Cleavage of BID during cytotoxic drug and UV radiation-induced apoptosis occurs downstream of the point of Bcl-2 action and is catalysed by caspase-3: a potential feedback loop for amplification of apoptosis-associated mitochondrial cytochrome c release

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Abstract

BID, a pro-apoptotic Bcl-2 family member, promotes cytochrome c release during apoptosis initiated by CD95L or TNF. Activation of caspase-8 in the latter pathways results in cleavage of BID, translocation of activated BID to mitochondria, followed by redistribution of cytochrome c to the cytosol. However, it is unclear whether BID participates in cytochrome c release in other (non-death receptor) cell death pathways. Here, we show that BID is cleaved in response to multiple death-inducing stimuli (staurosporine, UV radiation, cycloheximide, etoposide). However BID cleavage in these contexts was blocked by Bcl-2, suggesting that proteolysis of BID occurred distal to cytochrome c release. Furthermore, addition of cytochrome c to Jurkat post-nuclear extracts triggered breakdown of BID at Asp-59 which was catalysed by caspase-3 rather than caspase-8. We provide evidence that caspase-3 catalysed cleavage of BID represents a feedback loop for the amplification of mitochondrial cytochrome c release during cytotoxic drug and UV radiation-induced apoptosis. Cell Death and Differentiation (2000) **7**, 556–565.

Keywords: apoptosis; BID; Bcl-2; caspase; cell-free; cytochrome c

Introduction

Apoptosis is a mode of cell death that is involved in situations where elimination of specific cells in necessary for the development, maintenance, and survival of multicellular organisms.[1] In recent years, evidence has accumulated to argue that apoptosis is orchestrated by a panoply of cysteine proteases – the caspases – that cooperate to dismantle the cell upon receipt of a death-inducing stimulus.[2–4]

Stimuli that trigger apoptosis are diverse and appear to engage the cell death machinery in a variety of ways.[5] However, evidence is emerging to suggest that the signals generated by numerous death-promoting agents all converge at some point on mitochondria and trigger release of cytochrome c, as well as other factors such as apoptosis inducing factor (AIF), from this organelle.[6–11] Thus, although pro-apoptotic signals that provoke cytochrome c release from mitochondria may do so by a variety of means, events that take place distal to this step may be relatively conserved. This view is supported by the observation that the Bcl-2 protein can efficiently block apoptosis in response to diverse death-promoting signals, possibly due to its ability to regulate the cytochrome c release checkpoint in the cell death pathway.[6,7] Whereas cell death-associated events that take place upstream of this checkpoint may be stimulus-specific, downstream events are likely to be stimulus-independent and may be largely dictated by the interaction between cytochrome c and its cytosolic target, Apaf-1.[12–15]

The importance of the cytochrome c/Apaf-1 arm of the cell death pathway is underscored by the phenotype of *Apaf-1* knockout mice, in which large numbers of redundant cells accumulate during foetal development – resulting in embryonic lethality.[13,14] Despite the ubiquity of cytochrome c release in apoptosis, the mechanisms that regulate this event are relatively poorly understood.[9] Recent studies have presented data to argue that BID, a death promoting member of the Bcl-2 family, promotes cytochrome c release in the Fas/CD95- and TNF-initiated cell death pathways.[16–20] In these contexts, receptor engagement results in early activation of caspase-8 through proximity-induced transprocessing that is facilitated by the FADD and TRADD adaptor proteins. Active caspase-8 cleaves cytosolic BID, resulting in translocation of the C-terminal portion of BID to the mitochondria, followed by integration into the outer mitochondrial membrane.[16–19] Insertion of BID into the mitochondrial membrane was found to be a potent instigator of cytochrome c release from mitochondria, via

an as yet unknown mechanism. Significantly, whereas Bcl-2 and Bcl-x did not prevent cleavage of BID in the context of death receptor-initiated apoptosis, these proteins were capable of blocking the subsequent release of cytochrome *c* from mitochondria, in agreement with their perceived role as regulators of mitochondrial integrity.[16−19]

Mitochondrial cytochrome *c* release in non-death receptor pathways is less well understood. Recent data suggests that the pro-apoptotic Bcl-2 family proteins Bax and Bak may promote cytochrome *c* efflux, possibly due to their ability to bind and regulate components of the multiprotein permeability transition pore complex.[21−25] The role of BID, if any, in cytochrome *c* release in the context of non-death receptor-induced cell deaths (cytotoxic drugs, radiation) remains unknown. Here, we show that BID is cleaved in response to divergent, non-death receptor associated, death stimuli (staurosporine, UV radiation, cycloheximide, etoposide), suggesting that this may contribute to the cytochrome *c* release that is seen during apoptosis induced by these agents. However, BID cleavage in these contexts was found to occur downstream of the point of Bcl-2 action, suggesting that this event occurred distal to cytochrome *c* release into the cytosol. In line with these observations, addition of cytochrome *c* to cell-free extracts of Jurkat cells triggered BID cleavage which was catalysed by caspase-3 as opposed to caspase-8. Immunodepletion of caspase-3 from extracts abolished cytochrome *c*-inducible BID cleavage. Similarly, cytochrome *c*-initiated BID cleavage was also absent in extracts derived from MCF-7 cells that are naturally devoid of caspase-3.[26] Addition of cytochrome *c* to cell extracts containing healthy mitochondria provoked cytochrome *c* release from these mitochondria. These data suggest that cytochrome *c* release from mitochondria can be amplified through a caspase-3/BID feedback loop, irrespective of the initial trigger for cytochrome *c* redistribution to the cytosol.

Results

BID is cleaved during apoptosis initiated by diverse stimuli

Previous studies have established that apoptosis-promoting stimuli such as staurosporine and UV radiation trigger release of cytochrome *c* from mitochondria and that this release can be regulated by Bcl-2.[6,7,29] Recent studies have implicated BID in the cytochrome *c* release that is seen during FasL and TNF-induced apoptosis.[16−20] However, it is unclear whether BID plays a similar role in non-death receptor-mediated cell deaths. To address this question, we induced apoptosis in Jurkat T lymphoblastoid cells using a range of pro-apoptotic stimuli and monitored BID protein levels as cells entered apoptosis.

Figure 1 shows that levels of intact BID dramatically decreased after exposure to staurosporine, UV radiation, cycloheximide and etoposide suggesting that BID is cleaved in non-death receptor cell death contexts. Interestingly, we consistently observed much more significant decreases in the levels of intact BID during apoptosis induced by non-death receptor stimuli, when
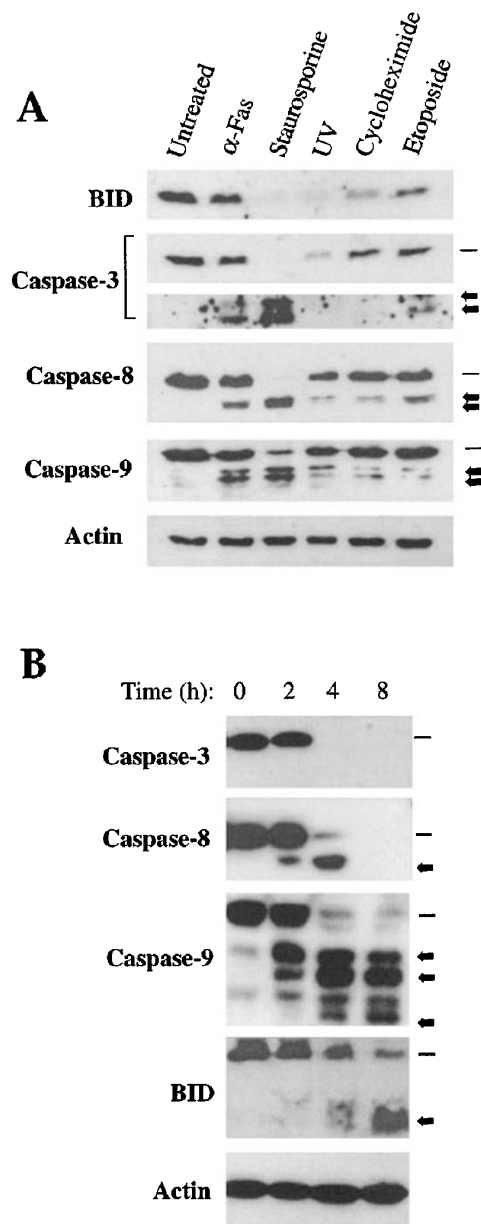

Figure 1 BID cleavage occurs during apoptosis induced by diverse stimuli. (**A**) Jurkat cells were treated either with anti-Fas IgM mAb (100 ng/ml), staurosporine (500 nM), cycloheximide (10 μM), etoposide (10 μM), UV radiation (20 min), or were left untreated, as indicated. Cells were then incubated for either 8 h (staurosporine) or 16 h (all other treatments), prior to preparation of cell lysates for Western blotting. Blots were probed for the indicated proteins using appropriate antibodies as described under Materials and Methods. Equal protein loadings were confirmed by probing for actin. Where cleavage products were detectable, these are indicated by arrows. (**B**) Jurkat cells were incubated in the presence of 500 nM staurosporine and cell lysates were prepared at the indicated times. Cell lysates were Western blotted followed by probing for the indicated proteins

compared with the decreases seen in response to Fas ligation, despite the fact that populations with similar numbers of apoptotic cells were compared. Figure 1 also illustrates that there was little correlation between the extent of BID cleavage and the degree of caspase-8

activation in the same samples, as assessed by the appearance of processed forms of caspase-8, suggesting that BID cleavage in the non-death receptor pathways may be mediated by another caspase.

Staurosporine and UV-induced BID cleavage is blocked by Bcl-2

It has been demonstrated that cleavage of BID in the context of Fas- or TNF-induced apoptosis is not blocked by Bcl-2 or Bcl-x, rather, these death repressor proteins block BID-induced release of cytochrome *c*.[16–18] This implies that cleavage of BID occurs upstream of the point of Bcl-2 action in death receptor pathways. To ask whether this was also true in the context of non-death receptor initiated cell deaths, we examined BID cleavage in CEM[vector] *versus* CEM[bcl-2] cells that were induced to undergo apoptosis by exposure to staurosporine or UV radiation.

As illustrated in Figure 2, CEM[bcl-2] cells were resistant to both staurosporine- and UV-radiation induced apoptosis, as previously reported.[30,31] Both stimuli triggered multiple caspase activation events in CEM[vector] cells but all of these activation events were very substantially inhibited in CEM[bcl-2] cells, suggesting that these caspases are activated downstream of the point of Bcl-2 action. In line with these observations, multiple caspase substrate cleavage events (fodrin, PARP, U1snRNP, lamin B, topoisomerase I) were also blocked in cells overexpressing Bcl-2 (data not shown). Surprisingly, UV- and staurosporine-induced cleavage of BID was also completely blocked in CEM[bcl-2] cells, suggesting that BID cleavage occurs downstream of the point of Bcl-2 action in these pathways (Figure 2B). This contrasts sharply with observations made in the context of Fas and TNF-initiated apoptosis where Bcl-2 and Bcl-x failed to block cleavage of BID, but blocked its downstream effects by antagonising BID-mediated release of cytochrome *c* from mitochondria.[16–18]

Cytochrome *c* triggers cleavage of BID in cell-free extracts

Because staurosporine- and UV-initiated BID cleavage was completely repressed by Bcl-2, this suggested that cleavage of BID in these contexts was occurring downstream of the mitochondrial event(s) that Bcl-2 regulates. Bcl-2 has been shown to block the release of mitochondrial cytochrome *c* in a variety of cell death contexts – including death initiated by staurosporine and UV radiation[6,7] – thereby attenuating the Apaf-1-mediated activation of caspase-9 and the subsequent caspase cascade.[12,15,32,33] Thus, we asked whether BID was cleaved downstream of the point of cytochrome *c* release, as the Bcl-2 data would predict.

To explore this question, we utilised a cell-free system based on Jurkat post-nuclear extracts that can support cytochrome *c*-initiated apoptotic events, such as caspase activation, cleavage of caspase substrates and condensation of nuclei.[15] Figure 3 illustrates that introduction of cytochrome *c* and dATP into these extracts initiated multiple caspase activation events, as previously re-
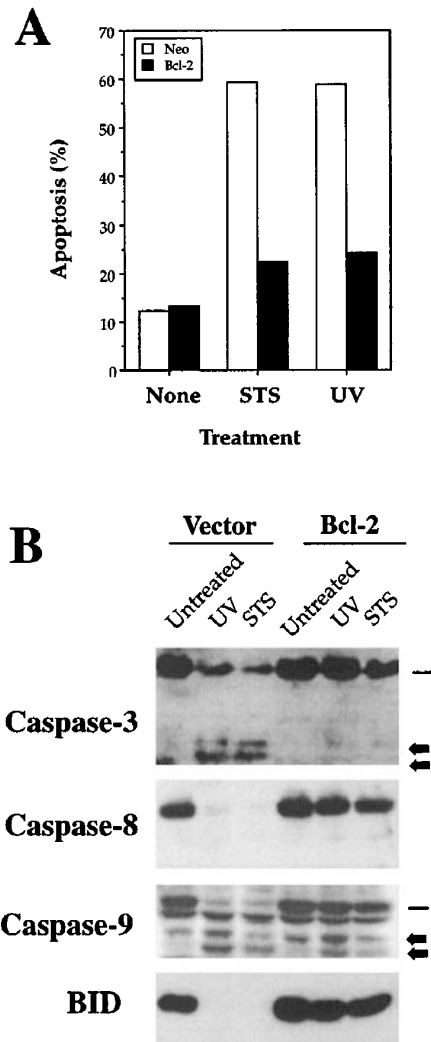

Figure 2 Bcl-2 blocks staurosporine- and UV-induced apoptosis upstream of caspase activation and BID breakdown. (**A**) CEM cells, stably transfected with either Bcl-2 or vector plasmid, were exposed to 500 nM staurosporine (STS), or UV radiation (20 min), followed by incubation for 6 h at 37°C. Apoptosis was quantitated by staining cells with annexin V-FITC, which detects phosphatidylserine externalisation, followed by flow cytometric analysis. Each data point represents counts made on 10 000 cells and results are representative of five separate experiments. (**B**) Cell lysates, prepared from cells treated as in (**A**), were blotted for the indicated proteins. Where cleavage products were detectable, these are indicated by arrows. Results are representative of five separate experiments

ported.[15] Significantly, cytochrome *c* also triggered cleavage of BID to yield a cleavage product of ~15 kDa, confirming that BID cleavage can take place upon entry of cytochrome *c* into the cytosol (Figure 3B).

In the cell death pathway that is engaged by the Fas/CD95 and TNF receptors, BID has been shown to undergo caspase-8-mediated proteolytic cleavage at Asp-59 to liberate a 15 kDa C-terminal cleavage product that translocates to the mitochondria and triggers release of cytochrome *c*.[16–18] To explore whether BID was cleaved at a similar site in response to cytochrome *c* we used a D59A mutant of BID that has been shown to resist caspase-8-
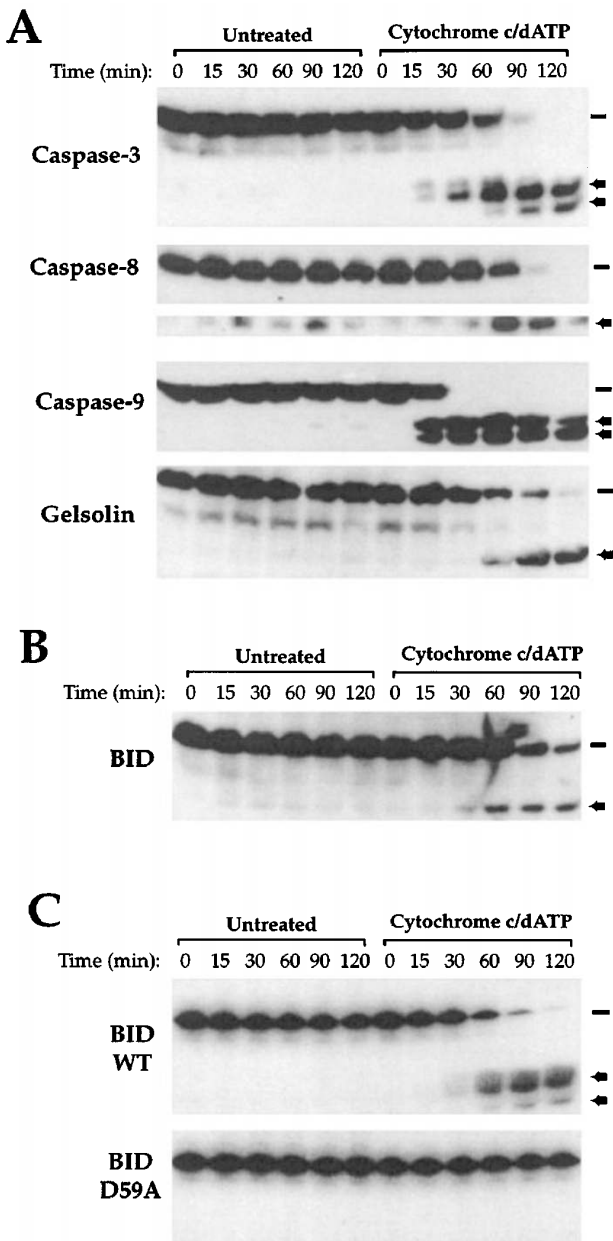
A

B

C

Figure 3 Cytochrome *c* initiates BID cleavage in cell-free extracts. (**A**) Time course of cytochrome *c*/dATP-induced caspase activation and gelsolin cleavage in Jurkat cell-free extracts. Cell-free extracts were prepared and reactions were set up as described under Materials and Methods. Reactions were incubated at 37°C in the presence or absence of cytochrome *c* (50 μg/ml) and dATP (1 mM), as shown. (**B**) Time course of BID cleavage under conditions identical to those used in (**A**). BID was detected by Western blotting. (**C**) Cleavage of ^{35}S-labelled BID and ^{35}S-labelled BID D59A mutant, prepared by *in vitro* transcription and translation, under similar conditions to those used in (**A**). Intact BID and BID cleavage products were visualised by fluorography. Results are representative of three separate experiments

mediated cleavage.[17] Figure 3C illustrates that whereas wild-type BID was efficiently cleaved upon introduction of cytochrome *c* and dATP into Jurkat extracts, the D59A mutant was not, suggesting that cleavage occurred at this site.

Cytochrome *c*-initiated cleavage of BID is catalysed by caspase-3

Cytochrome *c* triggers multiple caspase activation events due to its ability to promote activation of caspase-9 via Apaf-1.[15,32−34] Upon activation, caspase-9 then provokes a hierarchical caspase cascade resulting in the activation of caspases -3, -7, -2, -6, -8, and -10.[15] To determine which of the caspases were responsible for the cleavage of BID in the cascade initiated by cytochrome *c*, we used a combination of approaches. First, we explored the effects of three different caspase inhibitory peptides (z-VAD-fmk, VEID-CMK, DEVD-CHO) on cytochrome *c*-initiated BID cleavage (Figure 4A). These experiments suggested that a caspase-3-like activity was required for the cleavage of BID in this context.

To test this further, we depleted caspase-3, caspase-6 or caspase-7 from Jurkat extracts, using antibodies specific to each of these caspases, and explored whether the extracts could still support cytochrome *c*-initiated BID cleavage. Figure 4B,C illustrates that, whereas depletion of caspase-6 or caspase-7 had no significant effect, extracts devoid of caspase-3 failed to support cleavage of BID. As further confirmation of the role of caspase-3 as the caspase responsible for the cleavage of BID in the cytochrome *c* pathway, we used extracts of MCF-7 cells that are devoid of caspase-3 due to a deletion in exon 3 of the *CASP3* gene.[26] As previously reported, cytochrome *c* was able to initiate processing of caspase-9 and caspase-7 in MCF-7 extracts, but failed to support processing of caspase-6, as this event requires caspase-3.[15] In line with these observations, MCF-7 extracts also failed to support cytochrome *c*-induced BID cleavage. Taken together, these data strongly implicate caspase-3 as the caspase responsible for the cleavage of BID downstream of cytochrome *c* release.

Caspase-3 cleaves BID at Asp-59 and promotes cytochrome *c* release from mitochondria

To confirm that caspase-3 directly cleaves BID and to map the cleavage site, we exposed wild-type BID, as well as the D59A mutant, to recombinant caspase-3 or caspase-8. Figure 5 illustrates that recombinant caspase-3 could efficiently cleave wild-type BID over a wide range of concentrations, but the D59A mutant remained intact under the same conditions.

Caspase-8 has been shown to be capable of promoting cytochrome *c* release from mitochondria due to its ability to catalyse the cleavage of BID, the C-terminal portion of which then integrates into the outer mitochondrial membrane.[16−19] Because caspase-3 can cleave BID at an identical site, this suggested that caspase-3 should also be capable of triggering cytochrome *c* release. Thus, we exposed purified mouse liver mitochondria to similar concentrations of recombinant caspase-3 or caspase-8 in the presence or absence of Jurkat extract as a source of BID. These experiments revealed that both caspases could promote cytochrome *c* release to a similar extent, but only in the presence of BID-containing cytosol (Figure 5B.
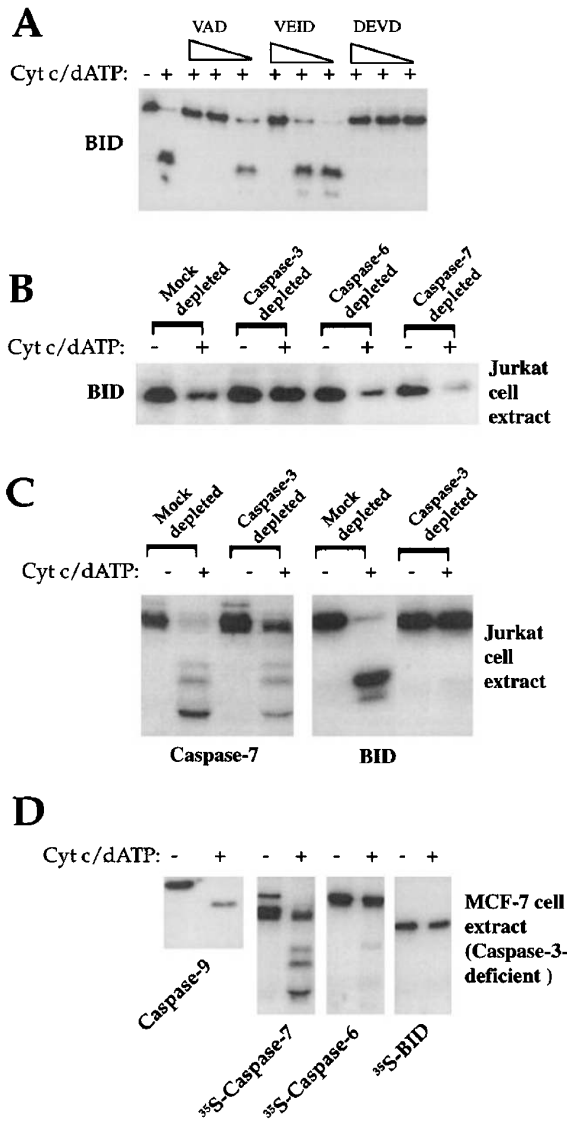
Cytochrome *c* release triggers a feedback amplification loop that promotes release of further cytochrome *c*

Our observations that cytochrome *c* can trigger cleavage of BID in a caspase-3-dependent manner suggest that cytochrome *c* should be capable of driving a feedback loop (via BID) that results in further release of cytochrome *c* from intact mitochondria. To explore this possibility, we exposed purified mouse liver mitochondria to cytochrome *c* under conditions where caspase-3 activation and BID cleavage
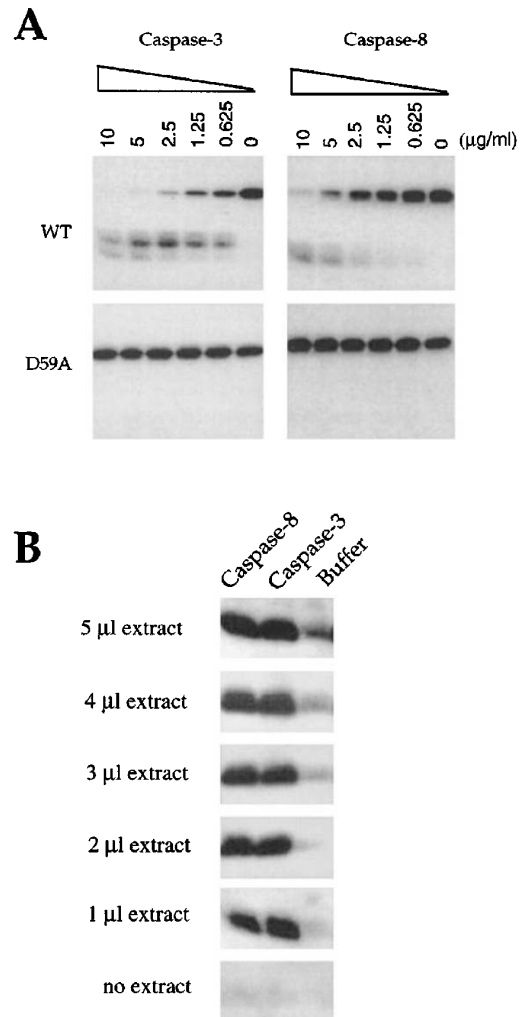
Figure 4 Cytochrome *c*-initiated processing of BID requires caspase-3. (**A**) Cytochrome *c*-induced BID breakdown requires a DEVD-inhibitable activity. Jurkat extracts, containing ^{35}S-labelled BID, were incubated for 2 h with cytochrome *c* (50 μg/ml) and dATP (1 mM), either alone, or in the presence of Z-VAD-FMK, VEID-CMK or DEVD-CHO (100, 10 and 1 μM), as indicated. Proteins were then separated by SDS–PAGE and visualised by fluorography. (**B**) Caspase-3, but not caspases -6, or -7, is required for cytochrome *c*-initiated BID cleavage. Jurkat extracts were either mock-depleted, or were immunodepleted with antibodies specific for caspases -3, -6 or -7, and then incubated for 2 h with or without cytochrome *c*/dATP, as shown. Levels of intact BID were then assessed by Western blot. (**C**) Caspase-3 is required for the breakdown of ^{35}S-labelled BID *in vitro*. Mock-depleted or caspase-3-depleted Jurkat cell-free extracts were incubated for 2 h in the presence or absence of cytochrome *c*/dATP, as shown. Extracts were supplemented with either ^{35}S-labelled caspase-7 or ^{35}S-labelled BID, as indicated. (**D**) BID is not cleaved in caspase-3-deficient MCF-7 cell extracts in response to cytochrome *c*. Extracts were incubated for 2 h in the presence or absence of cytochrome *c*/dATP, as indicated. Breakdown of ^{35}S-labelled BID, and processing of ^{35}S-labelled caspases -6 and -7 was determined by SDS–PAGE followed by fluorography. Caspase-9 processing was determined by Western blot

Figure 5 Caspase-3 cleaves BID at a site identical to that cleaved by caspase-8 and can also promote cytochrome *c* release from mitochondria. (**A**) ^{35}S-labelled BID wild-type (WT), or ^{35}S-labelled BID D59A mutant, were incubated for 2 h with the indicated concentrations of purified recombinant caspase-3 or caspase-8. Proteins were then separated by SDS–PAGE followed by fluorographic detection of radiolabeled proteins. Results are representative of two separate experiments. (**B**) Caspase-8 and caspase-3 both induced the release of mitochondrial cytochrome *c* in the presence of Jurkat cell extract. Purified mouse liver mitochondria were incubated for 60 min with the indicated volumes of Jurkat extract (~5 μg/μl) in a final reaction volume of 20 μl, either alone (buffer), or in the presence of 25 ng of purified recombinant caspase-3 or caspase-8. Mitochondria were then pelleted by centrifugation at 12 500 × *g* and the supernatants analyzed for released cytochrome c by SDS–PAGE and Western blotting

occurred. Figure 6A shows that addition of cytochrome *c* to Jurkat extracts containing freshly purified mitochondria resulted in the release of cytochrome *c* from these mitochondria in parallel with the activation of caspase-3. Mitochondria incubated for the same duration in the presence of extract alone failed to release cytochrome *c* (Figure 6A).

Whereas FasL and TNF-induced cytochrome *c* release have been reported to be caspase-dependent,[16–18,35] the release of cytochrome *c* in several other contexts (including staurosporine and UV-induced apoptosis) has been found to be at least partially caspase-independent.[11,29] This suggests that caspase-cleaved BID is not initially involved in provoking the cytochrome *c* release seen in these contexts. However, it remains possible that upon release of

cytochrome *c* via a caspase-independent mechanism, further cytochrome release is achieved via a caspase-3/BID-dependent feedback loop.

To explore this possibility, we examined the effect of the broad spectrum caspase inhibitor, z-VAD-fmk, on staurosporine- and UV-induced cytochrome *c* release in Jurkat cells. These experiments revealed that a substantial portion of the cytochrome *c* release seen in response to staurosporine and UV radiation was attenuated in the presence of z-VAD-fmk, providing evidence that a caspase-dependent feedback loop may be operative in these death pathways (Figure 6B). Thus, cytochrome *c*-initiated cleavage of BID is likely to represent a means of amplifying cytochrome *c* release in cell death pathways where the initial cytochrome *c* redistribution to the cytosol occurs in a caspase-independent manner.
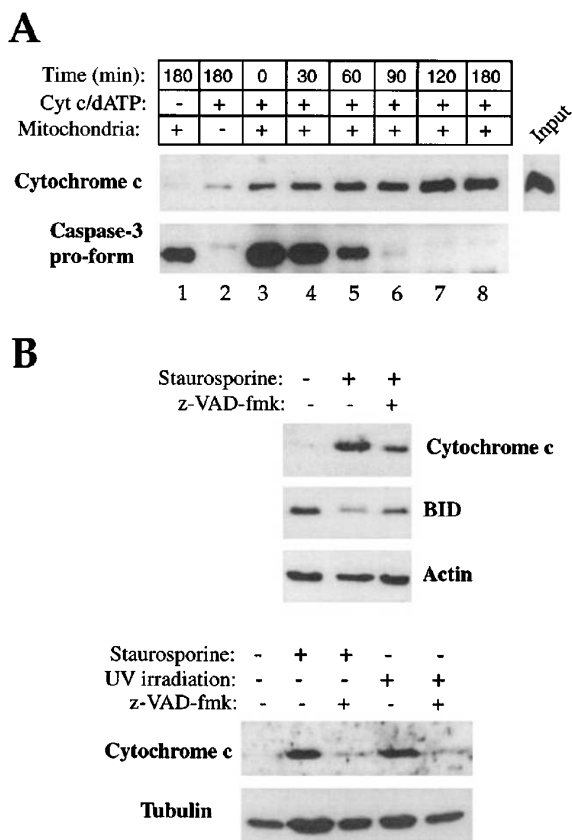
Discussion

BID has been implicated as the instigator of mitochondrial cytochrome *c* release early in the CD95/Fas and TNF pathways.[16–20,35] Whereas full-length BID is a relatively poor inducer of cytochrome *c* release, the C-terminal portion of BID that is generated upon cleavage by caspase-8 is very potent in this regard. Here we have shown that engagement of the cytochrome *c*/Apaf-1 pathway also results in BID cleavage. In contrast with the Fas and TNF pathways, BID proteolysis in this setting was mediated by caspase-3 and was blocked by overexpression of Bcl-2. In line with these observations, caspase-3 was found to be capable of promoting cytochrome *c* release from mitochondria, which correlated with its ability to cleave BID. These data suggest that the release of cytochrome *c* from mitochondria can drive an amplification loop that promotes further cytochrome *c* release and consequent caspase activation, thereby explosively amplifying the death signal. This loop may be particularly important in situations where the initial, caspase-independent, release of cytochrome *c* is relatively minor.

Evidence is accumulating to indicate that release of cytochrome *c* from the mitochondrial intermembrane space during apoptosis is a highly regulated event.[9,23,25,36] Entry of cytochrome *c* into the cytosol has severe consequences for the cell on two levels. On one level, the loss of this key component of the mitochondrial electron transport chain is likely to result in the disruption of ATP synthesis which will lead to cell death, regardless of whether the phenotype of this death is apoptosis or necrosis. On another level, cytochrome *c* can directly drive caspase-dependent death (apoptosis) due to its ability to act as a co-factor for the CED-4 homologue, Apaf-1.[12] Binding of cytochrome *c* to Apaf-1, in association with dATP or ATP, results in Apaf-1-mediated activation of caspase-9, probably via a clustering mechanism.[33,37–40] Caspase-9 then propagates a caspase cascade by directly processing caspases -3 and -7.[15,33] The latter caspases then activate other caspases as well as cleaving structural and regulatory proteins. All of these proteolytic cleavage events ultimately result in the full-blown apoptotic phenotype which triggers recognition and removal of the dying cell by phagocytes.

A

Time (min):	180	180	0	30	60	90	120	180	
Cyt c/dATP:	-	+	+	+	+	+	+	+	
Mitochondria:	+	-	+	+	+	+	+	+	Input

Cytochrome c

Caspase-3 pro-form

1 2 3 4 5 6 7 8

B

Staurosporine: - + +
z-VAD-fmk: - - +

Cytochrome c

BID

Actin

Staurosporine: - + + - -
UV irradiation: - - - + +
z-VAD-fmk: - - + - +

Cytochrome c

Tubulin

Figure 6 Evidence for a feedback loop that amplifies cytochrome *c* release during apoptosis. (**A**) Cytochrome *c* can induce further cytochrome *c* release from isolated mitochondria. Purified mouse liver mitochondria were incubated with Jurkat extract alone (lane 1), or with Jurkat extract containing cytochrome *c* (6.25 μg/ml) and dATP (1 mM) (lanes 3–8) for the time periods indicated. The amount of cytochrome *c* that represents the input (background) level is shown in lane 2. Following incubation for the indicated periods of time, the mitochondria were pelleted (12 500 × *g*, 7 min) and the supernatants were analyzed for released cytochrome *c* or caspase-3 processing, by Western blot, as indicated. (**B**) Caspase activity is required for maximal cytochrome release during staurosporine- and UV-induced apoptosis. Jurkat cells were either treated with staurosporine (500 nM), or were UV-irradiated (15 min), in the presence or absence of the broad-spectrum caspase inhibitor, Z-VAD-FMK (50 μM). After incubation for 5 h, cells were lysed and mitochondria were pelleted as described under Materials and Methods. Supernatants were then analyzed by SDS–PAGE followed by Western blotting for the detection of the indicated proteins. Where z-VAD-fmk was used, cells were pre-incubated with this peptide for 1 h prior to the initiation of apoptosis

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Whereas BID is thought to be the initial trigger for cytochrome *c* release in the Fas and TNFR death receptor pathways, the mechanism(s) by which cytotoxic drugs and other cellular stresses provoke the initial release of cytochrome *c* from mitochondria is unclear. A number of studies have implicated Bax as a trigger for (caspase-independent) mitochondrial cytochrome *c* release during apoptosis.[21–25,41,42] Irrespective of the initial means of triggering cytochrome *c* redistribution to the cytosol, the cleavage of BID downstream of this point introduces the possibility of a caspase-dependent feedback amplification loop, where caspase-3-cleaved BID is able to act back on the mitochondria causing further cytochrome *c* release – thereby perpetuating and amplifying the caspase cascade (Figure 7). In a scenario where there is an initial release of a small amount of cytochrome *c*, this may be sufficient to allow limited Apaf-1-mediated activation of caspase-9, followed by caspase-3 activation as a consequence. Cleavage of BID upon activation of caspase-3 may provoke a much more significant (caspase-dependent) release of cytochrome *c*. This scheme may provide an explanation for the ability of Bcl-2 to inhibit apoptosis in response to microinjection of cytochrome *c* into the cytosol, as Bcl-2 might simply have blocked a cytochrome *c*-driven

feedback loop.[43,44] Interestingly, MCF-7 cells (that lack caspase-3) are refractory to the effects of microinjected cytochrome *c*,[45] but introduction of caspase-3 restored susceptibility to this treatment, possibly as a result of restoration of the feedback loop.[45]

Because caspase-3 is responsible for cleavage of many of the structural and regulatory proteins that are broken down during apoptosis,[46,47] it is generally believed that caspase-3 is a downstream effector that exerts its effects during the final phase of death. However, the dramatic failure to delete numerous cells in the CNS of *casp-3*$^{-/-}$ mice suggests that caspase-3 plays a more upstream role in deciding the fate of certain cell populations (or the response to certain stimuli). In the latter cell types, it is plausible that the absence of caspase-3 alters the threshold at which cells engage their death machinery and cells lacking this caspase fail to die as a consequence.

In summary, we have shown that, in addition to its ability to instigate cytochrome *c* release in death receptor-mediated apoptosis, BID is also cleaved and activated as a consequence of cytochrome *c* release. In these circumstances the activated BID is able to participate in a feedback loop which may act to sustain and amplify the death signal. This feedback loop is regulated by Bcl-2, which prevents the cleavage of BID in response to cytochrome *c* release. This study provides a further insight into the control points at which death regulating molecules such as Bcl-2 can act, and also highlights the essential contribution of caspase-3 to the correct execution of the cell.
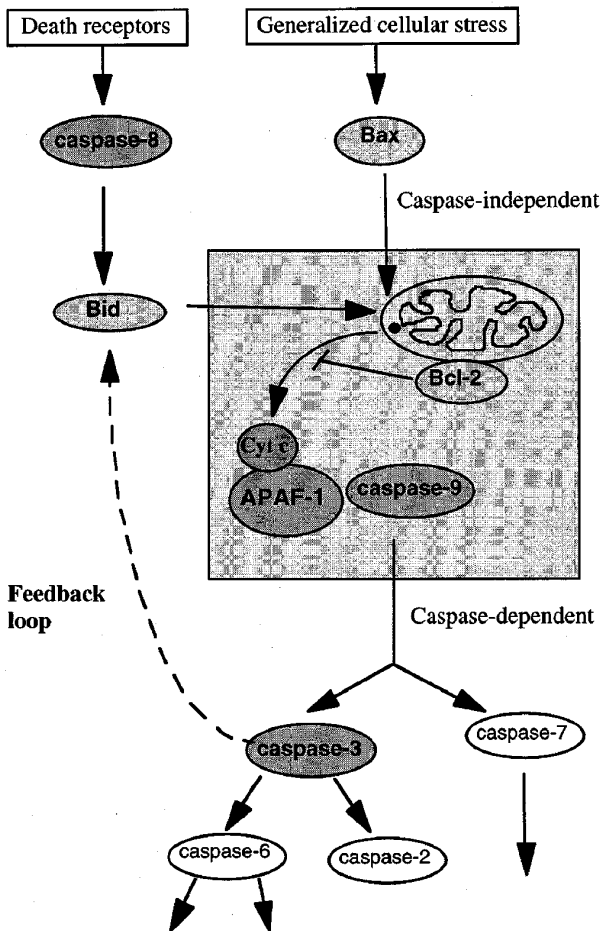
Materials and Methods

Materials

Anti-caspase-3 and anti-caspase-9 polyclonal antibodies were kindly provided by Drs. J Reed and D Green, respectively; anti-caspase-3, anti-caspase-7 and anti-gelsolin mouse monoclonal antibodies were purchased from Transduction Laboratories (Lexington, KY, USA); anti-caspase-8 antibody was purchased from Pharmingen (San Diego, CA, USA); purified rabbit polyclonal anti-caspase-6 antibody was purchased from Upstate Biotechnology (Lake Placid, NY, USA); anti-BID rat polyclonal antibody was kindly provided by Dr. J Yuan; anti-human/mouse BID goat polyclonal antibody was purchased from R&D Systems (Abingdon, UK); anti-BID rabbit polyclonal antibody was kindly provided by Dr. X Wang; anti-cytochrome *c* rabbit polyclonal antibody was kindly provided by Dr. D Newmeyer; anti-β-actin and anti-α-tubulin antibodies were purchased from ICN (Costa Mesa, CA, USA). Anti-Fas mAb clone CH-11 was purchased from Upstate Biotechnology. Ac-DEVD-CHO and Z-VAD-FMK peptides were purchased from BACHEM Bioscience (King of Prussia, PA, USA); VEID-CMK was purchased from Clontech (Palo Alto, CA, USA). Staurosporine, etoposide, cycloheximide and bovine heart cytochrome *c* were purchased from Sigma Chemical Co. (Poole, UK), and dATP was purchased from Boehringer Mannheim (Lewes, UK). Bacterially-expressed recombinant caspase-3 and caspase-8, kindly provided by Dr. G Salvesen, were produced and purified as described previously.[27]

Figure 7 Schematic representation of caspase-3/BID-mediated amplification of cytochrome c release

Cell culture and induction of apoptosis

Jurkat, CEM and MCF-7 cells were cultured in RPMI 1640 (Gibco, Paisley, UK) with 5% foetal calf-serum (Sigma). To induce apoptosis, Jurkat or CEM cells were suspended at 1×10^6 cells/ml, and incubated either alone or in the presence of anti-Fas CH-11 IgM (100 ng/ml), staurosporine (500 nM), cycloheximide (10 μM) or etoposide (10 μM), or exposed to UV radiation for 15 min. Where appropriate, the cells were pre-incubated for 1 h with z-VAD-fmk (50 μM).

Preparation of cell lysates for analysis by SDS–PAGE

Following incubation the cells were pelleted and washed twice in ice-cold phosphate buffered saline (PBS) PH 7.2, then resuspended at 4×10^7/ml in resuspension buffer (100 mM NaCl, 10 mM Tris pH 7.6, 1 mM EDTA). The cells were diluted to 2×10^7/ml in $2 \times$ SDS loading buffer and the cellular proteins separated by SDS polyacrylamide gel electrophoresis at 10^6 cells/lane.

Preparation of cell-free extracts

Post-nuclear extracts were generated from Jurkat T lymphoblastoid cells or MCF-7 cells essentially as previously described.[15] Briefly, cells ($2-5 \times 10^8$) were pelleted and washed $\times 2$ with PBS, pH 7.2. Cells were then transferred to a 2 ml Dounce-type homogenizer, were pelleted, and two volumes of ice-cold cell extract buffer (CEB) was added to the volume of the packed cell pellet (CEB; 20 mM HEPES-KOH, pH 7.5, 10 mM KCl, 1.5 mM MgCl$_2$, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 100 μM phenylmethylsulphonyl fluoride, 10 μg/ml leupeptin, 2 μg/ml aprotinin). Cells were allowed to swell under the hypotonic conditions for 15 min on ice, then disrupted with $\sim$40 strokes of a B-type pestle. Lysis was confirmed by examination of a small aliquot of the suspension under a light microscope. Lysates were then transferred to eppendorf tubes and were centrifuged at 15 000 $\times g$ for 15 min at 4°C to generate post-nuclear extracts. The supernatant was removed while taking care to avoid the nuclear and mitochondrial pellet. Supernatants were then frozen in aliquots at -70°C until required.

Depletion of caspases from cell extracts

Caspase-3, -6, or -7 were depleted from cell extracts using protein A/G-agarose-immobilized antibodies specific for these proteins, as follows. Aliquots (40 μl) of protein A/G agarose (Santa Cruz, CA, USA) were pre-coated with 5 μg of appropriate anti-caspase antibodies, or a control antibody (mouse or rabbit purified IgG; Sigma, UK), by incubation in a total volume of 300 μl in PBS, pH 7.2, for 3 h at 4°C under rotation. Antibody coated beads were washed three times in PBS before addition to Jurkat cell extracts (100 μl). Extracts were then incubated overnight under constant rotation at 4°C. Beads were removed from the extracts by centrifugation. Depletion of specific caspases from the extracts was confirmed by Western blotting, as previously described.[15]

Preparation of mouse liver mitochondria

Mitochondria were prepared fresh on the day of use and the entire procedure was undertaken on ice. Mouse livers (4–5 grams) were placed in mitochondria buffer (MTB); 250 mM mannitol, 1 mM EDTA, 5 mM KH$_2$PO$_4$, 5 mM MOPS/KOH pH 7.4) and chopped into fine pieces. Liver fragments were then washed 2–3 times in MTB to remove residual blood, followed by homogenization with 5–10 strokes

using a tight fitting teflon pestle. The homogenate was centrifuged at 500 $\times g$ for 10 min to pellet unbroken cells and debris, and the supernatant decanted into a fresh centrifuge tube. Mitochondria were pelleted by centrifugation for a further 7 min at 12 500 $\times g$. The mitochondrial pellet was resuspended in MTB, taking care to avoid any residual cells at the bottom of the tube, followed by pelleting again. Mitochondria were brought to a final volume of 1 ml in mitochondria storage buffer (MSB; 400 mM mannitol, 10 mM KH$_2$PO$_4$, 50 mM Tris pH 7.2, 5 mg/ml bovine serum albumen, 100 μM phenylmethylsulpho-nyl fluoride, 10 μg/ml leupeptin, 2 μg/ml aprotinin) and maintained on ice until required.

Coupled *in vitro* transcription/translation

^{35}S-methionine-labelled proteins were *in vitro* transcribed and translated using the TNT kit (Promega, Southampton, UK), as previously described.[15,28] Typically, 1 μg of plasmid was used in a 50 μl transcription/translation reaction containing 2 μl of translation grade [^{35}S]-methionine (1000 μCi/ml; NEN Life Sciences, Hounslow, UK).

Cell-free reactions

Cell-free reactions were typically set up in 10 or 20 μl reaction volumes. For 20 μl scale reactions, 10 μl of cell extract ($\sim$5 mg/ml) was brought to a final volume of 20 μl in CEB, with or without additions solubilized in the same buffer. Cell-free apoptosis was typically induced by addition of bovine heart cytochrome *c* and dATP to a final concentrations of 50 μg/ml and 1 mM, respectively. Extracts were then incubated at 37°C for periods of up to 3 h. When isolated mitochondria were included in the reaction, CEB was supplemented with mannitol (220 mM) and sucrose (68 mM).

Assessment of cytochrome *c* release in intact cells

To assess mitochondrial cytochrome *c* redistribution in intact cells, the cells were washed twice in PBS and resuspended in 100 μl MTB and broken open by centrifugation at 20 000 $\times g$ for 15 min at 4°C. The pellet was resuspended in the same supernatant and centrifuged again under the same conditions. Supernatants were then collected and frozen until required for analysis. In some experiments a different procedure was used. Here, the cells were transferred to a 2 ml Dounce homogeniser (after washing twice in PBS, pH 7.2), and were resuspended in CEB supplemented with 220 mM mannitol and 68 mM sucrose. After incubation on ice for 20 min, the cells were lysed by $\sim$50 strokes of the pestle and the debris pelleted by centrifugation at 14 000$-g$ for 15 min at 4°C. The supernatants were collected and normalised for protein content, then frozen until required.

SDS–PAGE and Western blot analysis

Proteins were subjected to standard SDS-polyacrylamide gel electrophoresis at 60–70 V and were transferred onto 0.45 μm PVDF membranes (Gelman, Northampton, UK) or 0.2 μm nitrocellu-lose membranes (Whatman, Maidstone, UK) overnight at 40–50 mA, followed by probing for various proteins using the antibodies described under Materials and Methods. Bound antibodies were detected using appropriate peroxidase-coupled secondary antibodies (Amerhsam Pharmacia Biotech, Little Chalfont, UK) followed by detection using the Supersignal chemiluminescence system (Pierce, Chester, UK), all as previously described.[15,28]

Acknowledgements

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