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Bcl-2 prevents activation of CPP32 cysteine protease and cleavage of poly (ADP-ribose) polymerase and U1-70 kD proteins in staurosporine-mediated apoptosis

Sandrine Estoppey[1], Ivan Rodriguez[1,2], Rémy Sadoul[1] and Jean-Claude Martinou[1,3]

[1] Glaxo Insititute for Molecular Biology, 14 chemin des Aulx, Plan-Les Ouates, Switzerland
[2] Centre Médical Universitaire, Dept of Pathology, Geneva CH
[3] corresponding author: Jean-Claude Martinou, Glaxo Institute for Molecular Biology, 14, chemin des Aulx, 1228 Plan-Les-Ouates Geneva, CH
tel: 22 706 9822; fax: 22 794 6965 Email: JCM26619 GGR. CO.UK

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Abstract

Members of the the Bcl-2 and ICE/ced-3 gene families have been implicated as essential components in the control of the cell death pathway. Bcl-2 overexpression can prevent programmed cell death (PCD) in different cell types. ICE/ced-3-like proteases are synthesized as pro-enzymes and are activated by limited proteolysis. When overexpressed in diverse cell types, they trigger PCD. Bcl-2 can inhibit PCD mediated by these proteases, although as yet it is not clear at what specific step in the cell death pathway the protein acts. Here, we demonstrate that CPP32/Yama/Apopain, a member of the ICE/Ced-3 gene family, is processed during staurosporine-induced apoptosis in HeLa cells and that concomitant with CPP32 activation, two other proteins, poly (ADP-ribose) polymerase (PARP) and the U1-70 K small ribonucleoprotein, also undergo proteolysis. Overexpression of Bcl-2 prevents cleavage of CPP32, PARP and U1-70 K and protects HeLa cells from PCD. These results demonstrate that Bcl-2 controls PCD, by acting upstream of CPP32/Yama/Apopain.

Keywords: apoptosis, CPP32, proteases, Bcl-2, staurosporine, poly (ADP ribose) polymerase, U1-70K ribonucleoprotein

Abbreviations: PCD, programmed cell death; PARP, poly (ADP-ribose) polymerase; ICE, interleukin 1β converting enzyme; U1-70K, U1 small nuclear ribonucleoprotein

Introduction

Programmed cell death (PCD) is a form of active cell death that occurs in many cell types during development and in pathology. PCD can occur in response to a variety of stimuli and is mediated through multiple pathways which are as yet poorly understood. Genetic studies on the nematode *C. elegans* have identified two genes, ced-3 and ced-4, which are major components of the cell death program (Ellis *et al*, 1991). Ced-9 shows structural homology with proteins of the Bcl-2 family (for a review see Reed, 1994), whereas ced-3 is homologous to IL-1β converting enzyme (ICE) (Kumar and Harvey, 1995; Yuan, 1995). Members of the Bcl-2 family can be subdivided in two subgroups according to whether they display anti-apoptotic or pro-apoptotic function. Interaction between these proteins constitutes a checkpoint (Oltvai and Korsmeyer, 1994) in the cascade of events that lead to PCD although at present the mechanism of action of these proteins is unknown. Cysteine proteases of the ICE family which currently includes CPP32/Yama/Apopain (Fernandes-Alnemri *et al*, 1994; Tewari *et al*, 1995; Nicholson *et al*, 1995), Nedd-2/Ich-1 (Kumar al, 1994; Wang *et al*, 1994), TX/Ich-2/ICEII (Munday *et al*, 1995; Faucheu *et al*, 1995; Kamens *et al*, 1995), ICE-rel-III (Munday *et al*, 1995), Mch2 (Fernandes-Alnemri *et al*, 1995) and ICE-LAP3 (Duan *et al*, 1996). These proteases are synthesized as proenzymes and are activated by cleavage of a N-terminal sequence and removal of an internal peptide thus generating two subunits of approximately 20 and 10 Kd. This was demonstrated for ICE and probably applies to all proteases of this family. The protease(s) responsible for this cleavage is unknown, although activation by autoprocessing is one possibility. Several members of the ICE family have been shown, *in vitro*, to cleave poly(ADP ribose) polymerase (PARP), a nuclear enzyme involved in DNA repair and control of genome integrity (Kaufmann *et al*, 1993, Lazebnik *et al*, 1994). In addition to PARP, ICE-like proteases are likely to have several other substrates including the 70 kD protein component of the U1 small nuclear ribonucleoprotein (U1-70K) (Casciola-Rosen *et al*, 1994), lamins, topoisomerase I (Martin and Green, 1995), and sterol regulatory element-binding protein-1 (Wang *et al*, 1995). Cleavage of these proteins appears to be an early event of PCD as it occurs well before any morphological signs of cell demise. All the proteases of the ICE family have been shown to induce PCD in several cell types and this effect can be blocked by co-expression of Bcl-2 (Kumar *et al*, 1994; Wang *et al*, 1994; Miura *et al*, 1993). Two viral proteins, CrmA and p35 from cowpoxvirus and baculovirus respectively are potent inhibitors of the catalytic activity of ICE-like (Xue and Horvitz, 1995; Bump *et al*, 1995). However, the mechanism by which Bcl-2 inhibits the killing function of these proteases is unknown. Here, we have investigated whether activation of CPP32 occurs during PCD and whether Bcl-2 can interfere with this process. These studies were performed in the well characterized model system of staurosporine-induced apoptosis (Jacobson *et al*, 1993, 1994). We report that, in HeLa cells undergoing staurosporine-triggered apoptosis, CPP32 is

activated by cleavage and that overexpression of Bcl-2 prevents cleavage of CPP32 and protects cells. These results demonstrate that, in this model of PCD, Bcl-2 acts before activation of CPP32.

Results and Discussion

Bcl-2 inhibits programmed cell death induced by staurosporine in Hela cells

Staurosporine has been shown previously to trigger apoptosis in different cell types. This compound was tested on Hela cells expressing either endogenous levels of Bcl-2 (control cells) or stably transfected with an expression vector which drives overexpression of Bcl-2. These latter cells contain about three times more Bcl-2 than control cells based on Western blot analysis (Figure 1). Control and Bcl-2 overexpressing cells were treated with 1 μM staurosporine and cell survival was measured at different times after addition of the compound. Counting of condensed nuclei after Hoechst labeling was used to quantify cell death. We found that approximately 50% of control cells underwent apoptosis within 24 h following addition of staurosporine (Figure 2). In contrast, cells overexpressing Bcl-2 were more resistant to the compound and less than 10% of them died during the same period. These data are in agreement with those previously published by Jacobson *et al* (1993, 1994).

Bcl-2 prevents cleavage of CPP32 induced by staurosporine treatment

Cleavage of CPP32 was analyzed by Western blotting in cell extracts from control and Bcl-2-overexpressing cells (Figure 3A and B). A monoclonal antibody directed against p20 of CPP32 recognized a band of about 30 kD which corresponds to full length CPP32. Following a 15 h exposure to staurosporine, the intensity of this band slightly decreased in control cells with concomitant appearance of a lower molecular weight band of about 20 kD. This band, which probably corresponds to the p17 subunit of CPP32, was first detected 2 h after addition of staurosporine to the cells and its intensity peaked 15 h later (Figure 3A). As expected the antibody used in these experiments did not detect the p12 subunit of CPP32. The cleavage of CPP32 preceded appearance of apoptotic cells (*cf.* Figure 2) suggesting that CPP32 activation might take part in the cell death process, rather than just being a consequence of it. In contrast, in Bcl-2 overexpressing cells, the cleavage of CPP32 did not occur (Figure 3B). The intensity of the band corresponding to p32 remained almost constant for 24 h and no 17 kD band was detected. These results demonstrate that Bcl-2 prevents cleavage of CPP32.

Bcl-2 prevents cleavage of PARP and U1-70 kD in staurosporine-treated HeLa cells

CPP32 has been previously shown, *in vitro*, to cleave PARP into a fragment of 85 kD that is retrieved in apoptotic cell extracts (Tewari *et al*, 1995; Nicholson *et al*, 1995). We therefore analyzed by immunoblotting the cleavage of PARP in control and Bcl-2-overexpressing cells using an antibody that was kindly provided by G. De Murcia. In cell extracts from untreated control and Bcl-2 overexpressing cells, we detected a 116 kD band which corresponds to intact PARP (Figure 3A and B). In addition, this antibody detected a faint band running

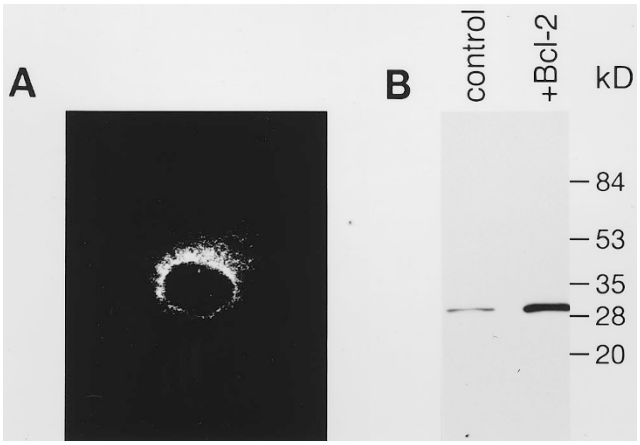

Figure 1 Hela cells overexpressing Bcl-2. (**A**) Immunofluorescence analysis of Bcl-2 in HeLa cells overexpressing Bcl-2. (No staining was detectable in untransfected cells (not shown). (**B**) Immunoblotting analysis of Bcl-2 in control and Bcl-2 overexpressing cells. HeLa cells were stably transfected with a plasmid vector encoding human Bcl-2. Levels of Bcl-2 were quantified by immunoblotting in pools of cells overexpressing Bcl-2 and in HeLa cells transfected with an empty vector (control cells). Note that Bcl-2-overexpressing cells contain about three times more Bcl-2 than control cells.

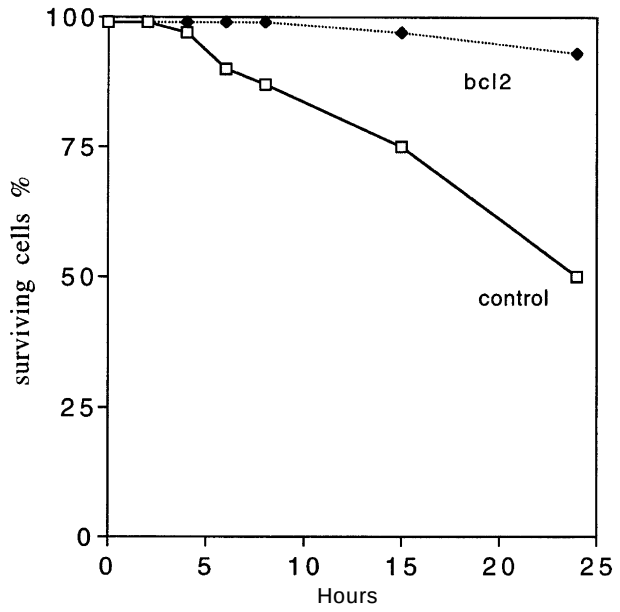

Figure 2 Effects of staurosporine on survival of control and Bcl-2-overexpressing HeLa cells. HeLa cells were plated in Petri dishes and grown in DMEM medium containing 10% FCS. Staurosporine was added at a concentration of 1 μM and cell survival analysed at different times after addition of the compound as described in (in Jacobson *et al*, 1993, 1994). The results represent the number of apoptotic nuclei determined by Hoechst staining and are representative of three different experiments.

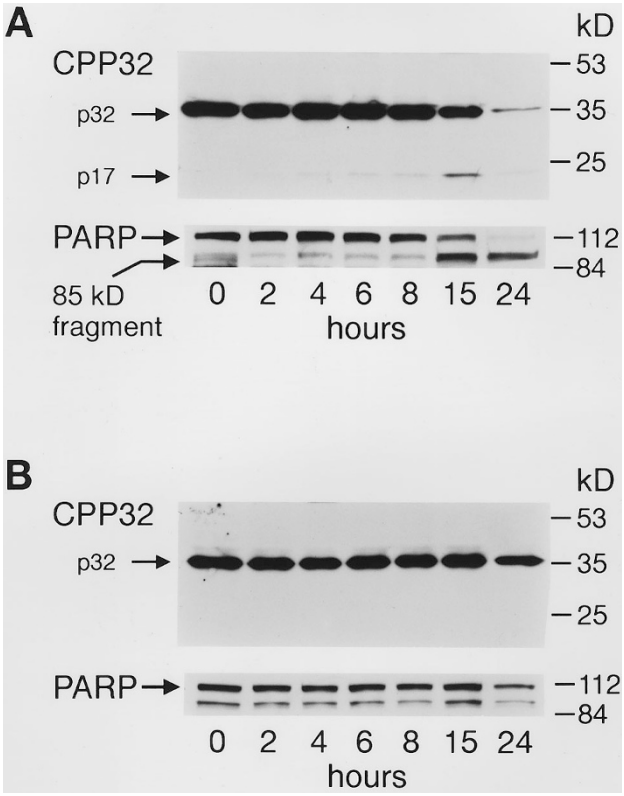

Figure 3 CPP32 and PARP activation in control and Bcl-2 overexpressing HeLa cells. Control (**A**) and Bcl-2 overexpressing HeLa cells (**B**) were exposed for different times to 1 μM staurosporine. Cells were then lysed directly in sample buffer. Lysates from 10^6 cells were electrophoresed on a reducing 4–20% SDS-polyacrylamide gel and proteins were then transferred by electroblotting on to nitrocellulose membranes. CPP32 and PARP were detected with appropriate antibodies.

around 85 kD which may correspond either to the 85 kD fragment of PARP, or to a non specific band. The first possibility would suggest the presence of a constitutive level of PARP proteolysis in cultured HeLa cells. The important result to note in these experiments was the reduction in the level of the 116 kD fragment in normal cells treated with staurosporine for 15 h. This disappearance was almost complete by 24 h and was accompanied by a strong increase in intensity of the 85 kD band (Figure 3A). This demonstrates, in agreement with previous studies using other cell types (Tewari *et al*, 1995; Lazebnik *et al*, 1994), that PARP is converted to the 85 kD fragment during PCD. Cleavage of PARP was inhibited by Bcl-2 overexpression (Figure 3B). Indeed, after a 24 h staurosporine exposure, levels of PARP contained in Bcl-2-overexpressing cells were similar to those of untreated cells. Also in these cells, the 85 kD band detected in untreated cells did not increase when cells were exposed to the compound.

In addition to PARP, we examined the status of the U1-70 kD small ribonucleoprotein by immunoblotting and immunocytochemistry (Figure 4A–E). U1-70K has been reported to be cleaved, probably by an ICE-like protease, into a fragment of 40-kD during apoptosis (Casciola-Rosen *et al*, 1994; Tewari *et al*, 1995). Immunoblotting of HeLa cell extracts with a mouse monoclonal antibody to U1-70K revealed a band migrating at around 70 kD (Figure 4A). A significant decrease in the intensity of this band was found after 15 h of treatment with staurosporine. In Bcl-2 overexpressing cells, this decrease in U1-70 kD levels did not occur. Immunocytochemistry studies revealed that all cells displayed a speckled-labeling pattern which is in agreement with previous reports (Spector *et al*, 1992) (Figure 4B). In almost all control cells cultured in the presence of staurosporine for 8 h, the staining had disappeared (Figure 4D). However, a small percentage of cells displayed a strong nuclear staining with a pattern totally different from that observed in cells cultured in normal conditions (Figure 4C). It was composed of two or three intensely labeled large dots. A Hoechst staining revealed that the morphology of these nuclei as well as that of cells lacking U1-70K was less homogenous than normal, but as yet was not condensed (Figure 4E). Hoechst staining also showed that the large dots did not contain DNA. In Bcl-2-overexpressing cells the pattern staining of U1-70K did not change after staurosporine treatment (not shown).

Conclusion

To date many members of the ICE gene family have been identified but, as yet, very little is known about their role in PCD. Here we have shown that CPP32 is processed during PCD in HeLa cells undergoing staurosporine-triggered apoptosis. We have evidence that CPP32 is also activated in FAS-mediated cell death (Chinnaiyan *et al*, 1996; our unpublished data). This suggests that CPP32 could be an effector common to many PCDs. Recently, a new protease of the ICE gene family, named ICE-LAP3 has been identified and found to be activated during FAS and TNF-mediated PCD (Duan *et al*, 1996). This protease is highly homologous to CPP32 and it is not clear yet whether it acts in the same or in a different pathway. ICE (Hogquist *et al*, 1991), ICE-LAP3 and CPP32 are, today, the only proteases known to be activated during PCD. Activation of CPP32 in staurosporine-induced apoptosis, like that of ICE-LAP3 for cytokine-mediated cell death, precedes the characteristic changes in cellular morphology, such as nuclear condensation. Interestingly, in our experiments, we found that proteolysis of PARP and U1-70K proteins was concomitant with CPP32 activation. This suggests, but does not prove, that CPP32 is responsible, directly or indirectly, for cleavage of these proteins. Concerning proteolysis of U1-70K, it was striking to note that loss of detection of the protein in the nucleus was preceded by a change in the pattern of nuclear staining. Disappearance of U1-70K immunostaining is likely to be due to the lack of immunoreactivity of the antibody against the cleaved fragments of the protein. Indeed by Western blotting the antibody was unable to detect the previously described 40 kD proteolytic fragment (Casciola-Rosen *et al*, 1994; Tewari *et al*, 1995). U1-70K immunostaining also confirmed that during apoptosis, the nucleus is the place of profound structural changes since the staining pattern of the protein was changed from a fine punctate staining to globular dots. We have also evidences that subnuclear domains, including

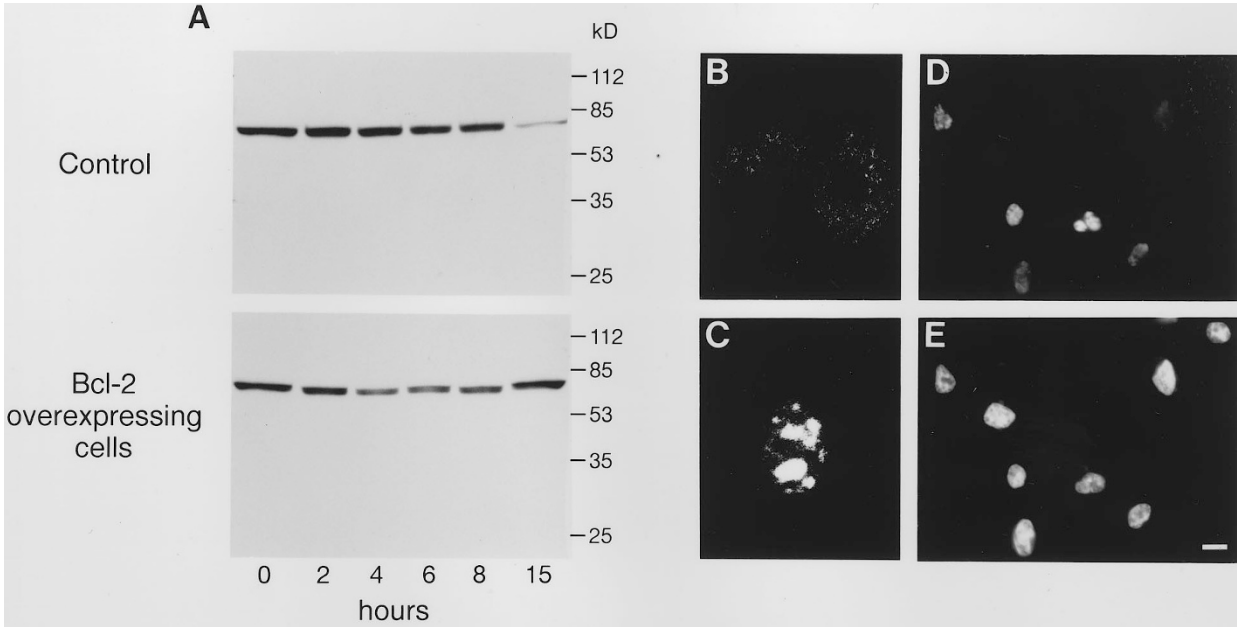

Figure 4 U1-70K cleavage (**A**) Western blot analysis of U1-70K Equal amounts of proteins from HeLa cells cultured in the presence of staurosporine were processed for Western blot analysis of U1-70K as described in Figure 3. The protein was detected by means of a mouse monoclonal to recombinant U1-70K. (**B – E**) Immunofluorescence localization of U1-70K in HeLa cells treated with or without staurosporine. (**B**) Untreated HeLa cells display a speckled-labeling nuclear pattern; the cytoplasm does not stain. (**C**) Control HeLa cells treated for 15 h with staurosporine: note that the staining reveals large nuclear bright dots. (**D – E**) View of a field containing several cells treated for 15 h with staurosporine. Note the difference in the intensity of U1-70K staining between cells (**D**). (**E**) Hoechst staining reveals that U1-70K negative cells display a nucleus which as yet is not condensed. Bar is 7 μm for **B**,**C** and 30 μm for **D**,**E**.

coiled bodies, are affected during staurosporine-induced PCD (unpublished data). Whether these structural modifications are specific to staurosporine or are events common to all PCD needs further investigations.

In this paper, we have also shown, in agreement with published data from other laboratories (Enari *et al*, 1995; Martin *et al*, 1995; Chinnaiyan *et al*, 1996), that overexpression of Bcl-2 prevents activation of CPP32 and proteolysis of PARP and U1-70K. Bcl-2 overexpression can block some, but not all, of the pathways leading to PCD. For example, Bcl-2 seems to provide little protection against FAS/APO-1 transduced apoptosis in lymphocytes (Strasser *et al*, 1995). In contrast, CrmA a potent inhibitor of ICE, and probably of other members of the family when overexpressed, confers protection to these cells. These results demonstrate that the mechanism of protection conferred by Bcl-2 is not the same to that conferred by inhibitors of the ICE family. The mechanisms by which Bcl-2 achieves protection remains to be elucidated.

Materials and Methods

Establishment of stable HeLa cell lines that constitutively overexpress Bcl-2

Human Bcl-2 cDNA was excised from the PGK-bcl-2 construct described by Martinou *et al* (1994) with SfiI, blunt ended and inserted into a neomycine resistance containing plasmid under the control of phosphoglycerate kinase promoter. HeLa cells were transfected with either the empty plasmid vector or with the plasmid encoding Bcl-2 by a calcium phosphate procedure (Promega). Pools of stably transfected cells were selected in media containing G418. The cell pools were grown in DMEM, 10% FCS and 0.4 mg/ml G418 and subjected to staurosporine. Cell viability was measured as described in (Jacobson *et al*, 1993, 1994).

Immunocytochemistry

Human Bcl-2 was detected using a monoclonal antibody to human Bcl-2 (Cambridge Research Biochemicals) as previously described (Martinou *et al*, 1994).

Western blot analysis

Mouse monoclonal antibody to CPP32 was purchased from Transduction Laboratories. This antibody was made against the first 219 amino-acids of CPP32. Polyclonal and monoclonal antibodies to PARP and U1-70K respectively have been described previously (Schreiber *et al*, 1995; Nelissen *et al*, 1994). Whole cell lysates were resolved by SDS – PAGE, transferred to nitrocellulose, and processed for immunoblotting. Both antibodies were used at a dilution of 1:1000. Peroxidase-labeled goat anti-mouse and goat anti-rabbit antibodies (Dako, DK) were used as secondary antibodies. The ECL detection system (Amersham) was used to develop blots.

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