

Conformational control of Bax localization and apoptotic activity by Pro168

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In healthy cells, Bax resides inactive in the cytosol because its COOH-terminal transmembrane region (TMB) is tucked into a hydrophobic pocket. During apoptosis, Bax undergoes a conformational change involving NH₂-terminal exposure and translocates to mitochondria to release apoptogenic factors. How this process is regulated remains unknown. We show that the TMB of Bax is both necessary and sufficient for mitochondrial targeting. However, its availability for targeting depends on Pro168 located within

the preceding loop region. Pro168 mutants of Bax lack apoptotic activity, cannot rescue the apoptosis-resistant phenotype of Bax/Bak double knockout cells, and are retained in the cytosol even in response to apoptotic stimuli. Moreover, the mutants have their NH₂ termini exposed. We propose that Pro168 links the NH₂ and the COOH terminus of Bax and is required for COOH-terminal release and mitochondrial targeting once this link is broken.

Introduction

The Bcl-2 family members Bax and Bak are crucial mediators of apoptosis during development and disease. Mice deficient in both proteins exert gross developmental defects and are not viable (Lindsten et al., 2000; Ranger et al., 2001). Moreover, cells derived from these animals are resistant to numerous apoptotic stimuli, including overexpression of proapoptotic BH3-only proteins (Cheng et al., 2001; Wei et al., 2001; Letai et al., 2002). BH3-only proteins, which act as sensors of cellular stress, are activated by transcriptional up-regulation and/or posttranslational modification following an apoptotic stimulus (Puthalakath and Strasser, 2002). Once activated, these proteins translocate to mitochondria inducing the activation of Bax/Bak by yet unknown mechanisms (Eskes et al., 2000; Bouillet et al., 2001; Cheng et al., 2001; Letai et al., 2002; Moreau et al., 2003). As a consequence, Bax and Bak form oligomeric pores (Griffiths et al., 1999; Antonsson et

al., 2000, 2001) leading to the release of apoptogenic factors from mitochondria into the cytosol (Jürgensmeier et al., 1998; Rossé et al., 1998; Degenhardt et al., 2002).

Both Bax and Bak are tail anchored in the mitochondrial outer membrane (MOM) via a COOH-terminal hydrophobic transmembrane domain that is followed by basic residues (Wolter et al., 1997; Goping et al., 1998; Nechushtan et al., 1999; Suzuki et al., 2000; del Mar Martinez-Senac et al., 2001). Bak constitutively localizes to the MOM where, following an apoptotic stimulus, it is activated by conformational change and/or relieved from inhibitory proteins (Griffiths et al., 1999). In contrast, Bax resides primarily in the cytosol and requires an initial activation step to promote its translocation to the MOM (Wolter et al., 1997; Goping et al., 1998; Gross et al., 1998; Hsu and Youle, 1998; Capano and Crompton, 2002). The solution structure of Bax revealed that its transmembrane region (TMB) is folded back into a hydrophobic pocket formed by its BH1, BH2, and BH3 domains (Suzuki et al., 2000). This conformation not only prevents Bax targeting to the MOM but also inhibits the binding of regulatory proteins to the hydrophobic pocket.

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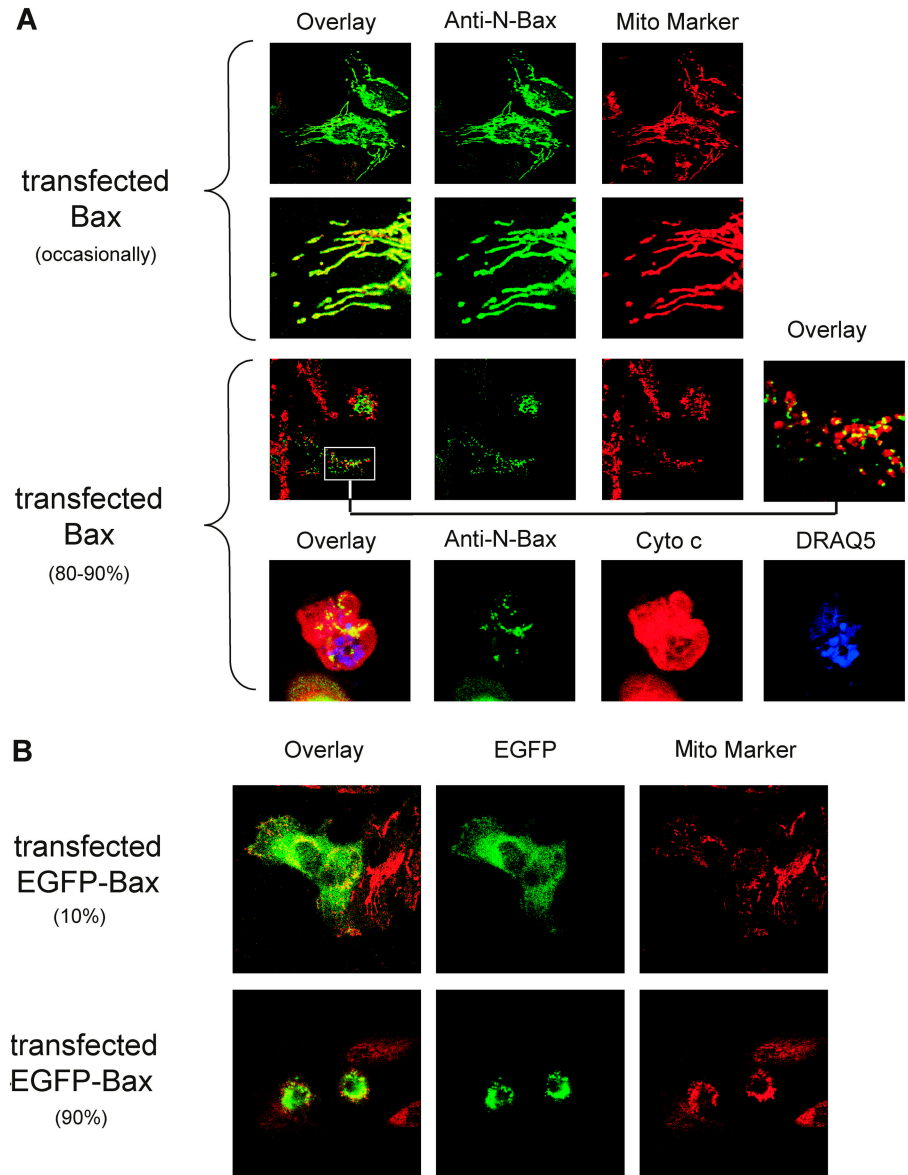
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Key words: apoptosis; Bcl-2 family; NH₂-terminal exposure; mitochondria; targeting

Abbreviations used in this paper: ART, apoptosis-regulating targeting domain; IVTT, in vitro transcription/translation; MEF, mouse embryo fibroblast; MOM, mitochondrial outer membrane; PI, propidium iodide; PPIase, prolyl cis/trans isomerase; TMB, transmembrane region.

Figure 1. Intracellular localization and cytotoxicity of transfected Bax and EGFP-Bax. (A) Confocal microscopy of HeLa cells transfected with Bax for 16 h using anti-N-Bax and an antibody against a mitochondrial protein (Mito Marker) for colocalization (Overlay). The fourth row shows an apoptotic cell (nuclear stain DRAQ5) with clustered Bax and cytochrome c released. (B) Direct fluorescence of EGFP-Bax in HeLa cells together with the mito marker.

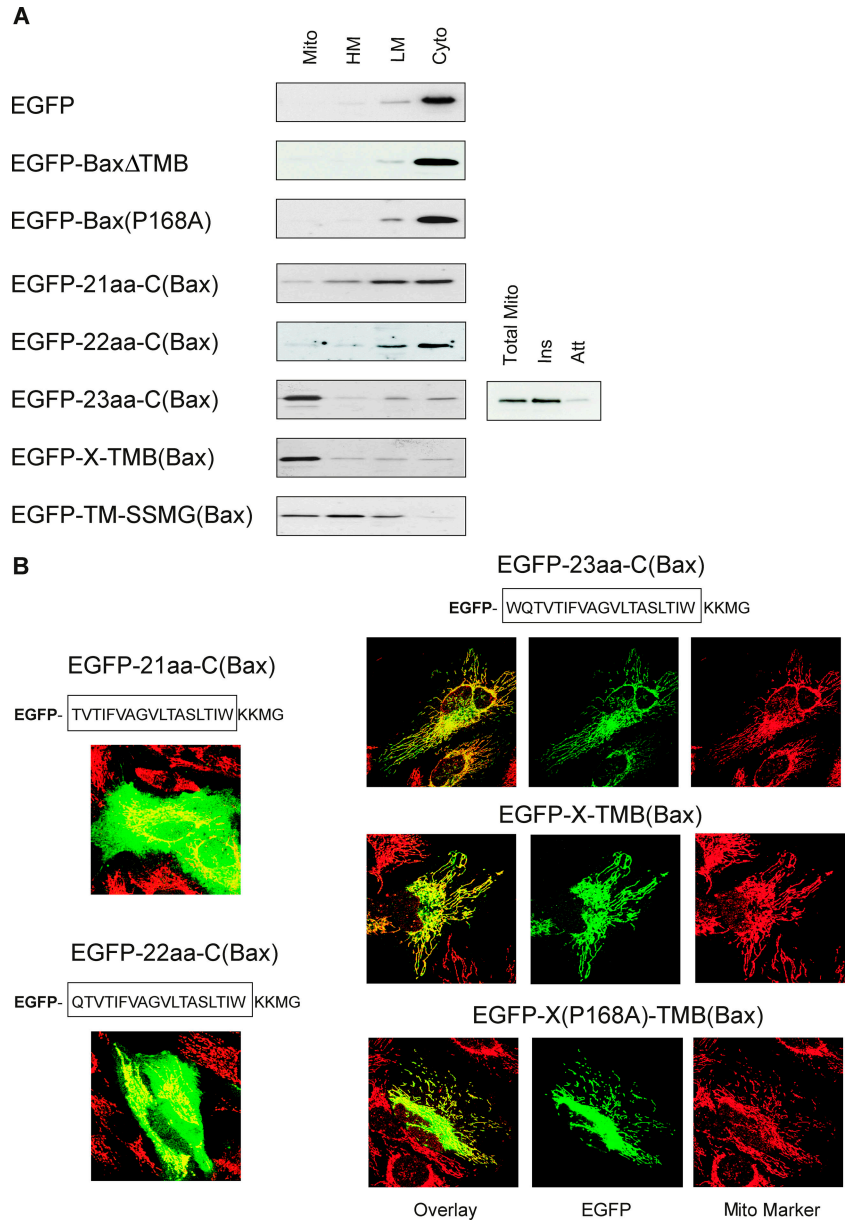


Although the COOH-terminal TMB of Bax possesses the properties of a transmembrane domain, it is still unknown whether once released from the hydrophobic pocket it also serves as a targeting signal for the MOM. Nechushtan et al. (1999) have shown that although the last 21 amino acids of the TMB fused to GFP were insufficient for mitochondrial targeting, they form a mitochondria targeting signal when Ser184 was deleted or mutated to Val or Ala. Ser184 stabilizes the inactive conformation of Bax by forming a hydrogen bond with Asp98 in the hydrophobic pocket (Suzuki et al., 2000). Therefore, two events may be necessary to target Bax to the MOM via the COOH-terminal TMB: (1) breaking the TMB-pocket interaction, and (2) increasing the hydrophobicity of Ser184 (mimicking the S184A mutation). Alternatively, Cartron et al. (2003) proposed that an NH₂-terminal sequence of Bax encompassing amino acids 20–37 mediates MOM targeting. Upon activation, Bax undergoes a conformational change during which it exposes an NH₂-terminal epitope, but the significance of this exposure is unknown (Hsu and

Youle, 1997, 1998; Nechushtan et al., 1999). As NH₂-terminal exposure occurs simultaneously with mitochondrial translocation, it may either unveil an NH₂-terminal targeting sequence or facilitate the release of the TMB from the hydrophobic pocket. Three proteins have recently been suggested to regulate targeting of Bax to the MOM by binding to the NH₂ terminus (Ku70; Sawada et al., 2003), the COOH terminus (humanin; Guo et al., 2003), or both (14-3-3 θ ; Nomura et al., 2003). The absence of these proteins increased Bax translocation and cytotoxicity but only in the presence of an apoptotic stimulus, indicating that these proteins stabilize Bax in the cytosol but that additional proteins and/or posttranslational modifications are required to trigger its conformational change and mitochondrial translocation.

In this work, we show that mitochondrial translocation of Bax is mediated by the 23 amino acids of the COOH-terminal TMB. Moreover, we define Pro168 in the loop preceding the TMB as a crucial amino acid that couples NH₂-terminal exposure to the release of the COOH-terminal TMB.

Figure 3. Mitochondrial targeting of Bax requires the 23 amino acids of the TMB region. (A) Anti-GFP Western blots of subcellular fractions from HEK293 cells expressing the indicated EGFP fusion constructs. To test the mitochondrial association of EGFP-23aa-C(Bax), isolated mitochondria were treated with sodium carbonate (pH 12) to remove loosely attached proteins (Att), and the pellet was extracted with detergent (right, Ins). (B) EGFP (green) and anti-mito marker (red) fluorescence analysis of HeLa cells transiently transfected with the indicated EGFP fusion constructs.



punctate mitochondrial staining as wild-type Bax (Fig. 1 A and not depicted), and was effectively targeted to mitochondria in vitro (see Fig. 8 A). However, when we also removed the TMB in the Bax Δ N20 mutant (Bax Δ N20 Δ TMB), the protein became cytoplasmic and nontoxic (unpublished data). These data show that Bax requires the TMB, and not the NH₂-terminal amino acids 20–37, for mitochondrial targeting. Moreover, through its interaction with the hydrophobic pocket, the TMB seems to protect Bax from inappropriate aggregation in the cytosol.

The COOH-terminal TMB region is both necessary and sufficient for mitochondrial targeting

It has been reported that the last 21 amino acids of the COOH-terminal TMB domain are insufficient for mitochondrial targeting, unless its hydrophobicity is increased by mutating Ser184 to Ala or Val (Nechushtan et al., 1999). Thus, a regulatory mechanism mimicking this mutation may

be required to make the COOH terminus competent for mitochondrial targeting. However, we envisaged the possibility that sequences upstream of these 21 amino acids, which still belonged to the TMB, could contribute to this targeting process (Fig. 2 A). Therefore, we fused the last 21, 22, and 23 amino acids of Bax to EGFP and expressed the chimeras in HeLa and HEK293 cells. In agreement with Nechushtan et al. (1999), transiently transfected EGFP-21aa-C(Bax) resided primarily in the cytoplasm (Fig. 3, A and B). Some of the fusion protein was found in membrane fractions, presumably because of the hydrophobicity of the 21 amino acids, but these membranes were not specifically mitochondrial (Fig. 3 A). When the COOH terminus was extended to 22 amino acids, the EGFP-22aa-C(Bax) fusion protein still displayed cytosolic fluorescence staining (Fig. 3 B) and the majority was found in the cytosol and on microsomes upon subcellular fractionation (Fig. 3 A). In contrast, the EGFP-23aa-C(Bax) construct, which includes the entire TMB domain of

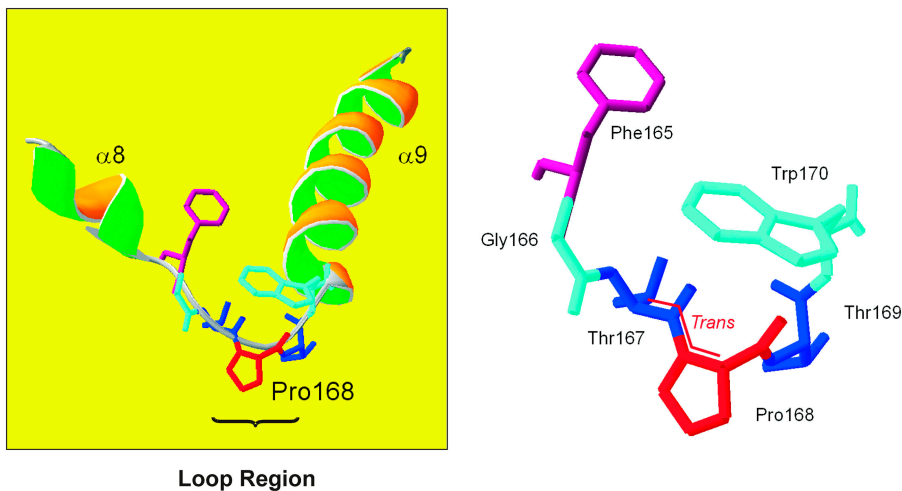


Figure 4. **Modeling of the solution structure of Bax in the region of X-TMB (Pro168).** Molecular modeling of Bax using the Deep View Swiss-PDB viewer (Schwede et al., 2003) and based on the Bax NMR solution coordinate data from Suzuki et al. (2000). Phe165–Trp170 are in a solvent-exposed loop of which Pro168 is at the turning point. Pro168 is in the trans configuration and is close to Trp170, the first amino acid in the TMB targeting sequence.

Bax (Fig. 2 A), specifically colocalized with mitochondrial markers (Fig. 3 B). At least one basic residue was required at the end of the TMB for correct targeting (see supplemental material and Fig. S4, available at <http://www.jcb.org/cgi/content/full/jcb.200309013/DC1>). As expected for a TMB domain that serves as a targeting and membrane insertion device, the EGFP-23aa-C(Bax) localized with and inserted into the MOM (Fig. 3 A). Extending the TMB targeting sequence to its upstream X-domain (EGFP-X-TMB(Bax); Fig. 2 A) did not alter mitochondrial targeting (Fig. 3, A and B), indicating that the 23 amino acids of the TMB are necessary and sufficient for mitochondrial targeting.

Pro168 regulates NH₂-terminal exposure and the release of the COOH-terminal TMB targeting sequence

We assumed that the release of the COOH-terminal TMB from its hydrophobic pocket requires a conformational change that is regulated by amino acids in the loop region directly upstream of the α -helical TMB (Fig. 2 A). This loop is solvent-exposed and may be modified or bound by regulatory proteins implicated in the release process (Fig. 4). All five amino acids within this loop region (X-domain, Phe165–Thr169) were mutated and their intracellular localization was determined after transient transfection of HeLa or HEK293 cells using NH₂-terminal antibodies. Most point mutations in the loop region did not affect the mitochondrial targeting of Bax, and transfectants had an identical appearance to those overexpressing wild-type Bax (unpublished data). However, when Pro168 was deleted (Bax Δ P168) or substituted for Ala (Fig. 2 A, Bax(P168A)), 80–90% of both HeLa (Fig. 5 A) or HEK293 transfectants (Fig. S2) displayed a diffuse cytoplasmic Bax staining, were stretched out, and had intact nuclei. Moreover, the Pro168 mutants were only slightly cytotoxic (Fig. 5 C). Strikingly, both Bax Δ P168 and Bax(P168A) were detected with NH₂-terminal antibodies, indicating that their NH₂-terminal epitopes were exposed in the cytoplasm. This exposure was confirmed by using the NH₂-terminal Bax antibody to immunoprecipitate stably expressed FLAG-tagged Bax or Bax(P168A) from HeLa cell extracts (Fig. 5 B). Only low levels of FLAG-Bax could be captured with the antibody, whereas FLAG-Bax(P168A) was effectively immunoprecipitated from the lysate although both proteins were ex-

pressed at similar levels (Fig. 5 B). These data show that the Bax Δ P168 and Bax(P168A) mutants had the NH₂-terminal epitope exposed but failed to translocate to mitochondria and induce apoptosis.

To further substantiate that the release of the COOH-terminal TMB was dependent on Pro168 and not on the exposure of the NH₂-terminal epitope, we deleted the NH₂-terminal 20 amino acids in the Bax(P168A) mutant. This double mutant confirmed our results, suggesting that Bax is targeted to the MOM via its COOH-terminal TMB rather than the NH₂-terminal amino acids 20–37. Bax Δ N20/P168A exhibited a diffuse cytoplasmic staining after transfection into HeLa cells (Fig. 5 A) and was as slightly cytotoxic in the HEK293/EGFP survival assay as Bax(P168A) (Fig. 5 C). Thus, Bax requires Pro168 for COOH-terminal unlashment, mitochondrial targeting, and cytotoxicity even when the NH₂ terminus is deleted.

Proline does not have the ability to form a classical hydrogen bond within the protein backbone. This property imposes some restrictions on the secondary and tertiary structures of proteins defining its role as a helix breaker. If Pro168 were to play a purely structural role in the TMB of Bax, its replacement by another helix-breaking amino acid such as glycine would not be expected to change the mitochondrial targeting and cytotoxic properties of Bax. However, a Bax(P168G) mutant was indistinguishable from Bax Δ P168 or Bax(P168A) with respect to survival activity, cytoplasmic retention, and NH₂-terminal exposure (Fig. 5, A and C). This was also the case when Pro168 was mutated to Glu, a helix-forming amino acid (Fig. 5, A and C, Bax(P168E)). These results suggest that the helix-breaking capacity of Pro168 is not involved in Bax COOH-terminal TMB release. To confirm that Pro168 was responsible for the release and not the targeting function of the TMB, we created an EGFP-X-TMB(Bax) (loop-TMB) fusion protein in which Pro168 was substituted for Ala. This EGFP-X(P168A)-TMB(Bax) protein was targeted to mitochondria as efficiently as the EGFP-X-TMB(Bax) construct (Fig. 3 B). Together, our data suggest that Pro168 is not part of the COOH-terminal TMB targeting sequence but is a crucial amino acid for regulating the release of this targeting sequence from the hydrophobic pocket. Most importantly, Pro168 inhibits NH₂-terminal ex-

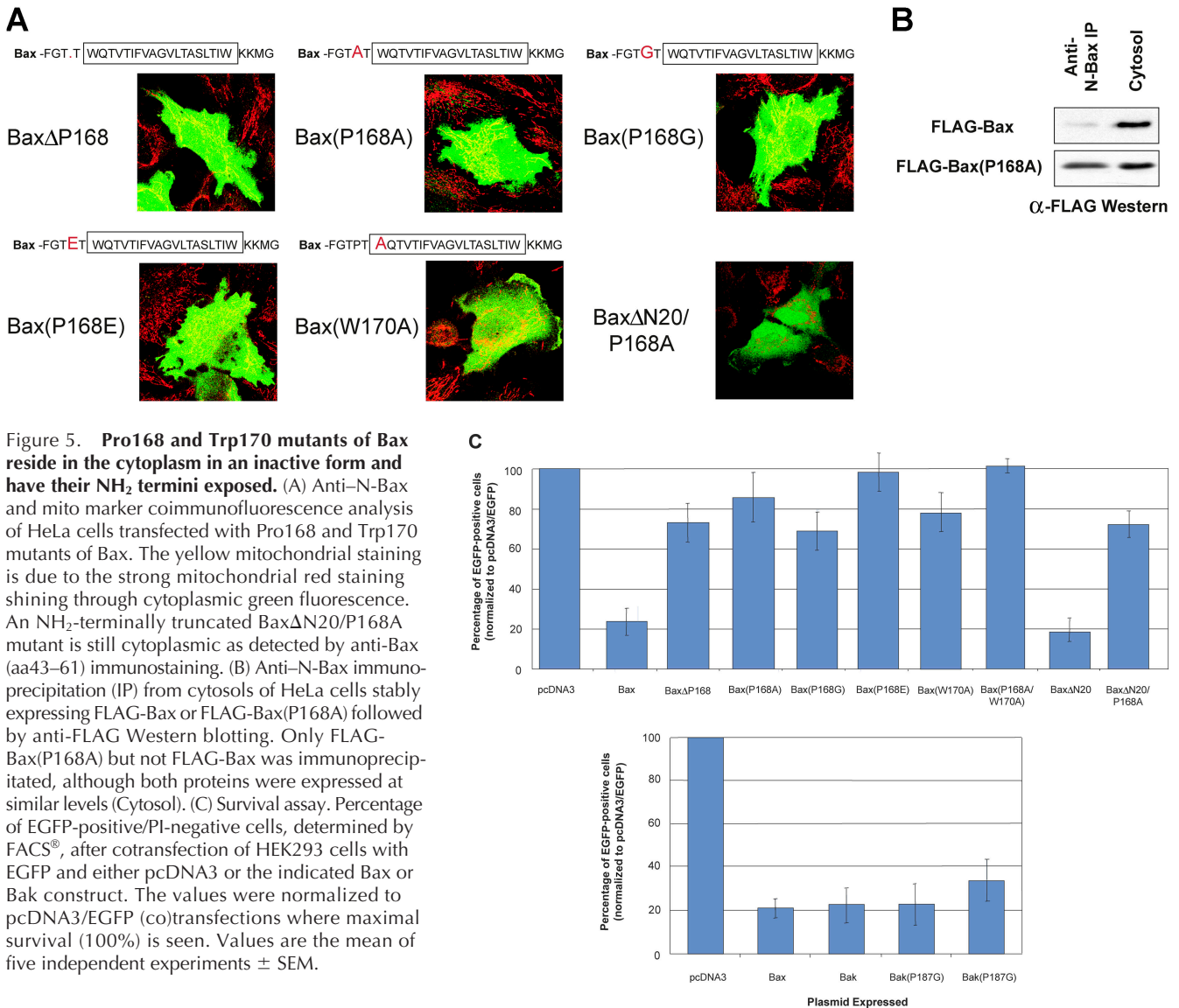


Figure 5. Pro168 and Trp170 mutants of Bax reside in the cytoplasm in an inactive form and have their NH₂ termini exposed. (A) Anti-N-Bax and mito marker coimmunofluorescence analysis of HeLa cells transfected with Pro168 and Trp170 mutants of Bax. The yellow mitochondrial staining is due to the strong mitochondrial red staining shining through cytoplasmic green fluorescence. An NH₂-terminally truncated BaxΔN20/P168A mutant is still cytoplasmic as detected by anti-Bax (aa43–61) immunostaining. (B) Anti-N-Bax immunoprecipitation (IP) from cytosols of HeLa cells stably expressing FLAG-Bax or FLAG-Bax(P168A) followed by anti-FLAG Western blotting. Only FLAG-Bax(P168A) but not FLAG-Bax was immunoprecipitated, although both proteins were expressed at similar levels (Cytosol). (C) Survival assay. Percentage of EGFP-positive/PI-negative cells, determined by FACS[®], after cotransfection of HEK293 cells with EGFP and either pcDNA3 or the indicated Bax or Bak construct. The values were normalized to pcDNA3/EGFP (co)transfections where maximal survival (100%) is seen. Values are the mean of five independent experiments ± SEM.

posure indicating that it somehow couples the NH₂ terminus to the loop-TMB region of Bax.

Pro168 mutants of Bax are not aberrantly folded and reside in the cytoplasm in a stable form

It is possible that mutations in Pro168 changed the structure of Bax in a way that the protein would aggregate and be rapidly degraded. To exclude this possibility, we determined the size of Bax protein complexes in the cytosol of FLAG-Bax- and FLAG-Bax(P168A)-transfected HeLa cells by gel filtration analysis. As shown in Fig. S5 A (available at <http://www.jcb.org/cgi/content/full/jcb.200309013/DC1>), endogenous Bax, overexpressed FLAG-Bax, and mutant FLAG-Bax(P168A) were all immunodetected in fractions corresponding to monomeric Bax (20–35 kD). No high molecular mass aggregates were observed for any of the Bax forms. Most importantly, both FLAG-Bax and FLAG-Bax(P168A) oligomerized in the presence of the nonionic detergent NP-40 indicating that they undergo the same conformational change as Bax (Fig. S5 B). In addition, the Pro168 mutants of Bax

were stable proteins. The steady-state expression of EGFP-Bax(P168A) and EGFP-Bax(P168G) proteins did not significantly change for 24–48 h after transfection (Fig. S6 A, available at <http://www.jcb.org/cgi/content/full/jcb.200309013/DC1>), nor were the proteins rapidly degraded in the presence of CHX (Fig. S6 B). In contrast, EGFP-Bax protein levels decreased (Fig. S6 B) and were almost lost at 24–48 h after transfection (Fig. S6 A), presumably due to the loss of EGFP-Bax-expressing cells by apoptosis (Figs. S3 and S6 B).

Pro168 mutants of Bax do not translocate to mitochondria after staurosporine treatment

Apoptotic stimuli such as the kinase inhibitor staurosporine provoke the exposure of the NH₂-terminal helix α1 and mitochondrial translocation of endogenous cytosolic Bax (Eskes et al., 1998). Similarly, staurosporine can trigger the mitochondrial translocation of transfected cytosolic GFP-Bax, and hence promotes apoptosis in cells that express low levels of GFP-Bax (Nechushtan et al., 1999). To investigate the contribution of Pro168 to the staurosporine-induced trans-

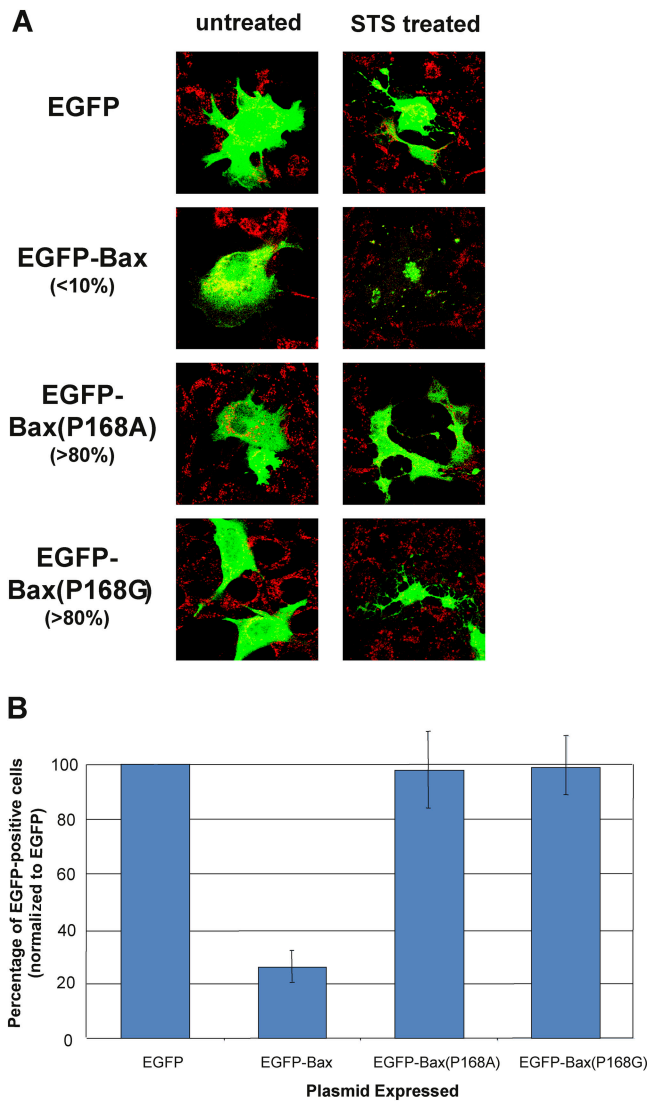


Figure 6. Pro168 mutants of Bax do not translocate to mitochondria in response to staurosporine. (A) EGFP (green) and anti-mito marker (red) fluorescence analysis of MEFs transfected with EGFP, EGFP-Bax, or two EGFP-Bax Pro168 mutants either untreated or treated with 1 μ M staurosporine (STS) for 4 h. Only 10% of the transfectants express cytosolic EGFP-Bax, whereas >80% exhibited cytosolic EGFP-Bax(P168A) or EGFP-Bax(P168G) fluorescence. In response to STS, the Pro mutants are retained in the cytosol and the cells remain stretched out. (B) Survival assay of the EGFP fusion proteins in HEK293 cells was performed as described in Fig. 5 C. Values were normalized to EGFP and are the mean of five independent experiments \pm SEM.

location of GFP-Bax, we generated Pro168 mutants of EGFP-Bax and studied their intracellular localization in mouse embryo fibroblasts (MEFs) in either the presence or absence of 1 μ M staurosporine by direct fluorescence (Fig. 6 A). In addition, we tested the apoptotic potential of the EGFP variants in HEK293 cells using FACS[®] analysis (Fig. 6 B). EGFP alone did not induce apoptosis and was highly expressed in the cytoplasm of both untreated and staurosporine-treated MEFs (Fig. 6). In contrast, most of the EGFP-Bax transfectants were lost (Fig. 6 B). The few surviving cells contained low levels of cytosolic EGFP-Bax of which all translocated to mitochondria in response to staurosporine

(Fig. 6 A). As shown for Bax(P168A) (Fig. 5 C), EGFP-Bax variants in which Pro168 was mutated to Ala or Gly were only mildly toxic (Fig. 6 B) and localized to the cytoplasm (Fig. 6 A, left) and a cytosolic fraction (Fig. 3 A). Strikingly, even after 4 h of staurosporine treatment, the majority of MEFs expressing EGFP-Bax(P168A) and EGFP-Bax(P168G) were still stretched out, contained intact nuclei, and retained the mutant proteins in the cytoplasm (Fig. 6 A, right). Thus, Pro168 is required for the release of the COOH-terminal TMB targeting sequence and the subsequent mitochondrial translocation and cytotoxicity of Bax even in the presence of an external apoptotic stimulus.

Pro168 mutants of Bax do not sensitize Bax/Bak DKO cells to drug-induced apoptosis

MEFs deficient in both Bax and Bak (DKO) are highly resistant to drug-induced apoptosis (Wei et al., 2001). If Pro168 mutants of Bax lack proapoptotic activity, they should not be able to sensitize these cells for apoptosis. We stably expressed EGFP-Bax and EGFP-Bax(P168A) to approximately the same levels in Bax/Bak DKO MEFs (Fig. 7 A) and determined the extent of etoposide-induced apoptosis by FACS[®] analysis of PI-positive cells. As shown in Fig. 7 B, EGFP-Bax resensitized the DKO MEFs for apoptosis to almost the level of wild-type MEFs. In contrast, the EGFP-Bax(P168A)-expressing cells remained resistant to the drug. These data confirm the lack of proapoptotic activity of Bax(P168A).

Disruption of the TMB-pocket interaction facilitates in vitro mitochondrial targeting

To further study the mechanisms of TMB unleashing, we developed an in vitro assay in which the cDNAs of Bax and its mutants were transcribed/translated in rabbit reticulocyte lysates in the presence of [³⁵S]methionine. The translation mixture was centrifuged to eliminate protein clusters formed during translation, and then added to isolated mitochondria (Fig. 8). Only ~20% of Bax synthesized in vitro was targeted to mitochondria. In contrast, Bcl-x_L, another member of the Bcl-2 family was effectively targeted to mitochondria in vitro (~60%; Fig. 8) presumably due to the constitutive exposure of its TMB (Kaufmann et al., 2003). We predicted that if inefficient mitochondrial targeting of Bax were due to the folding of the TMB into the hydrophobic pocket, any disruption of this interaction would enhance mitochondrial association. As expected, a Bax mutant in which the entire pocket was destroyed by deleting helices α 5 and α 6 (Bax Δ 5/6) was as efficiently targeted to mitochondria as Bcl-x_L (Fig. 8). Increased mitochondrial targeting was also observed with Bax(S184A), in which hydrogen bonding between the TMB and the pocket was disrupted, as well as with Bax Δ N20, which lacked the first 20 amino acids of Bax (Fig. 8 A). However, the targeting efficiency of these mutants was somewhat lower than for Bcl-x_L or Bax Δ 5/6. These results suggest that the NH₂ terminus interacts with the COOH-terminal TMB and that both the removal of the NH₂ terminus and a breakage of the TMB-pocket interaction facilitates mitochondrial targeting.

We used the in vitro transcription/translation (IVTT) assay to confirm the role of Pro168 in the unleashing and

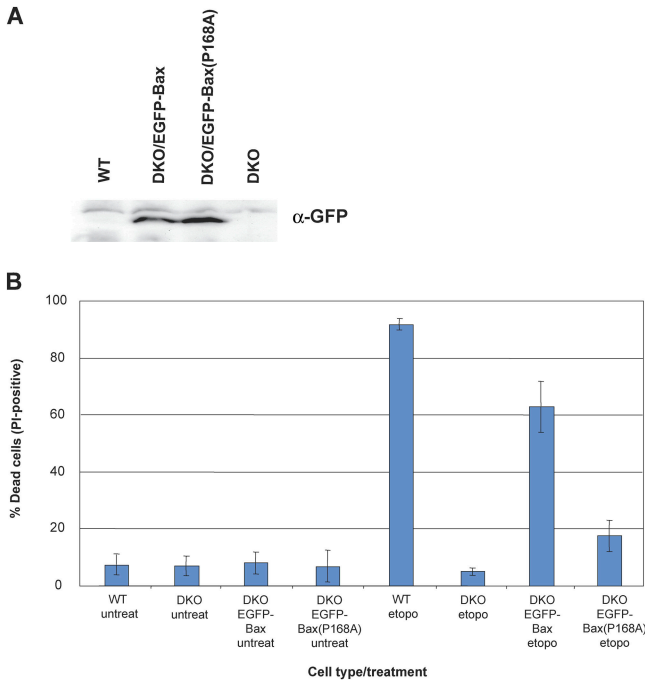


Figure 7. Proapoptotic activity of EGFP-Bax and EGFP-Bax(P168A) in Bax/Bak DKO MEFs. (A) Bax/Bak-deficient MEFs (DKO) were transduced with retroviruses carrying EGFP-Bax/pLPCx or EGFP-Bax(P168A)/pLPCx plasmids. Mixed populations of cells were FACS[®]-sorted, grown-up, and analyzed for the expression of the respective proteins by anti-GFP Western blotting. As controls, parental MEFs (WT) and DKO MEFs were used. (B) The cells described in A were left untreated or treated with 100 μ M etoposide (etopo) for 24 h and stained with 10 μ g/ml propidium iodide (PI), and the percentage of PI-positive dead cells was determined by FACS[®] analysis. The values are taken from fluorescence intensity $>10^2$ of the FACS[®] histogram and are the mean of four independent experiments \pm SEM.

mitochondrial targeting of the TMB of Bax. As variants of Bax with Pro168 mutations were observed to be cytoplasmic, they were expected to be targeted to the mitochondria to a lesser extent than Bax *in vitro*. Indeed, the amounts of Bax Δ P168, Bax(P168A), Bax(P168G), and Bax(P168E) recovered in the mitochondrial pellet were approximately half that of wild-type Bax under the same experimental conditions (Fig. 8 A and not depicted).

Trp170 cooperates with Pro168 for TMB unleasment

Trp170 is the first amino acid of the TMB mitochondrial targeting sequence (Fig. 3, A and B). In addition, as seen in the structure of Bax (Fig. 4), this amino acid is in close contact with Pro168. Therefore, we wondered whether or not Trp170 would assist Pro168 in unleashing the TMB by forming a binding pocket for a putative TMB releasing factor. For that purpose, we generated Bax mutants in which Trp170 was mutated to Ala. As shown in Fig. 5 (A and C), Bax(W170A) had its NH₂ terminus exposed, was retained in the cytoplasm, and was only mildly cytotoxic after transfection into HEK293 cells. A double mutant of Bax in which both Trp170 and Pro168 were mutated to Ala (Bax(P168A/W170A)) did not induce apoptosis at all (Fig. 5 C). These data indicate that Trp170 is not only part of the TMB targeting sequence but cooperates with Pro168 in regulating

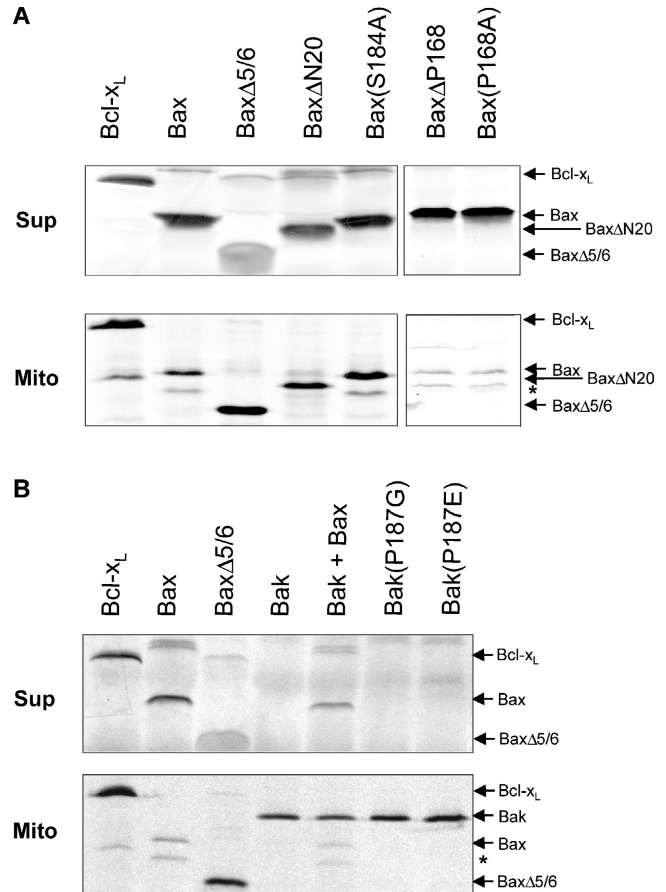


Figure 8. Targeting of *in vitro* translated Bax, Bak, and mutants to isolated mitochondria. Autoradiography of [³⁵S]methionine-labeled IVTT Bax, Bak, and mutants bound to mitochondria (Mito) or remaining in the supernatant after spinning down the organelles (Sup). Bcl-x_L is used as a control because it translocates efficiently to mitochondria *in vitro* (Kaufmann et al., 2003). In one lane, Bax and Bak were mixed 1:1 before adding to mitochondria (B). This mixture did not enhance Bax translocation. Asterisks denote a Bax protein with an alternative start codon.

NH₂-terminal exposure and the release of the TMB for mitochondrial targeting.

Proline-directed release of the COOH-terminal TMB does not occur with the related protein Bak

As shown in Fig. S1 C, endogenous Bak stably associates with mitochondria in nonapoptotic cells. Bak possesses a Pro residue, Pro187, at a similar position upstream of the TMB as Bax (Fig. 2 A). To determine if mitochondrial targeting of Bak was also dependent on a proline-mediated release of its TMB region, we determined the targeting properties and cytotoxicity of Bak and two Pro187 mutants, Bak(P187G) and Bak(P187E), using the *in vitro* mitochondrial targeting and the EGFP survival assays. In contrast to Bax, wild-type Bak and the Bak(P187G) and Bak(P187E) mutants were effectively targeted to mitochondria after *in vitro* translation (Fig. 8 B) and were cytotoxic (Fig. 5 C, bottom). As it was reported that Bak and Bax can form heterodimers or oligomers (Mikhailov et al., 2003), and this formation may disrupt the interaction between the TMB and the hydrophobic pocket of Bax, we further tested if Bak could assist Bax in the TMB un-

leashment and mitochondrial targeting. However, the extent of Bax translocation to mitochondria remained low when Bax and Bak were cotranslated *in vitro* (Fig. 8 B). Thus, proline-directed unleashing of the TMB mitochondrial targeting sequence is a regulatory mechanism specific for Bax and does not seem to play a role in the mitochondrial targeting of other Bcl-2 family members such as Bak.

Discussion

Our work provides novel insights into the mechanisms by which Bax translocates to the MOM where it carries out its function. First, we show that in nonapoptotic cells, Bax is not only cytosolic but also peripherally attached to mitochondria. Second, we define the last 23 amino acids of the COOH-terminal TMB domain as both necessary and sufficient for mitochondrial targeting and membrane insertion of Bax. Third, we identify Pro168 as a residue essential for regulating the conformational change of Bax leading to the exposure of the NH₂ terminus and the release of the TMB from its hydrophobic pocket.

Although Bax is reported to reside exclusively in the cytoplasm of tissue cells (Hsu and Youle, 1998; Letai et al., 2002), we confirm that there is an appreciable amount of the protein peripherally attached to mitochondria in cultured cell lines (Goping et al., 1998; Desagher et al., 1999). We suggest that this interaction of Bax with the MOM is not dependent on the TMB targeting sequence for the following reasons: (a) EGFP-TMB is inserted in the MOM rather than loosely attached (alkaline-sensitive; Fig. 3 A); (b) small amounts of endogenous Bax can also reside on other organelle membranes, such as the ER, but in an alkaline-sensitive form (Scorrano et al., 2003; Zong et al., 2003); and (c) the NH₂ terminus of alkaline-sensitive mitochondrial Bax is not exposed (Fig. S1 D). These findings suggest that endogenous Bax remains in an inactive conformation on the membranes of unstimulated cells before undergoing the structural changes required for membrane insertion and proapoptotic activity.

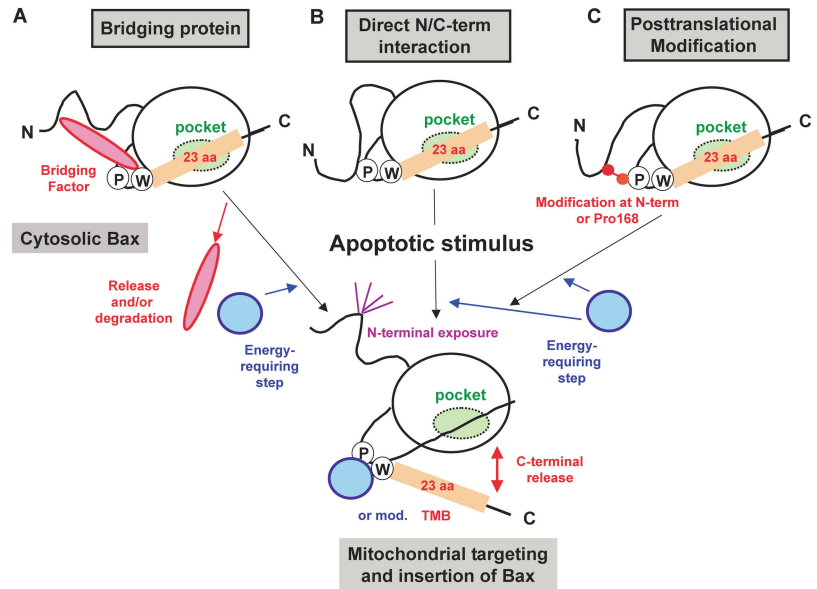
It has remained controversial whether or not the translocation of Bax to mitochondria in apoptotic cells is mediated via its NH₂ or COOH terminus. Recently, an NH₂-terminal sequence at amino acids 20–37 (α -helix 1) has been reported to act as a mitochondrial targeting signal when fused to GFP (Cartron et al., 2003). However, we think that this sequence does not mediate the rapid, early Bax translocation in apoptotic cells. First, it is preceded by an NH₂-terminal inhibitory domain called apoptosis-regulating targeting domain (ART; amino acids 1–20), which must first be cleaved off to expose the targeting signal (Goping et al., 1998; Gao and Dou, 2000). Indeed, a calpain-like protease has been shown to mediate ART cleavage (Wood et al., 1998; Gao and Dou, 2000; Choi et al., 2001; Toyota et al., 2003). Because proteolysis is a late event in apoptosis and is prevented by calpain inhibitors without affecting Bax translocation and cell death, it probably serves to accelerate, rather than initiate, Bax translocation and apoptotic signaling through mitochondria (Cao et al., 2003). Second, Bax mutants lacking the ART sequence (Bax Δ N20) still depended on the COOH-terminal TMB (not depicted) and Pro168 (Fig. 5 A, Bax Δ N20/P168A) for mitochondrial targeting. Third, Cartron et al. (2003) found

that the NH₂-terminal targeting sequence only mediates an alkaline-sensitive interaction of Bax with the mitochondrial membrane, indicating that the NH₂ terminus may contribute to the relatively nonspecific interaction of endogenous Bax in nonapoptotic cells. However, based on our results, the 23 amino acids of the COOH-terminal TMB mediate Bax translocation and MOM insertion in apoptotic cells.

In many apoptotic systems, Bax activation has been defined by the exposure of an NH₂-terminal epitope (Hsu and Youle, 1997, 1998; Nechushtan et al., 1999). Although this exposure coincides with Bax translocation to mitochondria, it has remained difficult to explain why it can occur hours before cytochrome *c* release and be reversible upon removal of the apoptotic stimulus (Gilmore et al., 2000; Valentijn et al., 2003). These findings indicate that NH₂-terminal exposure is not the commitment step in Bax activation. Why then is it important and what does it initiate? One possibility is that the NH₂ terminus interacts with the COOH terminus either directly or via a binding protein as previously proposed (Fig. 9; Ruffolo et al., 2000). Ku70 has recently been identified as an NH₂-terminal binding protein of Bax (Sawada et al., 2003). Overexpression of Ku70 retains Bax in the cytoplasm, supporting the notion that it may prevent the release of the COOH-terminal TMB. Similarly, a COOH-terminal binding protein, humanin, was shown to prevent Bax translocation after transfection into human cells (Guo et al., 2003). However, neither reducing endogenous Ku70 nor humanin expression sensitized cells for apoptosis or Bax translocation in the absence of an apoptotic stimulus. This finding implies that the loss of NH₂- or COOH-terminal binding proteins is not sufficient for Bax activation and that an additional, probably energy-requiring step, provided by an apoptotic stimulus is needed to provoke the release of the COOH-terminal TMB (Suzuki et al., 2000; Fig. 9). A putative trigger could be a member of the subfamily of BH3-only proteins because these proteins are known to be activated in response to apoptotic stimuli and to bind to the hydrophobic pocket of multidomain Bcl-2 family members with high affinity (Puthalakath and Strasser, 2002). Such a mechanism has been proposed for disengaging the COOH terminus from the hydrophobic pocket of the survival factor Bcl-w (Denisov et al., 2003; Hinds et al., 2003) and for relieving the COOH-terminal TMB of Bax by the NH₂-terminal domain (Ruffolo et al., 2000). However, Liu et al. (2003) recently presented the structure of Bcl-x_L complexed with a fragment of the BH3-only protein Bim and predicted, based on the structural homology between Bcl-x_L and Bax, that it is the α -helix 8 rather than the COOH-terminal TMB (α 9) that is displaced by the binding of Bim to Bax. Consistent with this view, Suzuki et al. (2000) suggested that the binding of a BH3-only protein to Bax does not confer enough energy for the release of the COOH-terminal TMB.

We provide evidence that Pro168 is a crucial amino acid for regulating both NH₂-terminal exposure and the release of the COOH-terminal TMB. First, Pro168 mutants of Bax had their NH₂ termini exposed in the cytoplasm in a non-toxic form. This exposure shows that NH₂-terminal exposure is neither sufficient for targeting nor for Bax activation and cytotoxicity. It is possible that the Pro mutants were detected with NH₂-terminal antibodies because endogenous cytoplas-

Figure 9. **Integrated model of mitochondrial association of Bax in healthy and apoptotic cells.** See Discussion for details.



mic Bax saturated an NH₂-terminal binding protein. However, Nechushtan et al. (1999) reported that transfection of an S184K mutant of Bax, which was constitutively retained in the cytoplasm, did not have its NH₂ terminus exposed. Moreover, as shown here, Pro168 mutants even remained in the cytoplasm in response to an apoptotic stimulus such as staurosporine, which is known to provoke Bax translocation. Thus, although COOH-terminal releasing factors (the energy-requiring step) induced by the apoptotic stimulus may have been present, the COOH-terminal TMB was probably not released due to a mutation of Pro168. Our data show that it is not the helix-breaking activity of Pro168 that determines COOH-terminal release, but that a binding protein or posttranslational modification on Pro168 could be the trigger. In this respect, Trp170, which is the first amino acid of the TMB targeting sequence, may contribute to a protein binding site as it is juxtaposed to Pro168 in the cytosolic structure (Fig. 4), and Bax variants mutated at Trp170 exhibited NH₂-terminal exposure and nonapoptotic cytoplasmic localization like the Pro168 mutants (Fig. 5).

What kind of modification or binding activity could operate on Pro168 and Trp170? Prolines can be the target amino acids for prolyl *cis/trans* isomerases (PPIases). These enzymes accelerate the slow *cis*-to-*trans* isomerization of prolines (Galat and Metcalfe, 1995). Pro368 of diphtheria toxin has been suggested to be a target site for a PPIase and to contribute to the conformational change required for the pore formation of the toxin in the membrane (Johnson et al., 1993). Diphtheria toxin is structurally related to Bax (Muchmore et al., 1996; Suzuki et al., 2000), and, thus, isomerization of Pro168 may be a crucial step for TMB unleasement, mitochondrial targeting, and membrane insertion. Molecular modeling of the soluble Bax structure revealed that the Pro168 is in its *trans* configuration (Fig. 4). Thus, PPIases could favor/accelerate the folding process of Bax after its synthesis on ribosomes. However, our preliminary data suggest that cyclosporine A and rapamycin, inhibitors against the classical PPIases cyclophilin and TOR, respectively, had no effect on Bax translocation and cytotoxicity. This finding does not

exclude the participation of another PPIase, such as Pin1, which has recently been shown to regulate the apoptotic activity of p53 (Zacchi et al., 2002; Zheng et al., 2002). Alternatively, Pro168 may bind or release specific proteins after apoptosis stimulation or be the target for a posttranslational modification such as hydroxylation (Kivirikko et al., 1989).

Together, we propose the following model for the mitochondrial translocation and activation of Bax in apoptotic cells (Fig. 9). In the cytosol of nonapoptotic cells, the NH₂ terminus is linked to Pro168 (and Trp170) either directly (Fig. 9 B) or via a binding protein (Fig. 9 A) or a posttranslational modification (Fig. 9 C) and prevents COOH-terminal unleasement. In response to an apoptotic stimulus, the interaction between the NH₂ and COOH terminus is disrupted by the degradation of the binding protein or other modifications. Although this process leads to the exposure of the NH₂ terminus and sensitizes Bax for activation, it is not sufficient for COOH-terminal release. An additional binding protein or a posttranslational modification is required that acts on Pro168 (and Trp170) and provides the energy to release the COOH-terminal TMB for mitochondrial targeting. Further work is required to identify these regulators of NH₂-terminal exposure and COOH-terminal release. They will be promising targets for new therapies against diseases in which Bax translocation needs to be stimulated (cancer) or inhibited (neurodegeneration).

Materials and methods

cDNAs and site-directed mutagenesis

All EGFP constructs were made in the pEGFP-C1, -C2, or -C3 vectors (Invitrogen). The cDNAs for FLAG-Bax, the various Bax and Bak mutants, and the EGFP fusion proteins were generated by PCR or standard cut and paste techniques as described in the online supplemental material. All constructs were verified by dideoxynucleotide sequencing.

Immunofluorescence analysis

HeLa cells, HEK293 cells, and MEFs were grown on 17-mm glass coverslips until they reached 70% confluence. The cells were transfected with 0.8 μg of plasmid DNA and 2.4 μl of Superfect for 3 h at 37°C and placed in growth medium. After 16 h, the cells were fixed in 4% PFA and perme-

abilized with 0.05% saponin and ice-cold acetone. The cells were incubated with anti-N-Bax (detecting amino acids 1–20; Upstate Biotechnology; 1:200) in the presence of either anti-cytochrome c (BD Biosciences; 1:50), anti-mitochondria (BP128; The Binding Site Ltd.; 1:300), or anti-hBcl-2 (clone 124; DakoCytomation; 1:100) as colocalization markers for 90 min followed by Alexa Fluor 488 (green)– and/or Alexa Fluor 546 (red)–conjugated goat anti-rabbit or anti-mouse secondary antibodies (Molecular Probes) for another 60 min. After postfixation in 4% PFA containing 2 µg/ml Hoechst 33342 dye (Molecular Probes) or DRAQ5 (Biostatus Ltd.), the antifading agent Slowfade (Molecular Probes) was added, and the cells were viewed under a laser scanning microscope (model LSM 410; Carl Zeiss MicroImaging, Inc.) using a 488-nm Argon Laser (for green fluorescence) and HeNe-Laser at 543 nm (for red fluorescence) and a HeNe-Laser at 633 nm for DRAQ5. Pictures were processed with Carl Zeiss MicroImaging, Inc. software.

Protein expression, subcellular fractionation, and sodium carbonate extraction

HEK293 cells were grown on 150-mm plates until ~80% confluent, and then transfected with 10 µg of plasmid DNA using 25 µl of Superfect (QIAGEN) as described by the manufacturer. After 3–6 h, the Superfect-DNA complexes were removed and the cells were cultured in fresh medium for another 24–48 h. The cells were homogenized; and fractionation into mitochondria, microsomes, and cytosol was performed by sucrose gradient centrifugation exactly as described in Kaufmann et al. (2003). Submitochondrial fractionation and sodium carbonate extraction of mitochondria were performed as described by Kaufmann et al. (2003). Nuclear fractions are not shown because they could not be reproducibly deprived of cosedimenting or aggregated mitochondria and unlysed cells. For protein stability experiments, 10 µg/ml cycloheximide was added to the cells at 24 h posttransfection for 1–4 h, and total extracts were prepared (Kaufmann et al., 2003).

Western blotting

30 µg of protein were immunodetected by anti-N-Bax (1:2,000), anti-N-Bak (1:2,000), anti-GFP/JL-8 (Living colors®; Invitrogen; 1:1,000), anti-KDEL (StressGen Biotechnologies; 1:1,000), anti-grp78/Bip (StressGen Biotechnologies, 1:1,000), anti-COX-I (Molecular Probes; 1:300), or anti-FLAG (Sigma-Aldrich, 1:1,000) primary antibodies followed by peroxidase-coupled, goat anti-rabbit or anti-mouse secondary antibodies (Sigma-Aldrich). Immunodetection was performed by ECL (Pierce Chemical Co.). Equal protein loading was confirmed by staining the membrane amido black or probing with an antibody against the ubiquitously expressed protein 14-3-3β (Santa Cruz, 1:2,000).

Generation of stable cell lines and immunoprecipitation

The FLAG-Bax and FLAG-Bax(P168A) and the EGFP-Bax and EGFP-Bax(P168A) cDNAs were cloned into the low expression retroviral vectors pLXIN and pLPCx, respectively (CLONTECH Laboratories, Inc.). Infectious retroviral viruses were produced by transient transfection of the packaging cell line Phoenix amphi (a gift from G.P. Nolan, Stanford University, Stanford, CA). HeLa cells were infected with FLAG-Bax and FLAG-Bax(P168A) viruses in the presence of polybrene, and stable populations were obtained by selection with G418 (2.5 and 5 mg/ml). To avoid clonal artifacts, selected cell populations, instead of clones, were further used. MEFs deficient in both Bax and Bak (DKO cells, provided by S.J. Korsmeyer, Dana-Farber Cancer Institute, Boston, MA) were infected with EGFP-Bax and EGFP-Bax(P168A) viruses and selected by FACS® sorting. Cytosolic fractions were immunoprecipitated using 5 µl anti-N-Bax and 50 µl of 50% (vol/vol) protein A-Sepharose. Immunocomplexes were captured on an end-over-end wheel at 4°C for 2 h and analyzed by anti-FLAG Western blotting as described in the previous paragraph.

IVTT

The TNT Quick T7-coupled reticulocyte lysate system (Promega) was used essentially as described by the manufacturer. IVTT and mitochondrial membrane association/insertion was performed exactly as described by Kaufmann et al. (2003).

Cell viability assay

A cotransfection assay was performed in HEK293 cells. Equal numbers of HEK293 cells were grown in six wells to 70% confluence and cotransfected with 2 µg of the desired cDNA and 0.5 µg EGFP-C2 (CLONTECH Laboratories, Inc.; 4:1 molar ratio) using the lipidic transfection reagent Metafectene (Biontex) as described by the manufacturer. After 24 h, the

cells were trypsinized, centrifuged, and resuspended in 1 ml PBS supplemented with 3% FCS and 10 µg/ml PI. Quantitative analysis was performed by analyzing 20,000 cells by FACS® and determining the number of EGFP-positive/PI-negative cell as a percentage of the total. Similar analyses were performed with EGFP-Bax wild-type and mutant constructs 24 h after transfection into HEK293 cells. The percentages obtained for cells transfected with mutant constructs were normalized to that of pcDNA3/EGFP or EGFP.

To test the apoptosis-rescuing effect of Bax and its mutants, wild-type MEFs, Bax/Bak DKO MEFs, and Bax/Bak DKO MEFs stably expressing EGFP-Bax and EGFP-Bax(P168A) were treated with 100 µM etoposide for 24 h and subjected to FACS® analysis in the presence of PI as described in the previous paragraph. Cell death was quantified by counting the PI-positive cells.

Online supplemental material

Details about the role of the two positive charges at the COOH terminus of Bax for mitochondrial targeting, the subcellular localization and cytotoxicity of endogenous and overexpressed Bax and Bak, the cytoplasmic localization of Pro168 mutants of Bax in HEK293 cells, the activity of the TMB-deleted Bax variants, the stability and integrity of the Bax(P168A) protein, as well as the methods for gel filtration analysis and the generation of the various Bax and Bak mutants and the EGFP fusion proteins by PCR or standard cut and paste techniques can be found as supplemental material. Online supplemental material is available at <http://www.jcb.org/cgi/content/full/jcb.200309013/DC1>.

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