Bcl-2 regulates amplification of caspase activation by cytochrome c

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Caspases, a family of specific proteases, have central roles in apoptosis [1]. Caspase activation in response to diverse apoptotic stimuli involves the relocalisation of cytochrome c from mitochondria to the cytoplasm where it stimulates the proteolytic processing of caspase precursors. Cytochrome c release is controlled by members of the Bcl-2 family of apoptosis regulators [2,3]. The anti-apoptotic members Bcl-2 and Bcl-x_L may also control caspase activation independently of cytochrome c relocalisation or may inhibit a positive feedback mechanism [4–7]. Here, we investigate the role of Bcl-2 family proteins in the regulation of caspase activation using a model cell-free system. We found that Bcl-2 and Bcl-x_L set a threshold in the amount of cytochrome c required to activate caspases, even in soluble extracts lacking mitochondria. Addition of dATP (which stimulates the procaspase-processing factor Apaf-1 [8,9]) overcame inhibition of caspase activation by Bcl-2, but did not prevent the control of cytochrome c release from mitochondria by Bcl-2. Cytochrome c release was accelerated by active caspase-3 and this positive feedback was negatively regulated by Bcl-2. These results provide evidence for a mechanism to amplify caspase activation that is suppressed at several distinct steps by Bcl-2, even after cytochrome c is released from mitochondria.

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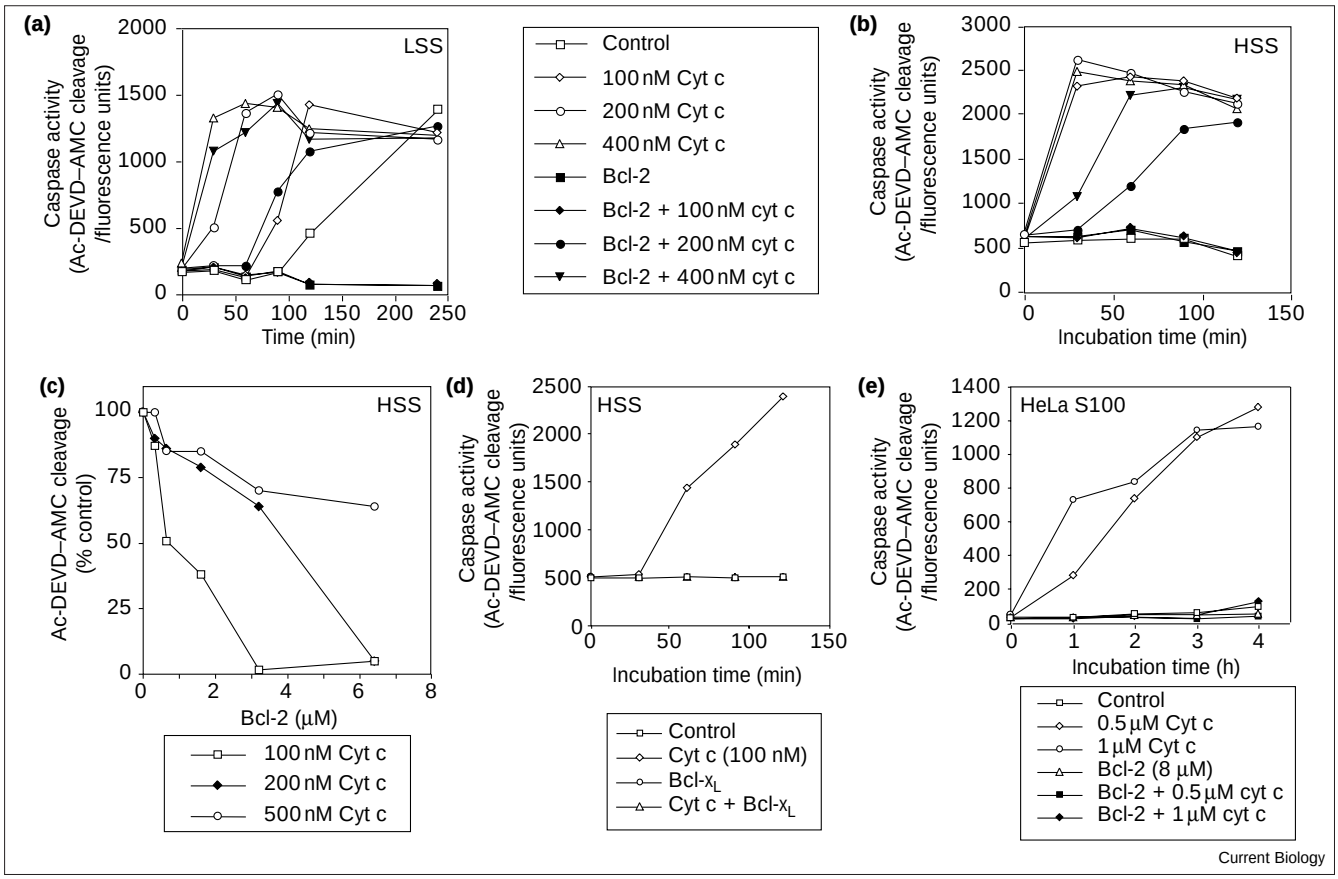
Results and discussion

In a cell-free system of *Xenopus* egg extracts that reproduces many aspects of apoptosis, cytochrome c release from mitochondria and activation of a caspase-3-like enzyme occur spontaneously after prolonged incubation. Both cytochrome c release and caspase activation are inhibited by the addition of Bcl-2 [2,10–14]. Previously it has been reported that exogenous cytochrome c bypasses the inhibitory effect of Bcl-2-containing cell lysate [2,13,14], suggesting that Bcl-2 only acts upstream of cytochrome c release in the mechanism of caspase activation. In this study, we have re-examined this question using a more quantitative analysis.

We found that purified recombinant Bcl-2 (4 μM) was indeed ineffective in blocking the acceleration of caspase activation in response to the addition of high levels (400 nM) of exogenous cytochrome c to low-speed supernatants (LSS) of *Xenopus* egg extracts (which contain membrane fractions including mitochondria). Bcl-2 delayed caspase activation that was accelerated by 200 nM cytochrome c, however, and completely blocked caspase activation in LSS containing 100 nM exogenous cytochrome c (Figure 1a). In high-speed supernatants (HSS) prepared from *Xenopus* LSS by centrifugation and removal of mitochondria and other heavy membranes, 4 μM Bcl-2 was still able to inhibit caspase activation induced by cytochrome c, completely blocking activation in the presence of 100 nM cytochrome c and delaying activation at 200–400 nM cytochrome c (Figure 1b). This shows that Bcl-2 can regulate caspase activation downstream of cytochrome c release and does not require association with mitochondria for this activity. The ratio of cytochrome c to Bcl-2 was important: at 100 nM cytochrome c there was a progressive inhibition of caspase activation with increasing concentrations of Bcl-2. A greater concentration of Bcl-2 was required to inhibit the effect of 200 nM cytochrome c, whereas caspase activation initiated by 500 nM cytochrome c was not blocked even by high concentrations of Bcl-2 (Figure 1c). The level of Bcl-2 therefore sets a threshold for the amount of cytochrome c required to activate cytosolic caspases.

The related protein Bcl-x_L also inhibited caspase activation in response to cytochrome c in *Xenopus* HSS (Figure 1d), indicating that it can function in a similar fashion to Bcl-2. In contrast, BH3 domains from the pro-apoptotic proteins Bax and Bak, which have been shown to promote cytochrome c release and caspase activation in *Xenopus* LSS [11], had no effect on caspase activation in the soluble extracts and failed to overcome the inhibition of caspase activation by Bcl-2 or Bcl-x_L (data not shown). Regulation of caspase activation by cytochrome c seems to be conserved in humans, as Bcl-2 (Figure 1e) or Bcl-x_L (data not shown) inhibited caspase activation in a post-mitochondrial

Figure 1



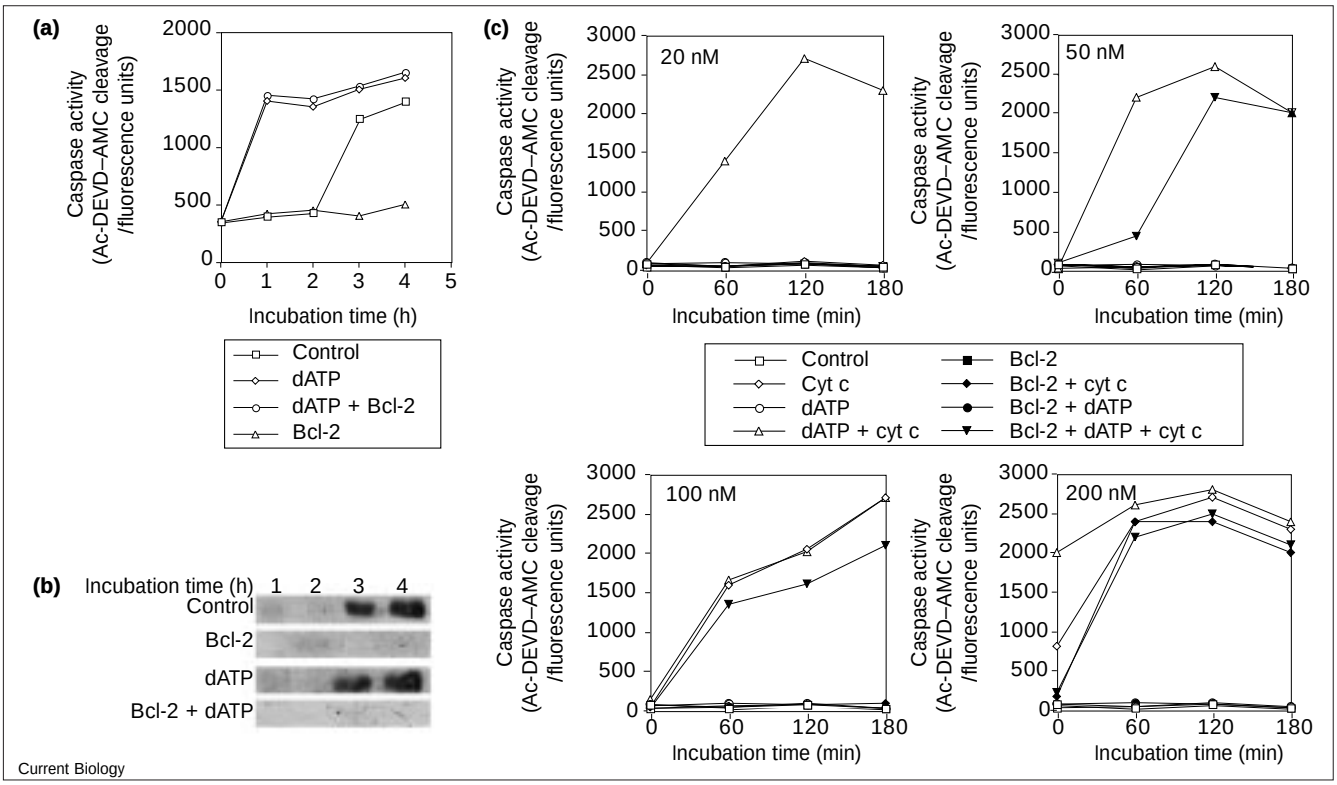
Bcl-2 and Bcl-x_L regulate caspase activation induced by cytochrome c (cyt c). Bcl-2 protein was added to the particular extract at the same time as cytochrome c and samples taken at the times shown. In this and subsequent experiments, caspase activity was assayed by monitoring the cleavage of the fluorogenic caspase substrate Ac-DEVD–AMC. (a) Whole *Xenopus* egg extract containing mitochondria (LSS). (b,c) Post-mitochondrial supernatant of *Xenopus* egg extracts (HSS). In (a,b), Bcl-2 was added at 4 μM and incubations carried out for the times shown; in (c), Bcl-2 was added at the concentrations shown and incubations carried out for 15 min. (d) The Bcl-2-related protein Bcl-x_L (4 μM) also inhibits caspase activation induced by cytochrome c in *Xenopus* HSS. (e) Post-mitochondrial (S100) extract of HeLa cells. Bcl-2 was added at 8 μM. Each panel is representative of at least three similar experiments.

supernatant derived from human HeLa cells. Bcl-2 blocks caspase-3 activation in these extracts by inhibiting the proteolytic processing of procaspase-3 [15].

Activation of mammalian caspase-3 by Apaf-1 is stimulated by dATP in a reaction that requires cytochrome c and caspase-9 [8,9,16]. In *Xenopus* LSS, 1 mM dATP produced a dramatic acceleration of caspase activation, suggesting that an Apaf-1-like factor is also involved (Figure 2a). However, dATP had little or no effect on the timing of cytochrome c release into the post-mitochondrial fraction. Although dATP uncoupled caspase activation from widespread cytochrome c release in this experiment, it remained possible that low levels of cytochrome c were released into the soluble fraction during incubation. Indeed, in *Xenopus* HSS, dATP alone had no effect on caspase activity, but produced a marked stimulation when added together with 20 or 50 nM cytochrome c (Figure 2c) — concentrations of cytochrome c that failed to activate caspases when added alone. When added at 100 nM or greater, cytochrome c activated caspases in HSS even without added dATP (Figure 2c). At 20 nM cytochrome c, 4 μM Bcl-2 was effective at preventing caspase activation even in the presence of dATP, while at 50–100 nM cytochrome c, the inhibitory effect of Bcl-2 could be partially overcome by dATP. Bcl-2 failed to block caspase activation initiated by 200 nM cytochrome c with or without dATP. These results indicate that stimulation of a soluble Apaf-1-like factor by dATP decreases a threshold in the amount of cytochrome c required to overcome the block on caspase activation by Bcl-2. However, dATP does not interfere with the ability of Bcl-2 to control cytochrome c release (Figure 2b), suggesting that these functions are separable.

In *Xenopus* LSS, when the release of endogenous cytochrome c from mitochondria was accelerated by the

Figure 2

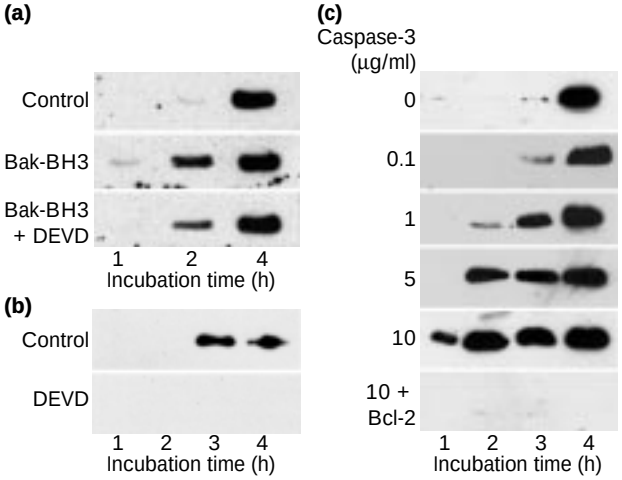


Addition of dATP stimulates caspase activation in *Xenopus* egg extracts. (a) Effects of dATP (1 mM) on caspase activation in *Xenopus* LSS; caspase activation was accelerated by dATP even in the presence of Bcl-2. (b) Levels of cytochrome c released from the LSS into the HSS under the indicated conditions as detected by western blotting: dATP thus accelerates caspase activation even when Bcl-2 prevents widespread release of cytochrome c into the post-mitochondrial supernatant. Cytochrome c was detected on western blots using a specific anti-cytochrome c antibody (Pharmingen) [11]. (c) Cytochrome c is required for dATP (1 mM) to stimulate caspase activation in *Xenopus* HSS, and dATP decreases the concentration of cytochrome c required to overcome inhibition of caspase activation by Bcl-2. In (c), cytochrome c was added at the concentration shown in each panel and Bcl-2 was added at 4 μM.

addition of the BH3 domain of Bak (Figure 3a; see also [11]), neither Ac-DEVD-CHO — a potent inhibitor of caspase-3 — nor the more general caspase inhibitor zVAD-fmk (data not shown) had any significant effect on the extent of cytochrome c release. Although this result does not exclude the possible involvement of a caspase that was not blocked by these inhibitors, it is consistent with previous evidence for a caspase-independent mechanism for cytochrome c release triggered by Bak and some other pro-apoptotic signals [2,4,13,14]. Ac-DEVD-CHO was effective in inhibiting the spontaneous release of cytochrome c, however, showing that caspase-3 or a similar activity plays a role in the release of cytochrome c under these conditions (Figure 3b). Furthermore, addition of purified active caspase-3 to the extracts accelerated the release of cytochrome c in a dose-dependent manner (Figure 3c). When very high levels of caspase-3 were added to egg extracts, Bcl-2 protein failed to prevent cytochrome c release (data not shown). If a lower concentration of caspase-3 was used (10 μg/ml), which is similar in activity to that generated by activation of endogenous caspases and still sufficient to markedly accelerate cytochrome c release, 4 μM Bcl-2 was able to inhibit the process completely, however (Figure 3c). Together, these results suggest that that the widespread release of cytochrome c from mitochondria is part of a mechanism for the feedback amplification of caspase activity that is under the control of Bcl-2.

In response to pro-apoptotic signals acting via mitochondria, caspase-3 may be activated initially by caspase-9 in a reaction dependent on cytochrome c and Apaf-1 [16]. Active caspase-3 would promote the widespread release of cytochrome c and amplification of the signal, presumably by cleaving regulatory protein(s). The amplification of caspase activation must be tightly controlled to prevent inappropriate apoptosis while allowing an effective response to pro-apoptotic stimuli. In addition to its role in controlling cytochrome c release from mitochondria, Bcl-2 sets a threshold in the amount of soluble cytochrome c required for caspase activation, possibly by sequestering cytochrome c in an inactive complex with Apaf-1 [17].

Figure 3



Release of cytochrome c from LSS of *Xenopus* egg extracts into the post-mitochondrial supernatant (HSS), following incubation with the indicated factors, as detected by western blot analysis of HSS. (a) Effect of 0.1 mg/ml Bak-BH3 domain on cytochrome c release in the presence or absence of 1 μM Ac-DEVD-CHO (DEVD). (b) Effect of Ac-DEVD-CHO on spontaneous cytochrome c release. (c) Effect of 4 μM Bcl-2 on cytochrome c release accelerated by increasing amounts of caspase-3.

This threshold effect, together with control of the feedback amplification of cytochrome c release promoted by active caspases (this study and [18]), provides for a dynamic process in which Bcl-2 acts at several steps to determine the response to pro-apoptotic signals initiating at mitochondria or elsewhere. The release of cytochrome c from mitochondria to a sub-threshold level and partial caspase activation would not necessarily lead to cell death. Once the threshold is exceeded, however, widespread caspase activation and apoptosis would ensue.

Materials and methods

Preparation of extracts and recombinant proteins

Xenopus egg extracts containing cycloheximide, cytochalasin B and an ATP-regenerating system were prepared as described previously [12]. HSS extracts were prepared by centrifugation of the LSS extracts at 100,000 × *g*. HeLa S100 extracts were prepared as described previously [19]. Caspase-3 was produced by expressing the p12 and p17 subunits separately in *Escherichia. coli* BL21 DE3. Active enzyme generated by mixing stoichiometric amounts of the subunits was stored in PBS at –70°C. Bcl-2, Bcl-x_L (both tagged at the amino terminus with a His_6 tag) and GST–Bak-BH3 were expressed in *E. coli* BL21 DE3 as described previously [11,19]. Horse cytochrome c was purchased from Sigma.

Caspase assays

Samples (2 μl) of each pre-incubation mixture were diluted 50-fold into 100 μl extraction buffer [11] containing 50 mM Ac-DEVD–AMC (Calbiochem), prewarmed to 23°C, then incubated at 23°C for 10 min, before stopping by addition of sodium acetate buffer which reduces the pH to 4.5. All assays were carried out in duplicate and read after dilution with 2 ml water in a Perkin–Elmer LS-5B fluorimeter using an excitation wavelength of 380 nm and an emission wavelength of 460 nm. Assays were carried in a 0.1 ml microcuvette thermostatted to 23°C and monitored in real time to determine that AMC release was linear with time.

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References

1. Thornberry NA, Lazebnik Y: Caspases: enemies within. *Science* 1998, 281:1312-1316.
2. Kluck RM, Bossy-Wetzel E, Green DR, Newmeyer DD: The release of cytochrome c from mitochondria: a primary site for Bcl-2 regulation of apoptosis. *Science* 1997, 275:1132-1136.
3. Yang J, Liu X, Bhalla K, Kim CN, Ibrado AM, Cai J, *et al.*: Prevention of apoptosis by Bcl-2: release of cytochrome c from mitochondria blocked. *Science* 1997, 275:1129-1132.
4. Rossé T, Olivier R, Monney L, Rager M, Conus S, Fellay I, *et al.*: Bcl-2 prolongs cell survival after Bax-induced release of cytochrome c. *Nature* 1998, 391:496-499.
5. Zhivotovsky B, Orrenius S, Brustugun OT, Døskeland SO: Injected cytochrome c induces apoptosis. *Nature* 1998, 391:449-450.
6. Hengartner MO: Death cycle and Swiss army knives. *Nature* 1998, 391:441-442.
7. Li F, Srinivasan A, Wang Y, Armstrong RC, Tomaselli KJ, Fritz LC: Cell-specific induction of apoptosis by microinjection of cytochrome c. Bcl-xL has activity independent of cytochrome c release. *J Biol Chem* 1997, 272:30299-30305.
8. Liu X, Kim CN, Yang J, Jemmerson R, Wang X: Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome c. *Cell* 1996, 86:147-157.
9. Zou H, Henzel WJ, Liu X, Lutschg A, Wang X: Apaf-1, a human protein homologous to ***C. elegans*** CED-4, participates in cytochrome c-dependent activation of caspase-3. *Cell* 1997, 90:405-413.
10. Newmeyer DD, Farschon DM, Reed JC: Cell-free apoptosis in ***Xenopus*** egg extracts — Inhibition by Bcl-2 and requirement for an organelle fraction enriched in mitochondria. *Cell* 1994, 79:353-364.
11. Cosulich SC, Worrall V, Hedge PJ, Green S, Clarke PR: Regulation of apoptosis by BH3 domains in a cell-free system. *Curr Biol* 1997, 7:913-920.
12. Cosulich SC, Green S, Clarke PR: Bcl-2 regulates activation of apoptotic proteases in a cell-free system. *Curr Biol* 1996, 6:997-1005.
13. Bossy-Wetzel E, Newmeyer DD, Green DR: Mitochondrial cytochrome c release in apoptosis occurs upstream of DEVD-specific caspase activation and independently of mitochondrial transmembrane depolarization. *EMBO J* 1998, 17:37-49.
14. Kluck RM, Martin SJ, Hoffman BM, Zhou JS, Green DR, Newmeyer DD: Cytochrome c activation of CPP32-like proteolysis plays a critical role in a ***Xenopus*** cell-free apoptosis system. *EMBO J* 1997, 16:4639-4639.
15. Swanton E, Savory PJ, Cosulich SC, Clarke PR, Woodman P: Bcl-2 regulates a caspase-3/caspase-2 apoptotic cascade in cytosolic extracts. *Oncogene* 1999, in press.
16. Li P, Nijhawan D, Budihardjo I, Srinivasula SM, Ahmad M, Alnemri ES, Wang X: Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. *Cell* 1997, 91:479-489.
17. Reed JC: Double identity for proteins of the Bcl-2 family. *Nature* 1997, 387:773-776.
18. Kuwana T, Smith JJ, Muzio M, Dixit V, Newmeyer DD, Kornbluth S: Apoptosis induction by caspase-8 is amplified through the mitochondrial release of cytochrome c. *J Biol Chem* 1998, 273:16589-16594.
19. Cosulich SC, Horiuchi H, Zerial M, Clarke PR, Woodman PG: Cleavage of Rabaptin-5 blocks endosome fusion during apoptosis. *EMBO J* 1997, 16:6182-6191.