BID-dependent and BID-independent pathways for BAX insertion into mitochondria

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Abstract

In the absence of an apoptotic signal, BAX adopts a conformation that constrains the protein from integrating into mitochondrial membranes. Here, we show that caspases, including caspase-8, can initiate BAX insertion into mitochondria *in vivo* and *in vitro*. The cleavage product of caspase-8, tBID, induced insertion of BAX into mitochondria *in vivo*, and reconstitution *in vitro* showed that tBID, either directly or indirectly, relieved inhibition of the BAX transmembrane signal-anchor by the NH$_2$-terminal domain, resulting in integration of BAX into mitochondrial membrane. In contrast to these findings, however, *Bid*-null mouse embryo fibroblasts supported Bax insertion into mitochondria in response to death signaling by either TNFα or E1A, despite the fact that cytochrome *c* release from the organelle was inhibited. We conclude, therefore, that a parallel Bid-independent pathway exists in these cells for mitochondrial insertion of Bax and that, in the absence of Bid, cytochrome *c* release can be uncoupled from Bax membrane insertion. *Cell Death and Differentiation* (2000) 7, 1101–1108.

Introduction

Induction of apoptotic pathways in response to death signals is critically dependent on the status of survival/death regulators within a cell. Prominent among these is the BCL-2 family of anti-apoptotic (BCL-2, BCL-X$_L$, BCL-w, MCL-1, A-1) and pro-apoptotic (BAX, BAK, BOK) members, whose activities and ability to form heterodimers is influenced by a third subgroup of the BCL-2 family, which includes mammalian BID, BAD, BIK, BIM, BLK, HRK, and *C. elegans* EGL-1.[1,2] The latter are proapoptotic and contain a minimal apoptotic domain, BH3, which targets these proteins for interaction with BCL-2 proteins. Several of these 'BH3 domain only' members, including BID, BAD, and BIM, are themselves influenced by specific signal transduction pathways,[3–7] which serve to link the BCL-2·BAX checkpoint to upstream cell-death initiating events. BCL-2 and BAX each contain a single transmembrane segment at their extreme COOH-terminus, which is responsible for targeting these proteins into membrane sites, including mitochondria,[8–10] where their opposing functions influence organellar integrity and function.[11] In situations where anti-apoptotic BCL-2 members are limiting, mitochondria undergo profound dysfunction in response to most death signals. This includes release of cytochrome *c* from the intermembrane space,[12,13] which triggers activation of downstream caspases,[14] and ultimately induction of permeability transition at the inner membrane, resulting in loss of the electrochemical potential and production of excess reactive oxygen species.[11] Recently, mitochondrial transformations have been directly linked to cleavage of cytosolic BID by caspases, including caspase-8, in the CD95/Fas and TNFR1 cell death pathways, at least in certain cell types in culture. The resulting product, tBID, targets the organelle and induces cytochrome *c* release.[4–6] This signaling event in the Fas/TNFR1 pathway is likely an important contribution to apoptosis only in type II cells in culture, where upstream induction of the pathway following receptor-mediated activation of caspase-8 may be amplified via mitochondrial transformations.[15] Further, such amplification by mitochondria may involve additional factors that operate in parallel to BID.[16]

BAX, like BID, is constrained from targeting membrane sites, including mitochondria, until the cell receives a death signal.[9,17,18] In the absence of such a signal, BAX adopts a conformation in which the COOH-terminal transmembrane signal-anchor domain of BAX cannot insert into membranes, and this is dependent at least in part on the NH$_2$-terminal ART (Apoptotic Regulation of Targeting) domain. This repression by ART is relieved by a death stimulus and the signal-anchor now inserts BAX into mitochondrial membrane.[9] Membrane insertion is accompanied by a conformational change in the protein, in which the NH$_2$-terminus of BAX now becomes exposed.[19] Alternatively, BAX translocation can be uncoupled from death signals by forced overexpression[18,20,21] or forced dimerization.[18] Such induced translocation of BAX results in mitochondrial permeability transition[18,22] and in some contexts causes cytochrome *c* release.[21,23] Moreover, BID and BAX can interact[19,24] and both molecules can induce loss of mitochondrial integrity by mechanisms inhibited by BCL-2 proteins.[4,5,18,19,25,26]

Here, we have studied the mechanism that stimulates BAX insertion into mitochondria following a death signal. We demonstrate that caspase-generated p15 tBID, either

directly or indirectly, releases inhibition of the COOH-terminal signal anchor of BAX by the NH_2-terminal ART domain, and mediates BAX membrane integration. In certain cell types, however, parallel pathway(s) exist to achieve the same end.

Results

Activation of Fas causes recruitment of initiator procaspase-8 into the death-inducing signaling complex (DISC) via the adaptor molecule FADD.[27-29] This stimulates autoactivation of procaspase-8[29,30] which then initiates an apoptotic pathway that, in type II cells in culture, involves a mitochondrial-dependent amplification of caspase activation.[15,31] Recent genetic analysis[32,33] has revealed that caspase-8 is an obligate and non-redundant constituent at the apex of this pathway. In Figure 1A, type II human KB epithelial cells were mock-treated or treated with agonistic anti-Fas antibody in the presence of cycloheximide,[34] and BAX in whole cell lysate or in a heavy membrane fraction enriched in mitochondria[9] was detected by immunoblotting. Activation of Fas resulted in neither an increase in levels of total cellular BAX (cell lysate) nor in the amount of BAX recovered with mitochondria. Extraction of these mitochondria with 0.1 Na_2CO_3, pH 11.5, however, which liberates proteins that are peripherally associated with the surface of membranes but retains proteins that are integrated into the lipid bilayer,[35] revealed significant differences. Whereas TOM20, a protein import receptor constitutively integrated into the lipid bilayer of the outer membrane by a single transmembrane domain,[36] was equally resistant to alkaline extraction in mitochondria obtained from cells with or without Fas stimulation, BAX resisted alkaline extraction only in mitochondria from Fas-stimulated cells. Membrane integration of BAX was abolished, however, when Fas-stimulation was conducted in the presence of the wide-spectrum caspase inhibitor, zVAD-fmk. We conclude, therefore, that upstream caspase-8 in the Fas pathway can initiate a caspase-dependent pathway for BAX integration into mitochondrial membrane. Furthermore, murine tBid, which is generated by cleavage of p22 Bid by caspase-8 in the Fas pathway, also stimulated BAX integration into mitochondrial membrane when expressed ectopically in human H1299 epithelial cells in the absence of Fas stimulation, whereas a BH3-defective mutant of tBid, in which leu at position 90 within helix 3 was replaced with gly,[5] did not (Figure 1B).
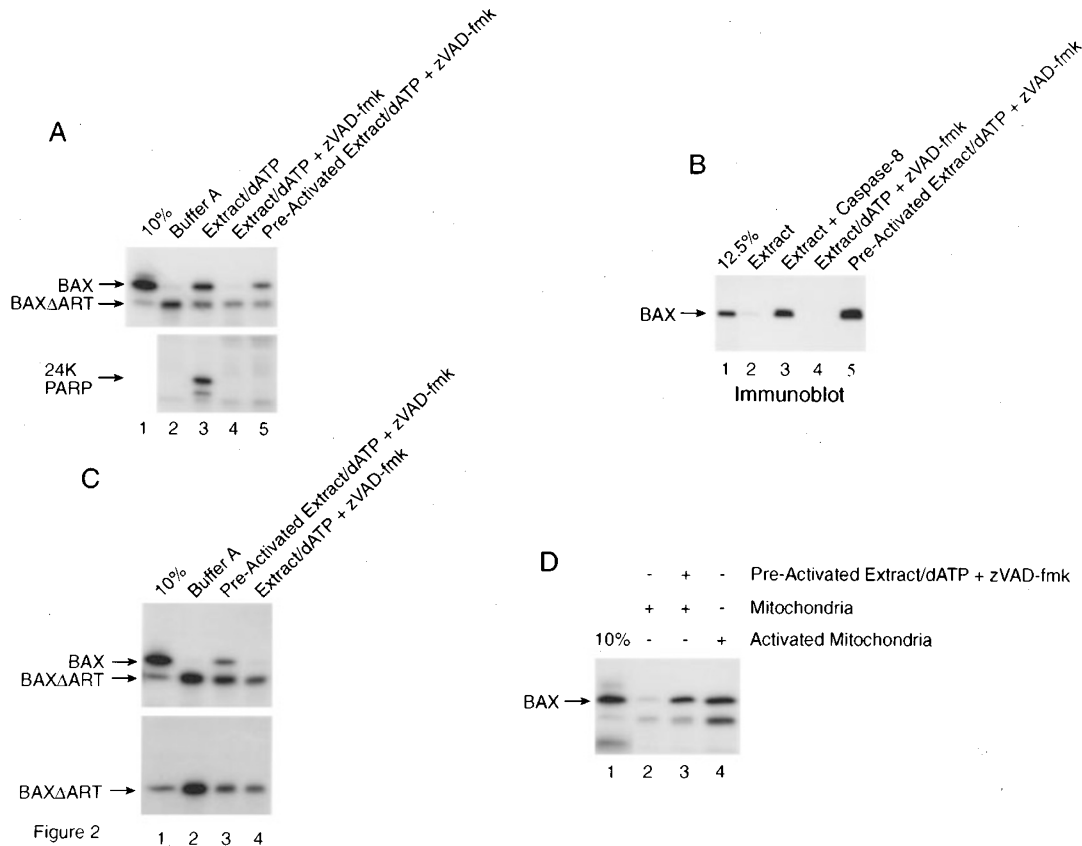
Reconstitution of caspase-dependent insertion of BAX into mitochondria *in vitro*

Consistent with the findings from Fas-stimulated KB cells, treatment of a control cytosol fraction from human HeLa epithelial cells[9] with caspase-8 induced the endogenous BAX in this fraction, when combined with purified rat heart mitochondria, to acquire resistance to alkali extraction, as assessed by immunoblotting (Figure 2B, lanes 1 and 2). Heart mitochondria were employed for these analyses because they can be isolated intact and contain negligible amounts of associated Bax, as determined by immunoblotting (not shown). As well, the treated extracts were capable of

subsequently cleaving exogenous PARP (not shown), indicating that caspases were in fact active. A similar amount of alkali-resistant BAX was observed upon dATP-activation of the endogenous caspases in HeLa extract at 37°C,[9,37] followed by incubation of the extract with mitochondria in the presence of the pan caspase inhibitor, zVAD-fmk (lane 5). In contrast, if zVAD-fmk was added to the extract prior to activation of endogenous caspases with dATP, BAX insertion into mitochondria was ablated (lane 4). Therefore, caspase(s) induce BAX membrane insertion by acting on a pre-existing constituent in the HeLa cell extract. Similar results were obtained for the influence of the zVAD-treated HeLa cell extract on membrane insertion of the ^{35}S-labeled, full-length BAX translation product (Figure 2A). Again, zVAD was
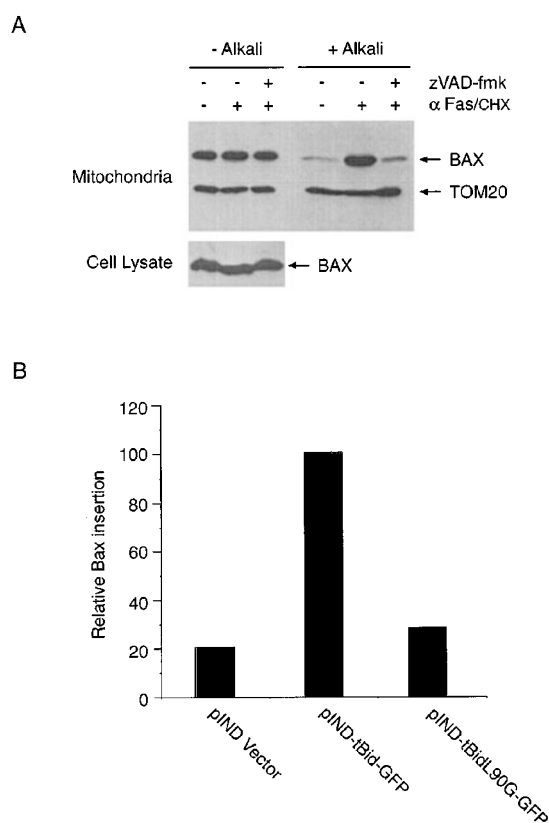
Figure 1 Induction of BAX insertion into mitochondrial membrane in human epithelial cells following stimulation of Fas or expression of murine tBid. (**A**) Human KB epithelial cells were mock-treated or treated with 0.5 μg/ml mouse monoclonal anti-human Fas (Upstate Biotechnology) and 10 μg/ml cyclohex-imide (CHX)[34] in the presence or absence of 50 μM zVAD-fmk for 14 h. Cells were homogenized and heavy membranes enriched in mitochondria recovered from the cell lysates,[9] subjected to SDS–PAGE either directly (−Alkali) or after extraction with 0.1 M Na_2CO_3 (+Alkali) (from twice the mitochondria as −Alkali),[9] and immunoblots developed with rabbit anti-BAX N-20 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and chicken anti-TOM20 and visualized by enhanced chemiluminescence. (**B**) Human H1299 epithelial cells were transfected with control vector, pIND-tBid-GFP, or pIND-tBid(L90G)-GFP and after 24 h, expression was induced with 5 μM ponasterone.[5] After 4 h, cells were recovered and the alkali-insoluble mitochondrial protein was analyzed by immunoblotting as in (**A**), and the bands quantified using a Power Macintosh 7200/120 and NIH Image v.1.61 image analysis software. BAX expression was normalized by dividing the BAX signal by the TOM20 signal, and setting the maximum value to 100

Figure 2 Caspase-dependent insertion of BAX into mitochondrial membrane in HeLa cell extracts requires a cytosolic factor(s). (**A**) Influence of caspase activation on BAX membrane insertion and PARP cleavage. Upper panel, [35S]BAX and [35S]BAXΔART translation products were incubated with isolated rat heart mitochondria for 60 min at 37°C under standard protein import conditions[9] in the presence of buffer (lane 2); extract/dATP, in which the caspases in the HeLa extract had been activated by dATP (lane 3); extract/dATP plus zVAD-fmk, in which the zVAD-fmk was added prior to caspase activation by dATP (lane 4); and pre-activated extract/dATP plus zVAD-fmk, in which the zVAD-fmk was added after activation of the caspases by dATP (lane 5). The mitochondria were subsequently collected, extracted with 0.1 M Na_2CO_3, pH 11.5,[9] and the integral membrane proteins analyzed by SDS–PAGE and fluorography.[9] Lane 1 represents 10% of input [35S]BAX. Lower panel, extracts were incubated as above in the absence of mitochondria and subsequently added to [35S]PARP translation product for 60 min at 30°C (lanes 2–5) and the 24 kDa apoptotic cleavage product of PARP detected by SDS–PAGE and fluorography.[9] (**B**) Influence of exogenous caspase-8. Purified mitochondria were incubated with untreated HeLa extract (lane 2); untreated extract plus 4 ng/μl caspase-8 (Pharmingen) (lane 3); extract/dATP plus zVAD-fmk (lane 4); and pre-activated extract/dATP plus zVAD-fmk (lane 5). The mitochondria were subsequently treated as in (**A**). Insertion of endogenous BAX in the extract was determined by immunoblotting with rabbit anti-BAX N-20 antibody (Santa Cruz), followed by enhanced chemiluminescence. Lane 1, BAX in 12.5% of the input HeLa extract. (**C**) Deletion of the BAX ART domain. As in (**A**) except that [35S]BAX and [35S]BAXΔART (upper panel) or [35S]BAXΔART (lower panel) translation products were incubated with mitochondria in the presence of buffer (lane 2); pre-activated extract/dATP plus zVAD-fmk (lane 3); and extract/dATP plus zVAD-fmk (lane 4). Lane 1, 10% of input translation product. (**D**) As in (**A**) except that [35S]BAX translation product was incubated with mitochondria in the presence of buffer (lane 2); pre-activated extract/dATP plus zVAD-fmk (lane 3); or the mitochondria were treated with pre-activated extract/dATP plus zVAD-fmk and the mitochondria re-isolated by centrifugation and resuspended and incubated for 60 min in the presence of buffer alone and [35S]BAX (lane 4)

inhibitory when added prior to dATP-dependent activation of extract caspases at 37°C (lane 4) but not when added after caspase activation (lane 5). In contrast, generation of the apoptotic 24 kDa caspase cleavage product of PARP was inhibited by zVAD-fmk in either circumstance (lanes 4 and 5). Of note, deletion of the NH_2-terminal 19 amino acid ART domain from BAX[9] allowed [35S]BAXΔART to bypass the requirement for the caspase-activated factor, and this was true if the BAXΔART translation product was presented to mitochondria either alone (Figure 2C) or together with full length BAX (Figure 2A,C).

Molecular seive chromatography indicated that most of the [35S]BAX translation product existed as a monomer (data not shown), consistent with the observations *in vivo* for the cytosolic form of the protein.[17,18] Incubation of this translation product with activated HeLa extract did not result in either cleavage of BAX or induction of a higher order structure. Though not conclusive, this suggests that the cytosolic factor influences BAX targeting either indirectly or at the level of the mitochondrion. Consistent with the latter, incubation of mitochondria with activated extract, followed by their re-isolation and subsequent incubation of these activated mitochondria in a standard BAX import reaction, in the absence of HeLa extract, revealed BAX insertion into mitochondrial membrane to a similar extent as for BAX import conducted with control mitochondria in the continued presence of activated extract (Figure 2D, lanes 3 and 4). This suggests that the caspase-regulated factor either associates with mitochondria or modifies a constituent of the organelle requisite for BAX membrane insertion, or both.
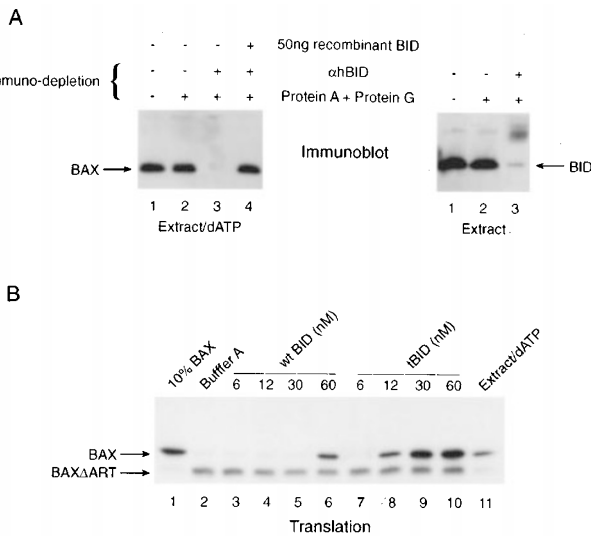
The caspase-regulated factor is BID

Partial purification of the caspase regulated factor in HeLa cell extracts revealed a BAX mitochondrial-insertion stimulating activity associated with a ~15 kDa protein (data not shown). BID's NH_2-terminal domain is removed by caspases, including caspase-8, to yield p15 tBID, which then targets mitochondria and induces mitochondrial dysfunction.[4–6] When BID was removed from HeLa cell extract by immunodepletion with antibody against BID prior to activation of caspases (Figure 3A, right panel lane 3), the ability of the endogenous BAX in these extracts to insert into mitochondrial membrane was lost upon subsequent caspase activation (left panel, lane 3). Addition of recombinant BID to these BID-depleted extracts reinstated BAX membrane insertion following activation of endogenous caspases (left panel, lane 4). Moreover, direct addition of low concentrations (12 nM) of recombinant tBID, generated by caspase-8 cleavage of full length BID,[5] to *in vitro* translated [35S]BAX could replace the requirement for activated extract and on its own stimulated [35S]BAX insertion into mitochondrial membrane (Figure 3B, lane 8). Full length BID was also stimulatory, but only at higher concentrations (lane 6). Likewise, full length BID can stimulate release of cytochrome *c* from mitochondria, but at concentrations higher than that of p15 tBID.[4–6] Consistent with the results using cell extracts (Figure 2A,C), however, membrane insertion of [35S]BAXΔART did not depend on and was not further stimulated by either BID or tBID, even at high concentrations of tBID (Figure 3B).

Effect of Bid gene deletion in mouse embryo fibroblasts

Previous analysis of embryonic fibroblasts from the $Bid^{-/-}$ mouse revealed only a slight delay in cell killing in response to TNFα compared to $Bid^{+/+}$ cells, yet a significant inhibition of cytochrome *c* release from mitochondria in the Bid-null cells was observed.[38] Similarly, $Bid^{-/-}$ mouse embryo fibroblasts were delayed in cell killing following expression of E1A oncoprotein (Figure 4A) yet, again, there was an inhibition of cytochrome *c* release from mitochondria as assessed by confocal microscopy, although the organelle did assume a condensed morphology as a consequence of E1A expression (Figure 4B). Analysis of the high-speed supernatant fraction from E1A-stimulated cells by immunoblotting likewise showed an inhibition of cytochrome *c* release to the cytosol in $Bid^{-/-}$ cells (Figure 4C). Of note, however, E1A expression resulted in Bax insertion into mitochondrial membrane to a similar extent in $Bid^{-/-}$ and $Bid^{+/+}$ cells over the time course examined (Figure 4D). This lack of difference between the two cell types was evident even though E1A stimulated Bid cleavage in the wild-type cells (Figure 4A, inset). Likewise, TNFα treatment stimulated Bax insertion into mitochondrial membrane to the same extent in $Bid^{-/-}$ and $Bid^{+/+}$ cells. Thus, in both cases cell death and Bax insertion into mitochondria can bypass the requirement for Bid in this murine cell type.

Discussion

Under normal physiological conditions, BAX membrane insertion is regulated and tied to specific signal transduction events.[9,17,18] In the absence of death signals, BAX adopts a conformation in which the COOH-terminal transmembrane-signal anchor is repressed and incapable of targeting the protein to mitochondria.[9,10] We show here that activation of caspases, both *in vivo* and *in vitro*, can over-ride this inhibition and BAX now inserts into mitochondrial membrane. This contribution by caspases may reflect an initiation and/or amplification of regulated BAX targeting. Further, we find that caspase-generated tBID is a direct stimulus of BAX insertion into mitochondrial membrane *in vitro* and can initiate BAX membrane insertion *in vivo* in the absence of other death signals. This dependence of BAX membrane insertion on tBID was bypassed, however, by deleting the BAX NH_2 ART domain. Deletion of the ART domain also enhanced the toxicity of BAX in transfected cells.[9] Therefore, at least in certain contexts, caspase-generated tBID is an upstream stimulus of BAX targeting that, directly or indirectly, can relieve repression of the COOH-terminal BAX transmembrane signal-anchor segment by the NH_2-terminal ART
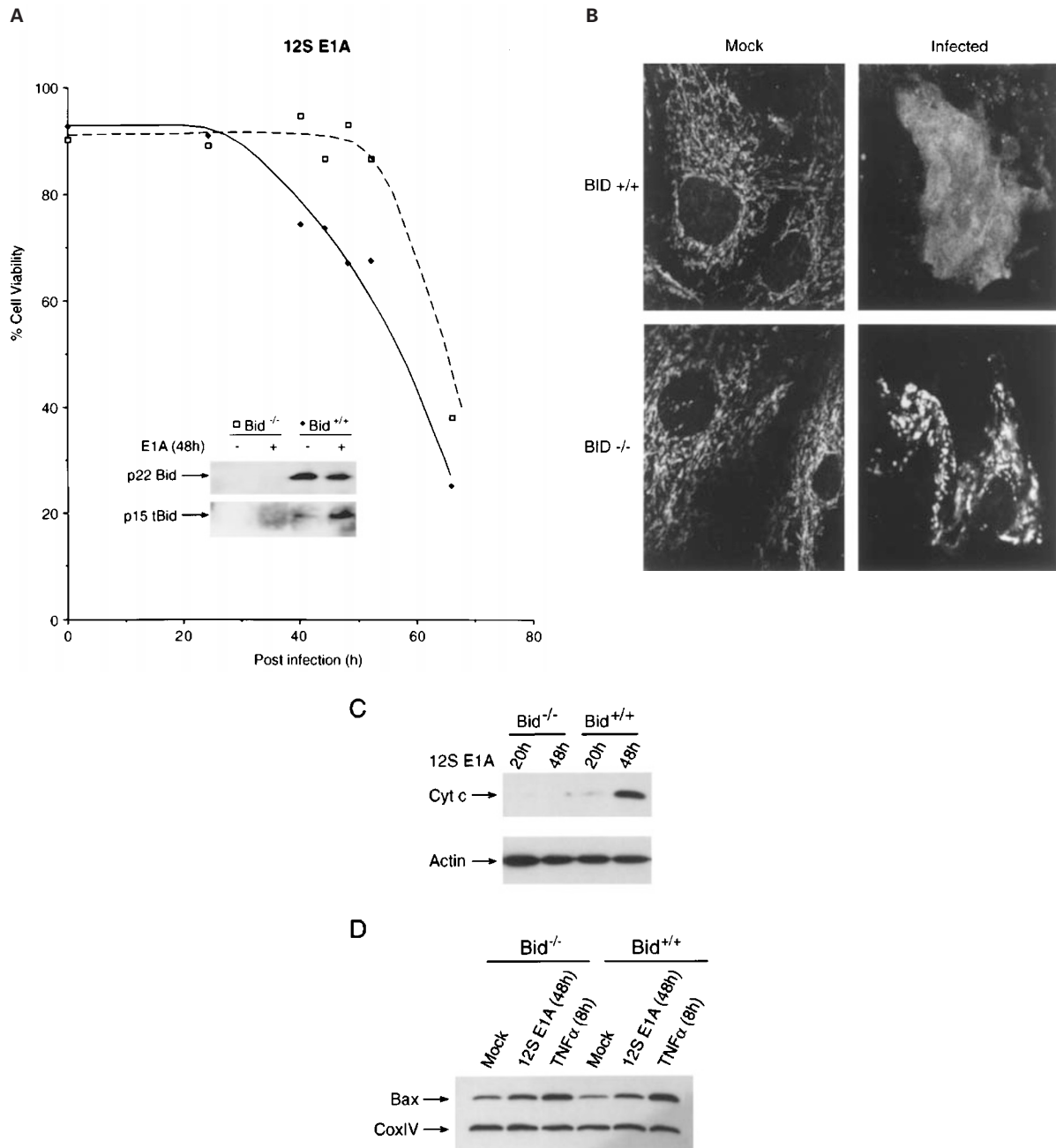

Figure 3 BID is required for caspase-dependent insertion of BAX into mitochondrial membrane in HeLa cell extracts. (**A**) Influence on endogenous BAX. Right panel. Untreated HeLa extract was incubated with Proteins A and G Sepharose in the presence (lane 3) or absence (lane 2) of rat anti-BID antibody, and immune complexes removed by centrifugation. Subsequently, a fraction of the immunodepleted extracts (lanes 2 and 3) and untreated extract (lane 1) were immunoblotted with the same anti-BID antibody and visualized by enhanced chemiluminescence. Left panel. Each of the extracts above were then activated by adding dATP. Rat heart mitochondria were subsequently incubated with extract/dATP (lane 1); mock immunodepleted extract/dATP (minus antibody) (lane 2); BID-immunodepleted extract/dATP (lane 3); and BID-immunodepleted extract/dATP with 50 ng of recombinant BID[5] added to it (lane 4). The mitochondria were then collected, extracted with 0.1 M Na_2CO_3, pH 11.5, and the insoluble protein analyzed by immunoblotting with rabbit anti-BAX N-20 antibody. (**B**) Translation products. [35S]BAX and [35S]BAXΔART translation products were incubated with mitochondria under standard protein import conditions[9] in the presence of buffer (lane 2); the indicated concentrations of either full length BID (lanes 3–6) or p15 tBID, which had been generated by cleavage of BID with caspase-8 (lanes 7–10)[5]; or extract/dATP (lane 11). The mitochondria were subsequently collected and analyzed as in Figure 2A

A

B

C

D

Figure 4 *Bid*^{+/+} and *Bid*^{−/−} mouse embryo fibroblasts. (**A**) Influence on cell killing by E1A. Primary embryo fibroblasts from *Bid*^{+/+} and *Bid*^{−/−} mice[38] were infected for the indicated times with adenovirus type 5 dl53OE1B[−] (expressing only 12 S E1A and no E1B products).[45] Cell viability was measured by exclusion of trypan blue. The data are an average of two independent determinations and are representative of multiple killing curves by E1A. After 48 h, infected (+) and mock-infected (−) cells were analyzed by immunoblotting with anti-mouse Bid (inset; the lower panel showing tBid was developed by chemiluminescence for twice as long as in the upper panel). (**B**) Influence of E1A expression on cytochrome *c* distribution. As in **A** except that cells, infected with or without dl52OE1B[−], were grown on glass cover slips for 48 h, fixed, and incubated with mouse monoclonal antibody 2G8.B6 against cytochrome *c* and anti-mouse IgG coupled to Texas Red, and visualized by immunofluorescence confocal microscopy. Representative images are shown. (**C**) As in (**A**) except that cells were infected for 20 or 48 h with adenovirus type 5 dl52OE1B[−] (expressing only 12 S E1A and no E1B products), and high-speed supernatant fractions were generated and analyzed by immunoblotting for cytochrome *c* and actin. (**D**) As in (**A**) except that cells infected with adenovirus type 5 dl52OE1B[−] for 48 h or with TNFα for 8 h[38] were homogenized and the heavy membrane fraction containing mitochondria was recovered and extracted with 0.1 M Na_2CO_3, pH 11.5.[9] The insoluble protein was subjected to SDS–PAGE and developed by immunoblotting with rabbit anti-BAX N-20 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and mouse anti-cytochrome *c* oxidase subunit IV (Cox IV) antibody[9]

domain,[9] permitting BAX integration into mitochondrial membrane (Figure 5). A number of point mutations within different regions in BAX, including the NH$_2$-terminal[39] and COOH-terminal domains,[10] and the putative pore-forming helices 5 and 6,[40] can also bypass the requirement for such regulation and permit constitutive targeting of BAX to

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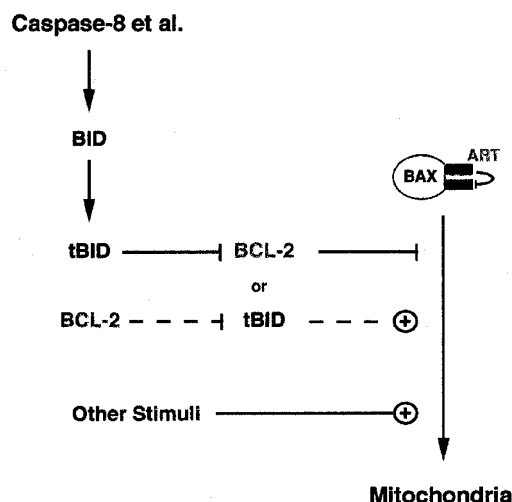
Figure 5 Working model for regulation of BAX insertion into mitochondria by BID. See the text for a description. The NH$_2$-terminal ART and COOH-terminal transmembrane domains of BAX are represented by upper and lower boxes, respectively

mitochondria, as can exposure of the wild-type protein to elevated pH.[39] As well, it will be interesting to learn if other BH3 domain-only proteins, such as BAD and BIM, can act like tBID and induce BAX targeting. It may be that the 'closed' inactive conformation of BAX, which correlates with inaccessibility of the NH$_2$-terminal domain to added protease,[9,19] can be perturbed or accessed by a variety of both intrinsic and extrinsic factors in addition to tBID, a situation that presumably accounts for the ability of $Bid^{-/-}$ mouse embryo fibroblasts to support Bax membrane insertion in response to different death signals. Additionally, however, our findings revealed that, in the absence of Bid, insertion of Bax into mitochondria did not result in release of cytochrome c from the organelle, indicating that the well-documented ability of Bax to stimulate cytochrome c release may depend on cooperation with Bid in vivo.

The role of BID and other potential regulators in controlling BAX targeting to mitochondria must be interpreted in the context of two conditions that enable BAX to bypass such regulation in vivo. Forced over-expression[20–22] and forced dimerization[18] of BAX both result in constitutive mitochondrial integration and cell death.[1] Overexpression might saturate an inhibitory pathway of BAX membrane integration, a role that has been ascribed to anti-apoptotic BCL-2/BCL-X$_L$ family members.[18] In this context, tBID might inactivate the BCL-2 death suppressors[4–6] (Figure 5). Likewise, forced dimerization might preclude the influence of BCL-2 suppressors on BAX distribution, or cause a conformational change in BAX that bypasses regulation of its targeting to mitochondria.

A second possibility is that tBID takes a more direct role in BAX integration into mitochondria.[19,41] For example, it may act as a receptor for BAX, inducing a conformational change in BAX and subsequent membrane insertion (Figure 5). In this scenario, BCL-2-related suppressors, if in excess, may bind and inactivate tBID. This model is consistent with studies in vitro showing that recombinant full-length BID can interact directly with BAX and support both BAX insertion into mitochondria and subsequent release of cytochrome c from the organelle.[19,41] It is not clear, however, how this model reconciles with manipulations to BAX (e.g., over-expression) that allows BAX to bypass the requirement for tBID as receptor or, conversely, with Bax membrane insertion being observed in stimulated Bid-null mouse embryo fibroblasts.

Finally, integration of tBID into mitochondria might exert influences beyond the regulation of other BCL-2 family members. For example, ion channel activity of tBID has been detected in vitro.[42] Also, the involvement of regulators in the endoplasmic reticulum that influence BAX activity has been recorded.[43] Further insights into the role of tBID in regulating BAX membrane insertion will undoubtedly emerge by elucidating the structural basis for this influence by tBID and by assessing the potential requirement for other factor(s) in this pathway.

Materials and Methods

General

Earlier studies describe the routine procedures for cell culture and infection with adenovirus type 5 dl52OE1B$^-$ expressing only 12S E1A and no E1B products),[44,45] and conducting immunocytochemistry by confocal microscopy, synthesizing [^{35}S]BAX transcription-translation product in reticulocyte lysate, and isolating mitochondria from rat heart and cultured cells.[9]

Insertion of BAX into mitochondrial membrane *in vitro*

Apoptotic cell extracts were prepared from HeLa cells exactly as described by Goping et al.[9] Twenty μl of extract (approximately 10 mg protein/ml), either alone or with 5 μl of ^{35}S-methionine-labeled Bax transcription-translation product or 5 μl of extract buffer, were incubated in a standard protein import reaction (50 μl) containing purified mitochondria from rat heart (1.0 mg protein/ml).[9] Alternatively, the cell extraction buffer (20 μl) alone replaced the extract in control reactions. The reaction mixtures were incubated for 60 min at 37°C in the absence of additives, or containing 1 mM dATP (extract/dATP), or containing 1 mM dATP and 50 μM tetrapeptide zVAD-fmk added either at the beginning (extract/dATP+zVAD-fmk) or at the end (pre-activated extract/dATP+zVAD-fmk) of the incubation period. The mitochondria were recovered by centrifugation[9] and were analyzed by SDS–PAGE and fluorography to detect [^{35}S]Bax or by immunoblotting with rabbit anti-BAX N20 antibody (Santa Cruz) to detect BAX derived from the HeLa cell extract. Analysis of rat heart mitochondria alone by immunoblotting revealed negligible Bax associated with the organelle. Alternatively, mitochondria isolated from reaction mixtures were resuspended (0.25 mg protein/ml) in freshly prepared 0.1 M Na$_2$CO$_3$, pH 11.5, and incubated for 30 min on ice. The membranes were then collected in an airfuge operating at 30 p.s.i. for 10 min prior to analysis by fluorography or immunoblotting.

Cytochrome *c*

Cells (4×10^6) were washed in PBS and suspended in 0.1 ml HIM buffer (200 mM mannitol, 70 mM sucrose, 1 mM EDTA, 10 mM HEPES, pH 7.5). After one cycle of freeze and thaw, the cells were homogenized with 25 strokes in a motorized Teflon-glass homogenizer operating at 500 r.p.m., and centrifuged at $800 \times g$ for 10 min to remove nuclei and cell debris. The supernatant was centrifuged at $100\,000 \times g$ for 10 min and aliquots from equivalent numbers of cells were subjected to SDS–PAGE and immunoblotting with mouse monoclonal 7H8.2C12 anti-cytochrome *c*.

Bid-null mouse embryo fibroblasts

Mouse embryo fibroblasts were prepared from 9.5 day-old embryos of mice carrying a homozygous deletion in the coding region of *Bid*.[38] They were cultured in Iscove's modified Dulbecco's medium containing 20% fetal calf serum, and infected with adenovirus type 5 *dl*52OE1B$^-$ (expressing only 12S E1A and no E1B products) or treated with TNFα, as described.[38,46]

Acknowledgments

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