peptide inhibitors (19–24). SPI-2 is the rabbot pox virus equivalent to the cowpox virus cytokine response modifier A (crmA). SPI-2, like crmA, has previously been shown to inhibit Fas-mediated apoptosis in a similar fashion to crmA (25–27).

We found that the two lytic pathways were clearly distinguishable based upon the patterns of inhibition of caspase 3 activation by the peptide inhibitors and also by SPI-2 inhibition.

**MATERIALS AND METHODS**

**Cell Lines**—Jurkat and L1210 target cells were grown in RPMI 1640 medium (Life Technologies, Inc.) supplemented with antibiotics, sodium pyruvate, b-mercaptoethanol, HEPES, and 5% fetal calf serum (RHF). Jurkats were stably transfected with BMGneo-SPI-2 and BMGNeo via electroporation (250 V, 250 microfarads) (28). Stably transfected Jurkats were selected in 1 mg/ml G418 (Life Technologies, Inc.) and cloned by limiting dilution. Once selected, the resulting cells were routinely grown in RHF containing 0.8 mg/ml G418.

Human lymphocytes were isolated from peripheral blood by centrifugation through Ficoll (Amersham Pharmacia Biotech). The harvested lymphocytes were plated at 4 × 10^5 cells/ml with a 1:5 ratio of breed virus-transformed RPMI-8666 cells (5000 rad) at a 5:1 ratio (lymphocytes:RPMI-8666). After 3 days in culture, the human CTL (hCTL) were separated from the dead RPMI-8666 cells by centrifugation through Ficoll. The hCTL were maintained in RPMI 1640 containing 10% fetal bovine serum and 90 units/ml recombinant interleukin 2 (Chiron). hCTL were restimulated on a weekly basis with irradiated RPMI-8666 cells at a ratio of 1:2 (lymphocytes:RPMI-8666). CTL cell lines from wild type, granzyme A knockout, and granzyme B knockout mice were initially generated in a mixed lymphocyte culture by obtaining splenocytes from the responder mice (all H-2b) and stimulating them with an equal number of H-2b γ-irradiated splenocytes from Balb/c mice. Cultures were grown in RPMI medium supplemented with 10% fetal calf serum and 90 units/ml interleukin 2. The effector cells lines were restimulated with irradiated Balb/c splenocytes on a weekly basis. One day before use in the DNA fragmentation assays, the CTL lines were stimulated with anti-CD3ε antibody 145–2C11 to ensure potent up-regulation of the granule-dependent lytic pathway.

**Reagents**—Murine anti-human Fas antibody was purchased from Upstate Biotechnology Inc. and routinely used at 100 ng/ml. Rabbit anti-caspase 3, which specifically recognizes the p17 subunit of caspase 3, was the gift of Dr. Donald Nicholson (Merck Frosst Center for Therapeutic Research, Quebec, Canada) and used at a dilution of 1:10,000 for Western blots. Donkey anti-rabbit horseradish peroxidase-conjugated secondary antibody (1:10,000 for Western blots) and used at varying concentrations ranging from 12.5 to 100 μM as indicated. ^51^Chromium and [3H]thymidine were obtained from NEN Life Science Products.

**Chromium Release and DNA Fragmentation Assays**—Cytolytic activity was assessed by measuring ^51^chromium release, and DNA fragmentation was assessed by measuring ^3H^thymidine release as described previously (29). Briefly, target cells were labeled with ^51^Cr for 60 min, or target cell DNA was labeled by overnight incubation with 1.5 μCi/ml [3H]thymidine in RHF. After labeling, cells were washed twice in label-free RHFM and incubated in fresh media for at least 1 h, after which they were washed two more times and counted. When necessary the target cells were pretreated with the peptide inhibitors zVAD-fmk and zDEVD-fmk at double the concentrations indicated to block any caspase 3 activity.

**Anti-Caspase 3 Western blots**—zVAD-fmk and zDEVD-fmk on DNA fragmentation resulting from granzyme B or Fas. In our experiments the Fas pathway was engaged by treating cells with anti-Fas IgM antibody. The granzyme B pathway was specifically assessed using granule-dependent allo-specific CTLs generated from granzyme A knockout mice (31) with allo-specific CTLs from granzyme B knockouts (32, 33) used as controls. The use of the two different knockout mice allowed us to be confident that the effects we observed were indeed due to granzyme B. As shown in Fig. 1, both zVAD-fmk and zDEVD-fmk were extremely potent inhibitors of DNA fragmentation induced by the granule-exocytosis pathway and by Fas ligation, confirming the importance of caspases in both these lytic mechanisms. Fas-based DNA fragmentation appeared to be more sensitive to both zVAD-fmk and zDEVD-fmk inhibition, with essentially total inhibition occurring at an inhibitor concentration of 12.5–25 μM, DNA fragmentation induced by allo-specific granzyme A^−/−^ CTL was less sensitive to both inhibitors and required 100 μM zDEVD-fmk for total inhibition. Granzyme B-deficient CTL caused no detectable DNA fragmentation, confirming that the DNA fragmentation seen with wild type and granzyme A knockout CTL was due to granzyme B activity and once more underscoring the importance of this enzyme in causing the rapid apoptotic nuclear events mediated by degranulation-dependent cellular cytotoxicity (6–8, 22, 32, 33).

**RESULTS AND DISCUSSION**

Initially, we assessed the effects of the caspase inhibitors zVAD-fmk and zDEVD-fmk on DNA fragmentation resulting from granzyme B or Fas. In our experiments the Fas pathway was engaged by treating cells with anti-Fas IgM antibody. The granzyme B pathway was specifically assessed using granule-dependent allo-specific CTLs generated from granzyme A knockout mice (31) with allo-specific CTLs from granzyme B knockouts (32, 33) used as controls. The use of the two different knockout mice allowed us to be confident that the effects we observed were indeed due to granzyme B. As shown in Fig. 1, both zVAD-fmk and zDEVD-fmk were extremely potent inhibitors of DNA fragmentation induced by the granule-exocytosis pathway and by Fas ligation, confirming the importance of caspases in both these lytic mechanisms. Fas-based DNA fragmentation appeared to be more sensitive to both zVAD-fmk and zDEVD-fmk inhibition, with essentially total inhibition occurring at an inhibitor concentration of 12.5–25 μM, DNA fragmentation induced by allo-specific granzyme A^−/−^ CTL was less sensitive to both inhibitors and required 100 μM zDEVD-fmk for total inhibition. Granzyme B-deficient CTL caused no detectable DNA fragmentation, confirming that the DNA fragmentation seen with wild type and granzyme A knockout CTL was due to granzyme B activity and once more underscoring the importance of this enzyme in causing the rapid apoptotic nuclear events mediated by degranulation-dependent cellular cytotoxicity (6–8, 22, 33, 34). Allo-specific CTL generated from wild type mice gave results that were essentially identical to that of the granzyme A^−/−^ CTL (data not shown).

Thus, both the Fas- and granzyme B-mediated lytic pathways involve caspase activation and can be inhibited by zVAD-fmk and zDEVD-fmk.

**Processing of caspase 3 into its fully active form is a two-step process, with initial activation occurring via caspase or granzyme B cleavage at aspartate 175, located between the large and small subunit domains (14, 34). This results in the creation of a 12-kDa (p12) small subunit and a 20-kDa (p20) fragment that is composed of the large subunit and the prodomain. Autocatalytic removal of the N-terminal prodomain at aspartate 9 then generates a 19-kDa (p19) large subunit (Fig. 2).**

**Since caspase 3 is a common target for both Fas and granzyme B-initiated apoptosis, and since it is clearly a key regulatory caspase for DNA fragmentation, we were interested in defining the effects of the zVAD-fmk and zDEVD-fmk inhibitors on the processing and molecular activation of this enzyme. As seen in Fig. 3, when Fas-sensitive Jurkat cells were treated with the general caspase inhibitor zVAD-fmk and then exposed to anti-Fas antibody, there was no detectable cleavage of caspase 3, even at the relatively low inhibitor concentration of 25 μM. Under these conditions, cleavage of poly(ADP-ribose) polymerase was not detected (data not shown). This indicated that a zVAD-fmk-sensitive caspase was required for the initial caspase 3 activation upon Fas ligation. In contrast, when the cells were first treated with the zDEVD-fmk, caspase 3 was cleaved to its intermediate subunit (25–26) and spontaneous release was determined by incubation of targets in the absence of CTL or anti-human Fas. All experiments were done in triplicate.**

**Direct Activation of Caspase 3 by Granzyme B**

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zDEVD-fmk-insensitive protease, but the processing was then arrested, and the enzyme would not autoprocess itself to the mature \(19\)-kDa large subunit (the antibody used does not recognize the \(12\)-kDa small subunit). This pattern was seen at both low (25 \(\mu\)M) and high (100 \(\mu\)M) concentrations of the inhibitor. A moderate inhibition on the generation of the \(20\)-kDa band was observed at 25 \(\mu\)M zDEVD-fmk, and a more pronounced inhibition was observed at 100 \(\mu\)M. This observation may reflect, at least in part, the ability of caspase 8, the most proximal caspase in the Fas system, to be inhibited by zDEVD-fmk (23). Thus, Fas ligation results in the activation of caspsases that lead to caspase 3 processing, which are exquisitely sensitive to zVAD-fmk and inhibited to some degree by zDEVD-fmk. We conclude that the inhibition of DNA fragmentation seen in Fig. 1 was due to complete abrogation of caspase 3 processing with zVAD-fmk and a combination of inhibition of both the activity and processing of caspase 3 by zDEVD-fmk.

We next evaluated the effects of these same inhibitors on caspase 3 activation by the granzyme B-mediated lytic pathway. These experiments were conducted at a low effector to target ratio (2:1) to more closely duplicate CTL attack in vivo. The results are depicted in Fig. 4. In direct contrast to the effects of zVAD-fmk on Fas-initiated caspase 3 activation, the \(20\)-kDa fragment was generated in the presence of both low and high concentrations of zVAD-fmk, indicating that this inhibitor did not block the initial processing of caspase 3 in the granzyme B-initiated pathway as it did in the Fas system. There did, however, appear to be a slight inhibition of the production of the \(20\)-kDa band, which we estimate by densitometric scanning of the combined \(20\)- and \(19\)-kDa bands to be approximately 30% in the 25 \(\mu\)M zVAD-fmk sample (Fig. 4 and data not shown). When the target cells were treated with 25 \(\mu\)M zDEVD-fmk inhibitor, generation of the \(20\)-kDa caspase 3 fragment was entirely unaffected, but the caspase 3 autoprocessing step that generates the \(19\)-kDa fragment was inhibited. At 100 \(\mu\)M zDEVD-fmk, there was evidence of some inhibition of the initial processing of caspase 3 to generate the \(20\)-kDa fragment, and the autoprocessing step that forms the \(19\)-kDa subunit was entirely blocked. Earlier experiments using wild type CTL gave identical results to those with the granzyme A\(^{+/+}\) CTL (data not shown). Therefore, the initial activation of caspase 3 is performed by a protease that is...
largely resistant to both zVAD-fmk and zDEVD-fmk. The most likely candidate is granzyme B itself.

It was interesting to note that the degree of inhibition of the production of the ~19-kDa mature subunit by the two concentrations of both zVAD-fmk and zDEVD-fmk reflects the degree of inhibition these peptides displayed in the DNA fragmentation experiments. That is, there was significant but incomplete inhibition at 25 μM and complete inhibition at 100 μM (see Figs. 1 and 4). This further illustrates the direct association between caspase 3 activity and DNA fragmentation. It should be noted that the clone of L1210 used was selected as the target for the granule effectors because of its low level of Fas expression (30).

The results presented in Fig. 4 clearly demonstrate that caspase 3 can be processed to the p20 subunit by a zVAD-fmk-insensitive protease. However, to establish that this first processing event is indeed due to the direct action of granzyme B, we analyzed the timing of the activation of caspase 3 in Jurkat cells in the presence of hCTL, which kills predominantly via the calcium-dependent granule-mediated pathway. The experiment was performed in both the presence and absence of the zVAD-fmk (Fig. 5, A and B). The earliest point at which the cleavage of caspase 3 was observed in either cells, treated or untreated with the peptide inhibitor, was 15 min. In the absence of zVAD-fmk, we saw the appearance of the p20 and p19, whereas in the presence of the inhibitor only p20 was seen at this early time. As noted earlier (Fig. 4), the appearance of p19 occurred only in the absence of inhibitor. We conclude that there is no difference in the kinetics of caspase 3 activation in the presence or absence of zVAD-fmk. Our results differ slightly from those of Talanian et al. (36), who reported some retardation in caspase 3 activation in the presence of caspase inhibitors. However, it should be noted that these investigators were using an in vitro system to induce lysis, whereas we have performed our experiments with both human and mouse CTL.

Thus with both human and mouse CTL, the initial cleavage of caspase 3 appears to be insensitive to the caspase inhibitor zVAD-fmk. Indeed a recently reported study demonstrates that caspase 3 cleavage to the p20 subunit is insensitive to zVAD-fmk as well as zDEVD-fmk and benzoyloxycarbonyl-Ile-Glu-Thr-Asp fluoromethyl ketone (zIETD-fmk), results that are entirely compatible with our own (35). In fact in the presence of zVAD-fmk, zDEVD-fmk, or zIETD-fmk, the level of the cleaved p20 subunit was found to increase, an observation that was also made in this study (Fig. 5), yet surprisingly the authors conclude that activation of caspase 3 is granzyme B-independent.

To test whether or not activation of caspase 3 is relevant to the granzyme B pathway, target cells were transfected with the rabbitpox virus serpin SPI-2, the rabbitpox virus equivalent to cowpox virus crmA, which is a potent inhibitor of caspase 1 and caspase 8 (3, 37, 38, 40, 41). Two independent, stably transfected clones were analyzed for sensitivity to Fas- and granule-induced killing. Both SPI-2-transfected clones (Fig. 6, A and B) demonstrated a significant resistance to DNA fragmentation (~75% inhibition) and membrane damage (~50% inhibition) when treated with anti-Fas antibody. Our results are in agreement with previous studies, which demonstrate that both crmA and SPI-2 are potent inhibitors of Fas-mediated cell death (25–27, 42). In contrast, the same cells demonstrated no protection from granule-mediated cell death induced by the presence of hCTL (Fig. 6, C and D). The involvement of the granzyme pathway in hCTL-induced killing was confirmed by sensitivity to calcium chelation. Thus, although SPI-2 is an effective inhibitor of the Fas pathway, it does not appear to impinge upon granzyme-mediated killing. This result is consistent with published data indicating that the SPI-2-related cowpox virus crmA gene product is a potent Fas inhibitor but has no effect on granule-mediated cell death (42). Previous studies have indicated that caspase 8 is inhibited by the SPI-2-related protein crmA both in vivo and in vitro (3, 40, 41). Thus, our experiments suggest that the activation of caspase 8 is not necessary for granule-mediated cell death since the presence of SPI-2 is unable to provide adequate protection. These results rule out the involvement of caspases 1 and 8, which previously have been shown to be involved in Fas killing and are inhibited by crmA (3, 37, 38, 40, 41). The data presented here also likely excludes involvement of caspase 9, which is inhibited by zVAD-fmk (12). Medema et al. (35) have recently reported the cleavage of caspase 8 during cell death mediated by the perforin and granzyme B pathway. Although caspase 8...
may be cleaved, we do not believe it plays a causative role in granzyme B-mediated death since in our experiments expression of SPI-2 or, as previously documented, expression of crmA (42), is unable to inhibit granzyme-mediated cell death. Previous studies from our laboratory have also indicated that the deletion of SPI-2 from the genome of rabbitpox virus has no effect on the ability of CTL to induce granule-mediated cell death on mutant rabbitpox virus-infected cells, a result that is consistent with the data presented in this study (25). An inhibition of granule-mediated cell death was only observed when both SPI-1 and SPI-2 were deleted from the genome, indicating that SPI-1 and SPI-2 may act in synergy to inhibit granule-mediated cell death (25).

Taken together, our results indicate that the production of mature, activated caspase 3 occurs by distinct pathways in apoptosis initiated by Fas and granzyme B. In Fas-mediated apoptosis, activation of caspase 3 occurs via an upstream caspase that is exquisitely sensitive to the peptide inhibitor zDEVD-fmk and the presence of the serine proteinase inhibitor zVAD-fmk and the presence of the serine proteinase inhibitor.

Granzyme B-dependent killing mechanism can overcome the inhibition mediated by viral proteins. The inherent plasticity in the granzyme death pathway is therefore vitally important for the survival of the host organism as a whole. The model must also be considered in the design of potential immunosuppressive drugs. It is unlikely that inhibitors of individual caspases or compounds that block activation of specific members by granzyme B would be useful. Rather, molecules that block the action of granzyme B on multiple substrates are much more likely to be effective.

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REFERENCES

1. Atkinson, E. A., and Bleackley, R. C. (1995) Crit. Rev. Immunol. 15, 359–384
2. Kagi, D., Vignaux, F., Ledermann, B., Burkii, K., Depreautere, V., Nagata, S., Hengartner, H., and Golstein, P. (1994) Science 265, 528–530
3. Muzio, M., Chinnaiyan, A. M., Kischkel, F. C., O’Burke, K., Shevchenko, A., Ni, J., Scaffidi, C., Bretz, J. D., Zhang, M., Gentz, R., Mann, M., Krammer, P. H., Peter, M. E., and Dixit, V. M. (1996) Cell 85, 817–827
4. Boldin, M. P., Goncharov, T. M., Golstev, Y. V., and Wallach, D. (1996) Cell 83, 803–815
5. Srinivasula, S. M., Ahmad, M., Fernandes-Alnemri, T., Litwack, G., and Alnemri, E. S. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 14486–14491
6. Shi, L., Kam, C. M., Powers, J. C., Aebersold, R., and Greenberg, A. H. (1992) J. Exp. Med. 176, 1521–1529
7. Shi, L., Kraut, R., P., Aebersold, R., and Greenberg, A. H. (1996) J. Exp. Med. 175, 553–566
8. Heuser, J. W., Wesselschmidt, R. L., Shresta, S., Russell, J. H., and Ley, T. J. (1994) Cell 76, 977–987
9. Murphy, M. E., Moult, J., Bleackley, R. C., Gershfeld, H., Weissman, I. L., and James, M. N. G. (1988) Proteins 4, 199–204
10. Odake, S., Kam, C. M., Narasimhan, L., Poe, M., Blake, J. T., Krahnenbuhl, O., Tschopp, J., and Powers, J. C. (1991) Biochemistry 30, 2217–2227
11. Caputo, A., James, M. N. G., Powers, J. C., Hodg, D., and Bleackley, R. C.
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(1994) Nat. Struct. Biol. 1, 364–367
12. Duan, H., Orth, K., Chinnaiyan, A. M., Poirier, G. G., Froelich, C. J., He, W.-W., and Dixit, V. M. (1996) J. Biol. Chem. 271, 16720–16724
13. Darmon, A. J., Nicholson, D. W., and Bleackley, R. C. (1995) Nature 377, 446–448
14. Martin, S. J., Amarante-Mendes, G. P., Shi, L., Chuang, T. H., Casiano, C. A., O’Brien, G. A., Fitzgerald, P., Tan, E. M., Bokoch, G. M., Greenberg, A. H., and Green, D. R. (1996) EMBO J. 15, 2407–2416
15. Quan, L. T., Tewari, M., O’Rourke, K., Dixit, V., Snipas, S. J., Poirier, G. G., Ray, C., Pickup, D. J., and Salvesen, G. S. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 1972–1976
16. Orth, K., Chinnaiyan, A. M., Garg, M., Froelich, C. J., and Dixit, V. M. (1996) J. Biol. Chem. 271, 16443–16446
17. Chinnaiyan, A. M., Hanna, W. L., Orth, K., Duan, H., Poirier, G. G., Froelich, C. J., and Dixit, V. M. (1996) Curr. Biol. 6, 897–899
18. Gu, Y., Sarnecki, C., Fleming, M. A., Lippke, J. A., Bleackley, R. C., and Su, M. S.-S. (1996) J. Biol. Chem. 271, 10816–10820
19. Schlegel, J., Peters, I., Orrenius, S., Miller, D. K., Thornberry, N. A., Yamin, T.-T., and Nicholson, D. W. (1996) J. Biol. Chem. 271, 1841–1844
20. Hasegawa, J., Kamada, S., Kamiike, W., Shimizu, S., Imazu, T., Matsuda, H., and Tsujimoto, Y. (1996) Cancer Res. 56, 1713–1718
21. Dubrez, L., Savoy, I., Hamman, A., and Solary, E. (1996) EMBO J. 15, 5504–5512
22. Darmon, A. J., Ley, T. J., Nicholson, D. W., and Bleackley, R. C. (1996) J. Biol. Chem. 271, 21709–21712
23. Srinivasula, S. M., Fernandes-Alnemri, T., Zangrilli, J., Robertson, N., Armstrong, R. C., Krebs, J., Srivivasula, S. M., Wang, L., Bullrich, F., Fritz, L. C., Trapani, J. A., Tomaselli, K. J., Litwack, G., and Alnemri, E. S. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 7464–7469
24. Medema, J. P., Toes, R. E. M, Scaffidi, C., Zheng, T. S., Flavell, R. A., Melief, C. J. M., Peter, M. E., Offerings, R., and Krammer, P. H. (1997) Eur. J. Immunol. 27, 3492–3498
25. Talanian, R. V., Yang, X. H., Turbov, J., Seth, P., Ghayur, T., Casiano, C. A., Orth, K., and Froelich, C. J. (1997) J. Exp. Med. 186, 1323–1331
26. Ray, C. A., Black, R. A., Kronheim, S. R., Greenstreet, T. A., Sleath, P. R., Salvesen, G. S., and Pickup, D. J. (1992) Cell 69, 597–604
27. Komiyama, T., Ray, C. A., Pickup, D. J., Howard, A. D., Thornberry, N. A., Peterson, E. P., and Salvesen, G. S. (1997) J. Biol. Chem. 272, 7797–7800
28. Takahashi, A., Hirata, H., Yonehara, S., Imai, Y., Lee, K.-K., Moyer, R. W., Turner, P. C., Mesner, P. W., Okazaki, T., Sawai, H., Kashi, S., Yamamoto, K., Okuma, M., and Sasada, M. (1997) Oncogene 14, 2741–2752
29. Tewari, M., Telford, W. G., Miller, R. A., and Dixit, V. M. (1995) J. Biol. Chem. 270, 22705–22708
30. Tewari, M., and Dixit, V. M. (1996) J. Biol. Chem. 270, 3255–3260