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Bcl-2 acts upstream of the PARP protease and prevents its activation

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Abstract

Apoptosis has recently been extensively studied and multiple factors have been implicated in its regulation. It remains unclear how these factors are ordered in the cell death pathway. Here we investigate the relationship between the inhibitor of apoptosis, bcl-2, and the PARP protease, prICE/CPP32, recently implicated in apoptosis. Using PARP proteolysis as an indicator of the activation of the PARP protease, we find that the chemotherapeutic agent, etoposide, induces apoptosis and PARP proteolysis in Molt4 cells as early as 4 h with cell death lagging behind this event. In contrast, Molt4 cells that over-express bcl-2 show no PARP proteolysis or cell death. In order to determine if bcl-2 inhibits the PARP protease or its activation, we developed a cell-free system. Using this system with extracts from etoposide-treated cells and purified bovine PARP, we demonstrate that extracts from bcl-2 over-expressing cells cause little or no PARP proteolysis. Whereas, extracts from control vector cells contain an active PARP protease. This protease is inhibited by the tetrapeptide ICE-like protease inhibitor, YVAD-chloromethylketone. Interestingly, this protease is not inhibited by the addition of purified bcl-2 protein. These results rule out that bcl-2 directly inhibits the active protease or that it has an effect downstream of prICE/CPP32 such as preventing access to the PARP substrate. These results also demonstrate a role of bcl-2 in interfering with an upstream signal required to activate the PARP protease and allow us to begin to order the components in the apoptotic pathway.

Keywords: apoptosis, Bcl-2, PARP, proteases

Abbreviations: ICE, interleukin-1β converting enzyme; prICE, protease resembling ICE; PARP, poly(ADP ribose) polymerase

Introduction

Apoptosis is a term given by Kerr *et al* (1972) to represent a process resulting in characteristic morphological changes in the cell and culminating in cell death. It is now known to be a regulated process that plays an important role in development and many physiological and pathological conditions.

Genetic studies in *C. elegans* have identified three genes which regulate apoptosis. Two of them, ced-3 and ced-4 are positive regulators of cell death, whereas the third, ced-9, is a negative regulator of apoptosis (Hengartner and Horvitz, 1994b).

The cloning and sequencing of ced-3 revealed that it had considerable identity (28%) to interleukin-1β converting enzyme (ICE) (Yuan *et al*, 1993). When ICE is over-expressed in mammalian cells, it is sufficient to cause the cells to undergo apoptosis (Miura *et al*, 1993); however, thymocytes or macrophages from mice which are homozygous knockouts in the ICE gene undergo apoptosis in a normal manner (Li *et al*, 1995), suggesting that ICE activity is not necessary for this event.

Recently, a host of other cysteine proteases of the ICE class have been cloned, and these include CPP32/yama/apopain (Fernandes-Alnemri *et al*, 1994; Tewari *et al*, 1995; Nicholson *et al*, 1995), ICE-LAP3/Mch3 (Duan *et al*, 1996; Fernandes-Alnemri *et al*, 1995b), TX/ICH-2/ICE_{rel}II (Faucheu *et al*, 1995; Kamens *et al*, 1995; Munday *et al*,1995), Nedd2/ICH-1 (Kumar *et al*, 1994; Wang *et al*, 1994), ICE_{rel}III (Munday *et al*, 1995) and Mch2 (Fernandes-Alnemri *et al*, 1995a). Of these, the strongest candidate for a downstream effector of the apoptotic machinery appears to be CPP32 in that it is activated by a wide variety of agents that induce cell death including TNF-α (Tewari *et al*, 1995) and Fas (Tewari *et al*, 1995). CPP32 also has a higher sequence identity to ced-3 than does ICE (35% vs. 28%) (Fernandes-Alnemri *et al*, 1994). Moreover, the removal of CPP32 from apoptotic extracts inhibits the ability of the extract to induce morphological changes in healthy nuclei. These changes could be restored by the addition of purified CPP32 to the system (Nicholson *et al*, 1995). Although CPP32 is a necessary component of the apoptotic machinery in this system, it alone was not sufficient to induce the morphological changes in the nuclei (Nicholson *et al*, 1995).

The only identified substrate for CPP32 at this point is poly(ADP ribose) polymerase (PARP), a nuclear enzyme involved in DNA repair. Proteolysis of PARP from a 116 kDa protein to a characteristic 85 kDa fragment was first reported as an early event in etoposide-induced apoptosis in HL-60 cells (Kaufmann, 1989). Thereafter, the protease responsible for this event was termed prICE (protease resembling ICE) based upon pharmacologic and biochemical data (Lazebnik *et al*, 1994). While this protease

has not been cloned, it appears to be the chicken homologue of the recently cloned mammalian protease, CPP32. The proteolysis of PARP is now a well recognized hallmark of apoptosis.

The third gene identified as a regulator of apoptosis from the *C. elegans* studies is ced-9. Ced-9, a negative regulator of apoptosis, is homologous to the mammalian gene bcl-2 (Hengartner and Horvitz, 1994a). Bcl-2 was initially discovered as an over-expressed protein in human lymphomas (as a result of a translocation) (Pegoraro *et al*, 1984), and it was later found to function in preventing cell death that would normally occur in response to a wide variety of agents, including TNF-α (Hennet *et al*, 1993), Fas (Itoh *et al*, 1993), and ceramide (Martin *et al*, 1995b; Zhang *et al*, 1996). Currently, the mechanisms underlying the protective effect of bcl-2 on apoptosis are poorly understood, especially in its relationship to proteases.

Herein, we have investigated the protective mechanism of bcl-2 using PARP proteolysis as an indicator of activation of prICE/CPP32 and of apoptosis. We find that in cells induced to undergo apoptosis with etoposide, bcl-2 prevents PARP proteolysis. Using a cell-free system for PARP proteolysis, we provide evidence that bcl-2 protects from cell death by acting upstream of the activation of prICE/CPP32. These results begin to order the cell death pathway.

Results

Effect of bcl-2 on PARP proteolysis and cell death

In order to understand the relationship of bcl-2 to the PARP protease in the cell death pathway, we initially investigated the effect of bcl-2 on PARP proteolysis induced by etoposide. Etoposide is a chemotherapeutic agent known to induce PARP proteolysis and to activate the PARP protease (Kaufmann, 1989). Molt4 control vector cells or Molt4 cells transfected with the mouse bcl-2 gene were treated with etoposide for 0–8 h. The data in Figure 1 (upper panel) demonstrate that etoposide induced PARP proteolysis between 2 and 4 h of treatment and caused complete proteolysis of PARP by 8 h. However, in the cells that had been transfected with bcl-2, no proteolysis of PARP was observed through 8 h. These results demonstrate that bcl-2 interferes with PARP proteolysis.

In order to define the relationship of PARP proteolysis to cell death in this system, cells were harvested at each time point and analyzed for viability by trypan blue exlusion. The data in Figure 1 (lower panel) indicate that little or no cell death occurred in the cells over-expressing bcl-2 while nearly 25% of the cells were dead in the control vector cell line after 8 h of treatment. It is interesting to note that the PARP substrate was completely proteolyzed after 8 h in the vector control cells while only 25% of the cell population had died. If the cells were incubated with etoposide for longer periods, there was a progressive increase in cell death until 95% of the cells were trypan blue positive after 24 h (data not shown). These results are consistent with prior observations showing that PARP proteolysis is an early event in apoptosis which precedes DNA fragmentation and complete cell death.

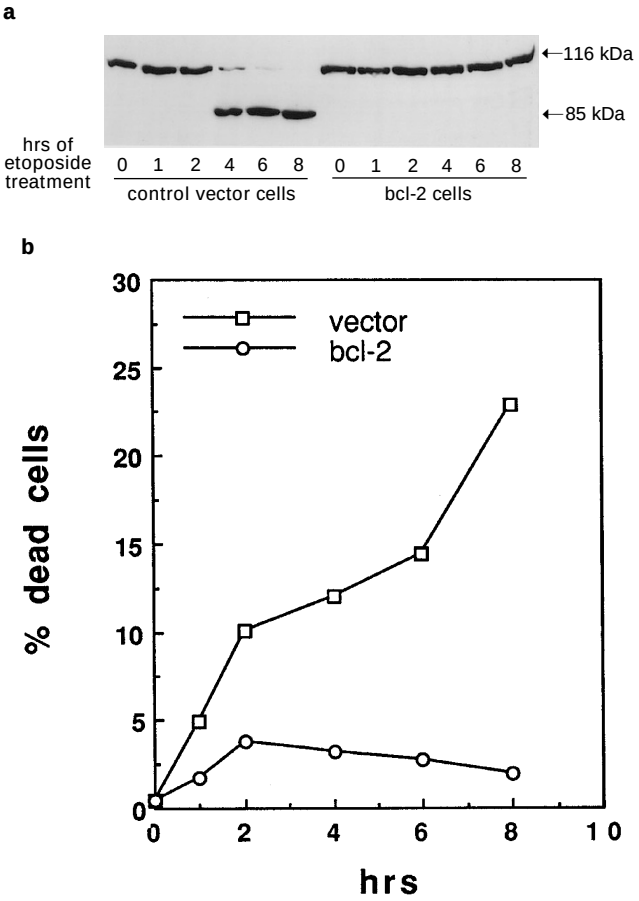

Figure 1 Effect of bcl-2 on etoposide-induced PARP proteolysis and cell death. Control vector or bcl-2 transfected Molt4 cells were treated at 5×10^5 cells/ml with 20 μM etoposide. At the indicated times the cells were harvested and either analyzed by western blot for PARP proteolysis (upper panel) or for viability by trypan blue exclusion (lower panel).

Characterization of a cell-free system for PARP cleavage

The above results raised the question of whether bcl-2 prevented PARP proteolysis by interfering with the activation of prICE/CPP32 (upstream) or with the action of prICE/CPP32 and/or availability of substrate (downstream). To delineate these relationships, we developed a cell-free system in which a cytosolic (100 000 $\times$ g) extract from cells treated with etoposide for 6 h was used as a source of the PARP protease. To this extract we added purified bovine PARP as the substrate. Figure 2 (upper panel) shows the kinetics of PARP proteolysis in the cell-free system. Proteolysis was detected as early as 5 min of incubation, and after 2 h of incubation the substrate was totally proteolyzed to the 85 kDa fragment. The extent of PARP proteolysis was dependent on the amount of cytosolic extract (i.e. active protease) that was added such that 1 μg of cytosol induced partial PARP cleavage and up to 60 μg of cytosolic extract induced nearly complete proteolysis (Figure 2, lower panel). Thus, the cell-free system recapitulates activation of the PARP protease in cells.

Using this cell-free system, we next evaluated the ability of bcl-2 to inhibit the generation of active protease. Control

vector or bcl-2 transfected Molt4 cells were treated with etoposide for 0–6 h. The cytosolic extract from these cells was then added to purified bovine PARP to investigate its proteolysis, and the cell-free incubation was carried out for 1 h. It was observed that the extracts isolated from etoposide-treated control vector cells induced significant PARP proteolysis after exposure of cells to etoposide for as early as 1–2 h. Complete proteolysis resulted using extracts from cells that had been stimulated with etoposide for 3 h (Figure 3, upper panel). When extracts from etoposide-treated cells over-expressing bcl-2 were evaluated, little or no proteolysis of PARP was observed (Figure 3, upper panel).

These results support two conclusions. First, they show that there is a lag period (1–3 h) between the activation of the protease in the intact cell and the point at which it gains access to the PARP substrate (compare Figures 1 and 3). As PARP is a nuclear protein and the PARP protease is a cytosolic enzyme, it is likely that the lag period represents the time necessary for the protease to enter the nucleus. Second, these data also suggest that bcl-2 either prevents activation of prICE/CPP32 or directly inhibits the protease once activated.

To investigate these possibilities further, we examined whether purified bcl-2 protein could inhibit the 'activated' protease in the cell-free system. The cytosolic extract from etoposide-treated control vector cells was incubated with baculovirus-derived bcl-2 (at concentrations up to 0.3 μM) prior to the addition of the PARP substrate. *In vitro*, this purified bcl-2 has been shown to exert anti-apoptotic activity at picomolar concentrations when used in similar cell-free systems where nuclear degeneration was used as an endpoint for assessing function (Martin *et al*, 1995a). Under these conditions we found no inhibition by bcl-2 of PARP proteolysis (Figure 3, lower panel). Identical extracts incubated with the tetrapeptide inhibitor of ICE-like proteases, YVAD-chloromethylketone, however, resulted in nearly complete inhibition of proteolysis (Figure 3, lower panel). These results demonstrate that the protease activity in these extracts is that of an ICE-like protease and that this protease, once activated, is not inhibited directly by bcl-2. These studies rule out a downstream effect of bcl-2 on prICE/CPP32 (such as preventing access of the protease to its substrate, or modification of PARP to become resistance to proteolysis), at least in terms of exogenously added PARP.

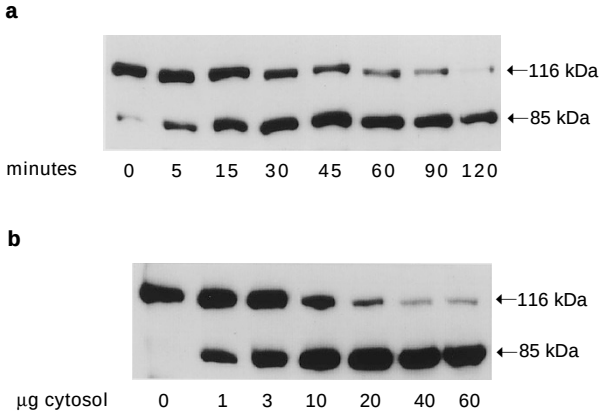

Figure 2 Kinetics and enzyme concentration dependence of PARP proteolysis in a cell-free system. In the upper panel, 25 ng of bovine PARP was added to 10 μg of cytosol from etoposide-treated cells and incubated for the indicated times at 37°C. Alternatively, 25 ng of bovine PARP was added to varying amounts of the cytosol for 1 h incubation period (lower panel). In both cases PARP proteolysis was detected by western blot analysis.

Discussion

In this study we have investigated the relationship of bcl-2 to prICE/CPP32 in the cell death pathway. A number of earlier studies had demonstrated an important role for the PARP protease in cell death and it had been hypothesized that bcl-2 acts upstream of the protease (Peter *et al*, 1996), but the role of bcl-2 in influencing this event had not been determined.

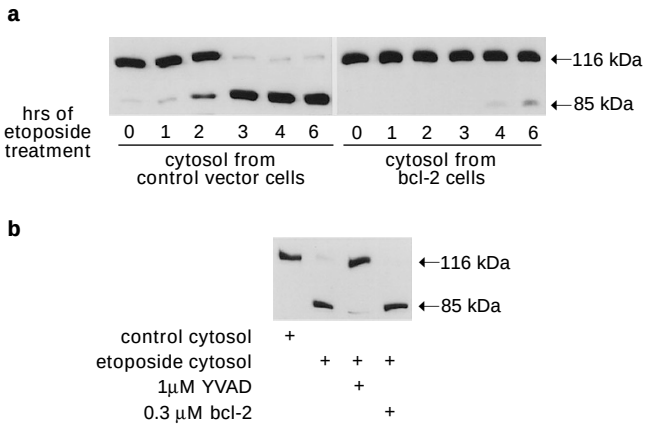

Figure 3 Evaluation of PARP proteolysis in cytosols from etoposide treated control vector and bcl-2 over-expressing cells. Bovine PARP (25 ng) was added to 10 μg of cytosol from control vector or bcl-2-transfected cells that had been treated with etoposide for the indicated period of time (upper panel). Reactions were allowed to proceed for 1 h at 37°C. In the lower panel, 10 μg of cytosol from vehicle control or etoposide-treated Molt4 cells was pre-incubated for 5 min at 37°C with no addition or with either YVAD-chloromethylketone or baculovirus-derived bcl-2 protein. Thereafter, 25 ng of bovine PARP was added for 1 h at 37°C. In both panels PARP proteolysis was analyzed by western blot.

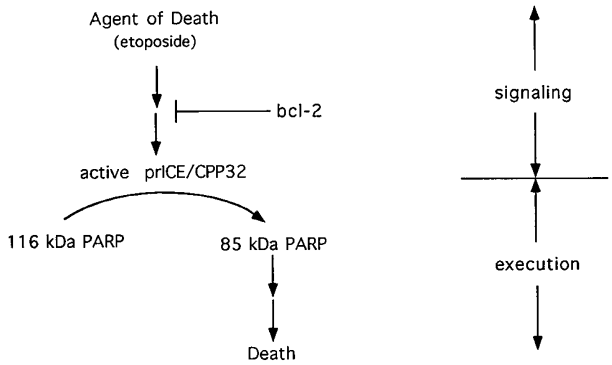

Figure 4 Scheme for ordering of the apoptotic machinery. As discussed in the text, bcl-2 acts upstream of the PARP protease and prevents its activation during the signaling stage of the cell death pathway. Once prICE/CPP32 is activated, the execution stage of cell death is initiated beginning with the proteolysis of PARP.

Genetic studies in *C. elegans* had indicated a functional interaction between ced-3 (prICE/CPP32) and ced-9 (bcl-2), although the exact mechanism of this interaction was still unknown. The ordering of this interaction has important implications for the understanding of the 'death' pathway and for pursuing the molecular mechanism of the action of bcl-2.

Our results demonstrate that bcl-2 inhibits PARP proteolysis by preventing activation of the PARP protease. This conclusion is supported by the following: (1) bcl-2 protects cells from etoposide-induced PARP proteolysis; (2) extracts from etoposide-treated cells overexpressing bcl-2 do not contain an active PARP protease; and (3) the addition of bcl-2 protein to an active protease extract did not inhibit its activity. These results demonstrate that bcl-2 does not prevent the protease from gaining access to its substrate in a cell-free system. The results also rule out the possibility that bcl-2 interferes with the preparation of substrate for proteolysis (for example, proteolysis of I-κB in response to TNF-α is a consequence of phosphorylation of the substrate I-κB and not activation of the proteases (Brown *et al*, 1995).

At least two reasons may exist as to why bcl-2 does not inhibit PARP proteolysis in our cell-free system but yet inhibits nuclear destruction in other cell-free systems (Martin *et al*, 1995a). The first is that bcl-2 could function to prevent the entry of the PARP protease into the nucleus. The second is that bcl-2 has previously been reported to inhibit a 'latent phase' of apoptosis which occurs in preparation for later destructive events (Newmeyer *et al*, 1994). In this 'latent phase' bcl-2 may inhibit small amounts of protease activity, but once the events have occurred which lead to amplification of protease activity, bcl-2 would no longer inhibit. The respective levels of protease activity may account for the functional differences of bcl-2 in our cell-free system and that of Martin *et al* (1995a).

The events which lead to the activation of prICE/CPP32 are unclear. In general, the class of ICE-like proteases are synthesized as pro-enzymes. For CPP32, activation occurs upon processing of the pro-enzyme to two subunits of 17 kDa and 12 kDa which constitute the heterodimeric, active enzyme. It is possible that CPP32 is activated by autocatalysis (similar to ICE) or alternatively, by a protease cascade. We are currently investigating these possibilities. In either event, our results predict that bcl-2 does not function downstream of the PARP protease as an antagonist of the execution of cell death. Instead, bcl-2 acts to antagonize the machinery that senses cellular injury and interferes with an upstream signal required to activate the PARP protease (see scheme in Figure 4). These results help to order the 'cell death' pathway and provide a biochemical landmark (PARP proteolysis) for investigating the molecular mechanism of action of bcl-2.

Materials and Methods

Cell culture

Molt4 cells were maintained in RPMI medium with 10% fetal calf serum. Molt4 cells that had been transfected with the mouse bcl-2 gene (the transfection procedure has been previously described) (Zhang *et al*, 1996) were maintained in the same medium with 420 μg/ml hygromycin. For treatments, cells of sub-confluent cultures were harvested and suspended at 5×10^5 cells/ml in RPMI with 2% fetal calf serum. After treatments, cells were harvested by centrifugation and solubilized in SDS sample buffer.

Cell fractionation

After treatment of the respective cell line with etoposide or vehicle, the cells were pelleted by centrifugation, rinsed once with phosphate-buffered saline, and suspended in 250 μl of cell-free buffer (10 mM HEPES, pH 7.4, 100 mM KCl, 3 mM NACl, 4 mM $MgCl_2$, 0.2 μM $CaCl_2$, 1 mM DTT). Cells were then lysed by N_2 cavitation for 10 min at 450 psi. The lysate was centrifuged at $100{,}000 \times g$ and the supernatant retained. After protein determination, aliquots were frozen on dry ice and stored at -80°C. PARP protease activity was retained in this fraction for at least 6 months.

Cell-free incubations

Reactions in the cell-free system were initiated by the addition of 25 ng of purified bovine PARP to 10 μg of the $100{,}000 \times g$ supernatant in a total volume of 15 μl of cell-free buffer. After 1 h at 37°C, the reactions were stopped by the addition of SDS sample buffer. For inhibitor studies, YVAD-chloromethylketone or affinity purified bcl-2 protein were pre-incubated with the $100{,}000 \times g$ supernatant for 5 min at 37°C prior to the addition of the PARP substrate.

Western blotting

Samples from the cell-free system or intact cells were boiled, and loaded onto 6% polyacrylamide gels. After transfer to nitrocellulose membrane, the membrane was incubated with a rabbit polyclonal antibody (1 : 2000) to an epitope in the automodification domain of PARP. Detection was accomplished using a goat anti-rabbit secondary antibody (1 : 5000) and the ECL detection system (Amersham).

Acknowledgements

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