Activation of the CPP32 Apoptotic Protease by Distinct Signaling Pathways with Differential Sensitivity to Bcl-xL*

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In the absence of growth factors, many types of mammalian cells undergo apoptosis. We and others have shown recently that growth factors promote cell survival by activating phosphatidylinositol 3-kinase (PI 3-kinase) in several cell types. In the present study, we have compared downstream elements of the apoptotic pathways induced by PI 3-kinase inhibitors and other stimuli. In U937 cells, both PI 3-kinase inhibitors (wortmannin and LY294002) and etoposide activated the CPP32 apoptotic protease by cleavage to active p17 subunits. In contrast, treatment with tumor necrosis factor α (TNF-α) resulted in the accumulation of a distinct active CPP32 subunit, p20. Furthermore, overexpression of Bcl-xL blocked DNA fragmentation, CPP32 activation and cleavage of poly(ADP-ribose) polymerase in U937 cells treated with both PI 3-kinase inhibitors and etoposide, but not in cells treated with TNF-α. Distinct patterns of CPP32 activation and differential sensitivities to Bcl-xL thus distinguish the cell death pathways activated by PI 3-kinase inhibition and DNA damage from that activated by TNF-α.

Programmed cell death occurs both during normal development and under certain pathological conditions in multicellular organisms (1, 2). In tissue culture cells, apoptosis can be induced by a variety of stimuli, including deprivation of growth factors, accumulation of DNA damage, and treatment with tumor necrosis factor α (TNF-α) as ligand (1, 3, 4). We and others have shown recently that growth factors prevent apoptosis by activating phosphatidylinositol 3-kinase (PI 3-kinase) in several cell types, including PC12 pheochromocytoma cells, Rat-1 fibroblasts, T lymphocytes, and hematopoietic progenitors (5–9). In the present study, we have investigated the effects of inhibition of PI 3-kinase on downstream regulators and effectors of apoptosis in comparison with other apoptotic stimuli.

Some of the downstream elements of the pathways that regulate apoptosis are conserved from nematodes to mammals (10). In Caenorhabditis elegans, the activities of two genes, ced-3 and ced-4, are essential for apoptosis. Ced-3 shows sequence similarity to the mammalian interleukin-1-β converting enzyme (ICE)-related proteases that are thought to trigger the execution phase of apoptosis (11). The ICE family of proteases consists of at least seven recently cloned enzymes (12, 13). When overexpressed in transfected cells, all of these proteases induce apoptosis. However, the physiological role of these multiple members of the ICE family in apoptosis induced by other stimuli is not clear. CPP32/Yama/apopain is the most widely investigated member of the ICE family. Its activity has been detected in both mammalian and chicken cell extracts, based on cleavage of its specific substrate, the poly(ADP-ribose) polymerase (PARP), at the onset of apoptosis (14–16). In addition, both inhibition and depletion of CPP32 abolished the ability of cytosolic fractions of apoptotic cells to induce changes characteristic of apoptosis when incubated with healthy nuclei in vitro (14). These data suggest that CPP32 initiates key events in apoptosis and may be an effector of apoptotic cell death. In contrast, mice in which ICE has been eliminated by homologous recombination develop normally, indicating that ICE is not required for programmed cell death during development (17, 18). Thymocytes of such ICE-deficient mice similarly undergo apoptosis normally in response to dexamethasone and ionizing radiation (17, 18). However, ICE-like thymocytes were resistant to apoptosis induced by anti-Fas antibody (17), indicating the possible involvement of ICE in some forms of apoptosis.

Another nematode gene, ced-9, prevents apoptosis in C. elegans, and lies genetically upstream of ced-3 and ced-4 (10). The Bcl-2 family of proteins is homologous of Ced-9 and regulates apoptosis in mammalian cells (19–22). Thus it can be hypothesized that members of the Bcl-2 family, such as Bcl-2 and Bcl-xL, control cell survival by regulating the activity of ICE family proteases.

In the present study, we have investigated the activation of CPP32 and ICE following inhibition of PI 3-kinase, exposure to TNF-α, and treatment with the DNA damaging agent etoposide in normal as well as Bcl-xL-overexpressing cells. All of these stimuli activate CPP32 in human promyelocytic leukemia cells by inducing cleavage of the procenzyme into active subunits. However, distinct patterns of CPP32 cleavage and differential sensitivity to Bcl-xL distinguish the apoptotic pathways induced by inhibition of PI 3-kinase and DNA damage from that induced by TNF-α.

MATERIALS AND METHODS

Cell Cultures—U937 cells and U937 cells overexpressing Bcl-xL (23, 24) (a generous gift of D. Kufe, Dana-Farber Cancer Institute) were cultured in RPMI 1640 supplemented with 10% fetal bovine serum.

Apoptosis Assays—Wortmannin (Sigma), LY294002 (Biomol Research), etoposide (Sigma), or TNF-α (Collaborative Research) were added directly to serum-containing medium of suspension cultures of 5 × 10⁶ or 10 × 10⁶ U937 cells (1 × 10⁶ cells/ml). An equal amount of solvent (Me2SO) was added to controls. At the end of the treatment period, cells were collected by centrifugation and soluble cytoplasmic DNA was extracted and analyzed by electron microscopy in 18% agarose gels containing ethidium bromide (5). TUNEL assays to detect DNA fragmentation in situ were performed with a kit from Boehringer Mannheim.

Immunoblot Analysis—For detection of CPP32 and ICE, either 10 or 20 μg of cytoplasmic cell lysate was electrophoresed in 12% denaturing or non-denaturing polyacrylamide gels, respectively, and then trans-

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1The abbreviations used are: TNF-α, tumor necrosis factor α; PI 3-kinase, phosphatidylinositol 3-kinase; ICE, interleukin-1-β-converting enzyme; PARP, poly(ADP-ribose) polymerase.
RESULTS

Activation of CPP32 in U937 Cells—Activation of ICE-like proteases requires proteolytic cleavage of the proenzymes into active subunits (12–15). To investigate the in vivo activation of endogenous ICE and CPP32 during apoptosis, we therefore analyzed the appearance of active subunits on immunoblots. To induce apoptosis by inhibition of PI 3-kinase, we treated U937 human promyelocytic leukemia cells with two specific and structurally unrelated inhibitors, wortmannin and LY294002 (25–27). The effects of these PI 3-kinase inhibitors were compared with those resulting from treatment with etoposide, which induces apoptosis as a result of DNA damage, and with TNFα.

PI 3-kinase inhibitors, etoposide, and TNFα all induced DNA fragmentation, with U937 cells being most sensitive to etoposide (Fig. 1A). In agreement with the DNA fragmentation results, U937 cells displayed a significant proportion of apoptotic cells in TUNEL assay, reaching 25% positive after treatment with 30 μM etoposide for 4 h (data not shown).

On Western blots, we detected both the inactive (p45) and active (p20) subunits of ICE in control as well as treated cells (Fig. 1B). There was no detectable increase in the amount of the active p20 subunit in response to treatment with either PI 3-kinase inhibitors, etoposide, or TNFα. Therefore, although ICE is already processed and possibly activated without treatment, it does not appear to be further processed during apoptosis of U937 cells.

In contrast to ICE, active subunits of CPP32 were not observed in control cells, but CPP32 became activated following treatment with PI 3-kinase inhibitors, etoposide, and TNFα (Fig. 1C). Consistent with the DNA fragmentation results, the extent of CPP32 cleavage induced by these agents differed, with cells being the most sensitive to etoposide. Importantly, the pattern of CPP32 cleavage induced by PI 3-kinase inhibitors and etoposide differed from that induced by TNFα. Treatment with PI 3-kinase inhibitors and etoposide resulted in a decrease in the level of CPP32 proenzyme accompanied by formation of the active p17 subunit. In contrast, treatment with TNFα resulted principally in formation of the p20 subunit, which also shows catalytic activity (28), with only a small amount of p17 being formed. Inhibition of PI 3-kinase, accumulation of DNA damage, and TNFα thus all activate CPP32, but the cleavage pattern induced by TNFα is different from that induced by the other apoptotic stimuli.

To further correlate the activation of CPP32 with apoptosis, we investigated the dose response and kinetics of CPP32 activation. Concentrations of LY294002 in the range of 12–50 μM, which is specific for PI 3-kinase inhibition (6, 25), resulted in increasing levels of both DNA fragmentation and CPP32 cleavage (Fig. 2A). Both DNA fragmentation and CPP32 cleavage similarly increased in parallel following treatment with etoposide at concentrations ranging from 2 to 60 μM (Fig. 2B).

In time-course experiments, both DNA fragmentation and the p17 subunit of CPP32 were first apparent following 3 h of treatment with LY294002 (Fig. 3A) and 2 h of treatment with etoposide (Fig. 3B). Both the effect of LY294002 and etoposide reached a peak between 3 and 4 h after administration of the drugs. DNA fragmentation and CPP32 cleavage were thus proportional to each other in both the dose-response and time-course experiments, suggesting a direct relationship between CPP32 activation and apoptosis.

The nuclear enzyme poly(ADP-ribose) polymerase (116 kDa) is a substrate for CPP32, possessing a specific recognition site (DEVD) whose cleavage results in the appearance of a well-defined fragment of 85 kDa (14–16). We therefore assayed PARP cleavage to evaluate the intracellular activity of CPP32 following treatment with apoptosis inducers. Cell extracts were analyzed by immunoblotting with a PARP monoclonal antibody.
that reacts with both the intact protein and the 85-kDa fragment (16). The time course of both LY294002- and etoposide-induced PARP cleavage was identical to that of DNA fragmentation and CPP32 activation (Fig. 3), suggesting that activation of CPP32 is responsible for PARP cleavage and eventually leads to activation of endonucleases producing DNA fragmentation.

**Differential Effect of Bcl-xL on Apoptosis and CPP32 Activation Induced by PI3-Kinase Inhibitors, Etoposide, and TNF α**

Since CPP32 appears to be involved in apoptosis in U937 cells, we sought to determine if activation of CPP32 was inhibited by members of the Bcl-2 family. To this end, we used U937 cells overexpressing Bcl-xL (U937(Bcl-x)) (23, 24), which is the strongest inhibitor of cell death of the Bcl-2 family (29, 30). U937(Bcl-x) cells were completely resistant to apoptosis induced by treatment with PI 3-kinase inhibitors and etoposide (Fig. 4A). In addition, overexpression of Bcl-xL in these cells blocked the formation of active CPP32 subunits (Fig. 4B), and abolished cleavage of PARP (Fig. 4C). It thus appears that Bcl-xL acts upstream of CPP32 activation to block apoptosis induced by either PI 3-kinase inhibition or DNA damage.

In contrast to treatment with PI 3-kinase inhibitors and etoposide, overexpression of Bcl-xL did not appear to inhibit apoptosis induced by TNF α. The extent of DNA fragmentation induced by TNF α in U937(Bcl-x) cells was similar to that induced in the parental U937 cells (Fig. 4A). Likewise, TNF α induced formation of p20 subunits of CPP32 and cleavage of PARP to similar extents in both U937 and U937(Bcl-x) cells (Fig. 4, B and C). Thus it appears that PI 3-kinase inhibitors and etoposide utilize a Bcl-xL-regulated pathway to activate CPP32 and induce apoptosis, while CPP32 activation and induction of apoptosis by TNF α is insensitive to Bcl-xL.

**DISCUSSION**

In the present study, we have investigated the regulation of the downstream elements of apoptosis elicited by PI 3-kinase inhibitors, DNA damage, and TNF α. All three agents induced activation of CPP32, as indicated by the formation of active subunits on Western blots. This was parallel to oligonucleosomal fragmentation of DNA and cleavage of the CPP32-specific cell death substrate PARP. Interestingly, however, the different agents induced two distinct patterns of CPP32 cleavage. PI 3-kinase inhibitors and DNA damage induced the formation of the p17 subunit of CPP32, while TNF α principally induced formation of the p20 subunit with only a small amount of p17. In agreement with these results, during the course of our studies it was reported that activation of Fas/APO-1 induces activation of CPP32 in Jurkat cells (28). Moreover, the precursor was processed first to p20, and then partially converted to p17 (28). Because CPP32 is cleaved at two recognition sites (14), cleavage of the C-terminal site produces two fragments (p20 and p12), and a second cleavage at the N-terminal site then converts p20 to p17. Thus, it appears that the CPP32 activator protease induced by TNF α in U937 cells has lower affinity for the N-terminal cleavage site. The significance, if any, of the difference in CPP32 subunits produced by different inducers is not known yet, since both p20 and p17 have catalytic activity (28). CPP32 thus appears to be part of a central pathway leading to programmed cell death that is activated by a variety of distinct apoptotic stimuli.

In contrast to CPP32, the ICE zymogen was significantly processed in nontreated control cells, but no further processing upon treatment with PI 3-kinase inhibitors, etoposide, or TNF α was observed, arguing against its central role in the regulation of cell death in U937 cells. This is consistent both with knockout mouse data indicating that ICE was not involved in apo-
ptosis evoked by most inducers and ICE−/− mice developed normally (17, 18), as well as with the effect of Fas/APO-1 activation in J urkat cells, where cleavage of interleukin-1β, a substrate for ICE, was not increased (28). However, these results do not exclude the participation of ICE in apoptosis of U937 cells, because presently unknown initiators or activators may modulate the activity of ICE in response to apoptotic stimuli.

Although CPP32 appears to be an important component of the cell death pathway in U937 cells, as well as in several other cell types (14, 15, 28, 31), the existence of multiple ICE family members might create redundancy in apoptotic signaling and CPP32 may be replaced or complemented by other proteases. This hypothesis is supported by the fact that high expression of CPP32 is restricted mostly to lymphoid and myeloid cells and their precursors (32). Also importantly, two enzymes closely related to CPP32 have recently been cloned (33–36). Both can cleave PARP normally (17, 18), as well as with the effect of Fas/APO-1 or anti-FAS in the breast carcinoma cell line MCF7 (39).

One interpretation to account for both the difference in CPP32 cleavage pattern and Bcl-xL sensitivity induced by TNFα compared to PI 3-kinase inhibitors and etoposide is that two different enzymes activate CPP32 during apoptosis. In this case, the protease responsible for TNFα-induced activation would poorly recognize the N-terminal cleavage site of CPP32 and would be resistant to Bcl-xL inhibition. Conversely, the protease activated by other agents would be able to cleave both sites with the same efficiency and would be sensitive to Bcl-xL. Alternatively, it is possible that there is only one CPP32 activator, with different signaling pathways converging on its activation. In such a scenario, the target of Bcl-xL would be upstream of the point where the TNFα pathway converges with the pathways stimulated by other agents, so treatment with TNFα would bypass the Bcl-xL block. However, it would then still be necessary to explain the difference in CPP32 cleavage pattern induced by TNFα compared to the other agents. It thus appears that the first possibility is more likely, although identification of the proteases responsible for CPP32 activation will be required to distinguish between these models.