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 apoptotic 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 (Juergensmeier 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.

The online version of this article contains supplemental material.

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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.
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 NH$_2$-terminal sequence of Bax encompassing amino acids 20–37 mediates MOM targeting. Upon activation, Bax undergoes a conformational change during which it exposes an NH$_2$-terminal epitope, but the significance of this exposure is unknown (Hsu and Youle, 1997, 1998; Nechushtan et al., 1999). As NH$_2$-terminal exposure occurs simultaneously with mitochondrial translocation, it may either unveil an NH$_2$-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 NH$_2$ terminus (Ku70; Sawada et al., 2003), the COOH terminus (humanin; Guo et al., 2003), or both (14-3-3/H9258; 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 NH$_2$-terminal exposure to the release of the COOH-terminal TMB.

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.
diffuse cytosolic cytochrome B, bottom) displayed punctate Bax fluorescence staining, with Bax (Fig. 1 A, bottom two rows) or EGFP-Bax (Fig. 1 B, top) detected on elongated structures that perfectly colocalized (Fig. 1 A, top two rows) and EGFP-Bax (not depicted) were found on the MOM (Fig. S1 B). Occasionally, both Bax and EGFP-Bax translocate efficiently to mitochondria and only residually remain soluble in the cytosol (Fig. S1 C). Neither cytosolic nor mitochondrial targeting of overexpressed Bax was detected on a mitochondrial marker by confocal microscopy. These cells did not yet exhibit morphological features of apoptosis, suggesting that Bax was in an early stage of activation. Only ≈10% of the EGFP-Bax transfectants displayed a diffuse cytosolic staining (Fig. 1 B, top). The expression of cytosolic EGFP-Bax was consistently low, indicating that soluble EGFP-Bax cannot accumulate to high levels (see Figs. S3 and S6A). These data show that upon overexpression, both Bax and EGFP-Bax translocate efficiently to mitochondria and do not require an exogenous apoptotic stimulus.

Figure 2. Bax lacking the TMB forms cytoplasmic aggregates. (A) Schematic structure and amino acid sequences of the COOH-terminal parts (TMB) of Bax and Bak. Pro168 of Bax and Pro187 of Bak are marked in bold and are situated at similar positions in a loop region in the X-domain ahead of the TMB. (B) Confocal microscopy of HeLa cells transfected with BaxΔTMB (using anti–N-Bax) or EGFP-BaxΔTMB (codeposition of EGFP and mito marker [red]). Note the cytoplasmic aggregates, which do not colocalize with mito marker.

**Results**

**Endogenous Bax is cytosolic and peripherally attached to mitochondria**

Analysis of purified subcellular fractions of HEK293 revealed that endogenous Bax mainly resides in the cytosol and on the MOM (Fig. S1, A and B, available at http://www.jcb.org/cgi/content/full/jcb.200309013/DC1). Most of the mitochondrial form was extracted with alkali treatment, indicating that Bax is peripherally attached to mitochondria (Fig. S1 C). Neither cytosolic nor mitochondrial Bax was detected by immunofluorescence with NH2-terminal–directed antibody, confirming that its NH2-terminal epitope was obstructed (Fig. S1 D; Hsu and Youle, 1997, 1998). As previously shown (Griffiths et al., 1999), Bax behaved differently from Bax as it was not found in the cytosol but instead stably inserted in the mitochondrial membrane (alkali-resistant; Fig. S1, A and C).

**Transfected Bax and EGFP-Bax effectively translocate to mitochondria and only residually remain soluble**

Next, we determined the subcellular distribution and proapoptotic activity of overexpressed Bax. Analysis by confocal microscopy showed that 80–90% of HeLa cells transfected with Bax (Fig. 1 A, bottom two rows) or EGFP-Bax (Fig. 1 B, bottom) displayed punctate Bax fluorescence staining, diffuse cytosolic cytochrome c, and fragmented nuclei. A similar finding was obtained with Bax-transfected HEK293 cells (Fig. S2, Bax, available at http://www.jcb.org/cgi/content/full/jcb.200309013/DC1). The punctate Bax fluorescence was in proximity to, but not overlapping with, mitochondrial markers (Fig. S3, Mito Marker). This finding is consistent with recent works demonstrating that in apoptotic cells Bax coalesces into clusters (Nechustin et al., 2001), which associate with mitochondrial fission sites (Karbowski et al., 2002). The majority of overexpressed Bax was found on the MOM (Fig. S1 B). Occasionally, both Bax (Fig. 1 A, top two rows) and EGFP-Bax (not depicted) were detected on elongated structures that perfectly colocalized with a mitochondrial marker by confocal microscopy. These cells did not yet exhibit morphological features of apoptosis, suggesting that Bax was in an early stage of activation. Only ≈10% of the EGFP-Bax transfectants displayed a diffuse cytosolic staining (Fig. 1 B, top). The expression of cytosolic EGFP-Bax was consistently low, indicating that soluble EGFP-Bax cannot accumulate to high levels (see Figs. S3 and S6A). These data show that upon overexpression, both Bax and EGFP-Bax translocate efficiently to mitochondria and do not require an exogenous apoptotic stimulus.

**Tailless Bax and EGFP-Bax form cytoplasmic aggregates and are not targeted to mitochondria**

To define the mitochondrial targeting sequence of Bax, we first removed the entire COOH-terminal transmembrane domain including the downstream basic-rich region (TMB) in both Bax and EGFP-Bax (Fig. 2 A). The mutants were transfected into HeLa cells and monitored for subcellular localization by fluorescence analysis (Fig. 2 B). In addition, we determined their cytotoxicity by transfecting them into HEK293 cells in the absence (for EGFP-BaxΔTMB) or presence (for BaxΔTMB) of an EGFP expression plasmid followed by FACS® analysis of EGFP-positive, propidium iodide (PI)–negative cells after 24 h (EGFP survival assay; Fig. S3, available at http://www.jcb.org/cgi/content/full/jcb.200309013/DC1). Both ΔTMB mutants lacked proapoptotic activity (Fig. S3), exhibited a cytoplasmic staining (Fig. 2 B), and were primarily found in a cytosolic fraction (Fig. 3 A). However, the mutants also formed aggregates, which did not colocalize with mitochondria (Fig. 2 B), ER, or lysosomes (not depicted). Strikingly, BaxΔTMB was detected with NH2-terminal antibodies, suggesting that the NH2 terminus was open but not competent for mitochondrial targeting. To test whether or not the NH2-terminal amino acids 20–37 could nevertheless contribute to mitochondrial targeting as previously reported (Cartron et al., 2003), we exposed this domain by removing the first 20 amino acids (BaxΔN20). This mutant was highly toxic upon transfection into HEK293 cells (see Fig. 5 C), exhibited a
punctate mitochondrial staining as wild-type Bax (Fig. 1 A and not depicted), and was effectively targeted to mitochondria in vitro (Fig. 8 A). However, when we also removed the TMB in the Bax/H9004N20 mutant (Bax/H9004N20/TMB), the protein became cytoplasmic and nontoxic (unpublished data). These data show that Bax requires the TMB, and not the NH$_2$-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). In contrast, the EGFP-23aa-C(Bax) construct, which includes the entire TMB domain of
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 (BaxΔP168) 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 B). Pro168 regulates NH2-terminal exposure by specifically colocalizing 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.

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posure indicating that it somehow couples the NH$_2$ 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. An NH$_2$-terminally truncated BaxDN20/P168A mutant is still cytoplasmic as detected by anti-Bax (aa43–61) immunostaining. Although both proteins were expressed at similar levels (Cytosol), the values were normalized to pcDNA3/EGFP (co)transfections where maximal survival (100%) is seen. Values are the mean of five independent experiments ± SEM.

Pro168 and Trp170 mutants of Bax reside in the cytoplasm in an inactive form and have their NH$_2$ termini exposed.

Figure 5.  (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 NH$_2$-terminally truncated BaxDN20/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.

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 NH$_2$-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-
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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 μM 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 ± SEM.

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 μM 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 ± SEM.

Disruption of the TMB–pocket interaction facilitates in vitro mitochondrial targeting.

To further study the mechanisms of TMB unleashment, 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. Only ~20% of Bax synthesized in vitro was targeted to mitochondria (Fig. 8). Only ~20% of Bax synthesized in vitro was targeted to mitochondria. In contrast, Bcl-xL, another member of the Bcl-2 family was effectively targeted to mitochondria (Fig. 8). Increased mitochondrial targeting was also observed with Bax(N184A), in which hydrogen bonding between the TMB and the pocket was disrupted, as well as with BaxΔN20, 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-xL or BaxΔ5/6. 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 unleashment and 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 μM 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 transfecteds 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 stetched 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).
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\(^{H9004P168}\), 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 unleashment

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 NH\(_2\) terminus exposed, was retained in the cytoplasm, and was only mildly cytotoxic after transfection into HEK293 cells, whereas the percentage of PI-positive dead cells was determined by FACS\(^{\circ}\) analysis. The values are taken from fluorescence intensity >\(10^2\) of the FACS\(^{\circ}\) histogram and are the mean of four independent experiments ± SEM.

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 Bak, 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 unleashment 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 (BaxΔN20) still depend on the COOH-terminal TMB (not depicted) and Pro168 (Fig. 5 A, BaxΔN20/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-xL complexed with a fragment of the BH3-only protein Bim and predicted, based on the structural homology between Bcl-xL and Bax, that it is the α-helix 8 rather than the COOH-terminal TMB (α9) 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-
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 unleashing, 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 unleashing. In response to an apoptotic stimulus, the interaction between the NH2 and COOH termini 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-
abitized with 0.05% saponin and ice-cold acetone. The cells were incubated with anti-N-Bax (decting amino acids 1–20); Upstate Biotechnol-
yogy; 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; 1:1000) 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 33,342 dye (Molecular Probes) or DRAQ5 (Biostatus Ltd.),
the anti-fading 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 fluores-
cence) and HeNe-Laser at 543 nm (for red fluorescence) and a HeNe-Laser
at 633 nm for DRAQ5. Pictures were processed with Carl Zeiss MicroIm-
ing, 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 me-
sium 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 mito-
chondria were performed exactly as described by the manufacturer.
Nuclear fractions were not shown because they could not be reproducibly
deposited of cosedimenting or aggregated mitochondria and unlysed cells. For pro-
tein stability experiments, 10 µg/ml cycloheximide was added to the cells
at 24 h posttransfection for 1–4 h, and total extracts were prepared (Kauf-
mann 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-1-8 (Living colors®; Invitrogen; 1:10,000), anti-
KDEL (StressGen Biotechnologies; 1:1,000), anti-gp78/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 per-
oxidase-coupled, goat anti-rabbit or anti-mouse secondary antibodies (Sigma-Aldrich). Immunodetection was performed by ECL (Pierce Chemi-
cal Co.). Equal protein loading was confirmed by staining the membrane
amido black or probing with an antibody against the ubiquitously ex-
pressed protein 14-3-3 (Santa Cruz, 1:2,000).

Generation of stable cell lines and immunoprecipitation
The Flag-Bax and Flag-Bax(P168A) cDNAs 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
retroviruses were produced by transfection of the packaging cell line Phoenix amphot cell line (gift from G.P. Nolan, Stanford University, Stan-
ford, CA). HEK293 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, se-
lected cell populations, instead of clones, were further used. MEFs defi-
cient 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 frac-
tions 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
especially 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 cotrans-
fected 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 supple-
mented with 3% FCS and 10 µg/ml PI. Quantitative analysis was per-
formed by analyzing 20,000 cells by FACS® and determining the number
of EGFP-positive/PI-negative cell as a percentage of the total. Similar anal-
yses 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 pcDNA32,
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-posi-
tive 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 cytotoxic-
ity of endogenous and overexpressed Bax and Bak, the cytoplasmic local-
ization 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 stan-
dard 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.

We thank G. Nunez for the Bcl-xl cDNA, G.P. Nolan for the Phoenix am-
pho cell line, S.J. Korsmeyer for the wild-type and Bax/Bak DKO MEFs, C.
Rhode for assistance with the FACS®, and T. Reinheckel and M. Follo for
critical comments on the manuscript.

This work was supported by grants from the Swiss National Science
Foundation (31-57236.99) and the Deutsche Forschungsgemeinschaft
(BG-1931).

Submitted: 3 September 2003
Accepted: 12 February 2004

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Results

At least one positive charge at the very COOH terminus is needed for mitochondrial sorting

It has been reported that two consecutive positive charges downstream of the hydrophobic COOH-terminal region of tail-anchored proteins are necessary for mitochondrial targeting and/or to anchor proteins into the membrane (Horie, C., H. Suzuki, M. Sakaguchi, and K. Mihara. 2002. Mol. Biol. Cell. 13:1615–1625). To understand the role of the two end-standing lysines in the TMB of Bax, we mutated them to serine residues either individually or simultaneously and fused the mutant constructs to EGFP (Fig. S4). As shown in Fig. S4, although the single mutants EGFP-TM-SMK(Bax) and EGFP-TM-KSG(M(Bax) still associated with elongated mitochondrial structures, the EGFP-TM-SMG(Bax) double mutant lost its specific mitochondrial localization and stained the nuclear envelope and the associated ER in a similar way to the ER marker Bcl-2. Subcellular fractionation of HEK293 cells transiently expressing EGFP-TM-SMG confirmed that the chimera was abundant in microsomal fractions (Fig. 3 A). Therefore, the TMB region of Bax requires at least one end-standing positive charge for mitochondrial targeting.

Materials and methods

Gel filtration analysis

Gel filtration was performed using an AEKTA explorer (Amersham Biosciences) on a Superose 6 (10/30) column equilibrated with MSH buffer (210 mM mannitol, 70 mM sucrose, 20 mM Hepes, 1 mM EDTA, pH 7.4, and protease inhibitors) in the absence or presence of 0.2% NP-40. The column was calibrated with gel filtration standard proteins giving the following elution volumes: Thyroglobulin, 669 kD; Apoferritin, 443 kD; BSA, 66 kD; and Carbonic Anhydrase, 29 kD (Sigma-Aldrich). 500 μg/H262 g of protein in MSH/H11006 NP-40 was loaded in a 200-μl injection loop, and the samples were run at 0.4ml/min. The eluate was monitored at 280 nm. 250-μl fractions were collected and pooled to 1 ml. The 1-ml samples were subjected to a standard TCA precipitation and resuspended in sample buffer (3% SDS). 100 μl of the sample was analyzed by anti-Bax or anti-FLAG immunoblotting.

cDNA constructs

The cDNAs for the various Bax mutants and EGFP fusion proteins were generated by PCR or cut and paste subcloning techniques as shown in the following sections.

Wild-type constructs

For EGFP-Bax/C3, hBax/pcDNA3 was digested with EcoRI–XbaI and subcloned sticky-blunt into EcoRI-BamHI–digested EGFP-C3. For EGFP-Bax/LPC, EGFP-Bax was digested with SphI-NheI and subcloned sticky-blunt into SphI-BamHI–digested LPC. For Flag-Bax/pcDNA3, PCR amplification on the hBax/pcDNA3 template using the upper primer 5’ ggaattcgcagatcatgacctacaagcagcagaagctctgctgggacag 3’ and lower primer 5’ gggccctctagatgcatgc 3’ and subcloned into the EcoRI and XbaI sites of pcDNA3. For Flag-Bax/pLxIN, hBax/pcDNA3 was digested with EcoRI-XbaI and subcloned sticky/blunt into EcoRI-digested pLxIN. His-hBak/pcDNA3 was received from J. C. Martinou (University of Geneva, Geneva, Switzerland) as hBax/pBS and subcloned into the BamHI and XhoI sites of pcDNA3. hBcl-xL/pcDNA3 was received from G. Nunez (University of Michigan, Ann Arbor, MI).

Mutant constructs

For BaxΔTMB/pcDNA3, PCR amplification was used on the hBax/pcDNA3 template using the upper primer 5’ gctggaattccgcatctgtcag 3’ and the lower primer 5’ atcggaaatctcagcggtgaagcgttcctg 3’, and subcloning into the EcoRI site of Bax/pcDNA3. For BaxΔN20/pcDNA3, an EcoRI site was introduced into hBax/pcDNA3 at amino acid 20. The construct was cut with EcoRI and ligated, leaving the Kozak and a start methionine intact. For BaxΔN20/P168A/pcDNA3, the BaxΔN20/pcDNA3 was digested with KpnI-Bsp I and ligated into KpnI-Bsp I-digested Bax(P168A)/pcDNA3. For BaxΔP168/pcDNA3, recombinant PCR was carried out by doing PCR amplification on the hBax/pcDNA3 template using the upper primer CB15 (5’ cggagctccgagcctcag) 3 and the lower primer 5’ ggtctgagctcagcagctcag 3’ and the upper primer 5’ atcggaaatctcagcggtgaagcgttcctg 3’, and subcloning into the KpnI and XhoI sites of pcDNA3. hBcl-xL/pcDNA3 was received from J. C. Martinou. For Bax(P168A)/pcDNA3, PCR amplification was used on the 5’ hBax/pcDNA3 template using the upper primer 5’
gacccaagctggccgacagctcggacactc 3′ and lower primer 5′ ggtctgcaagttcggcttccaaattagagagggc 3′ and subcloned into the HindIII and PmlI sites of hBax/pcDNA3. For Bax(P168G)/pcDNA3, PCR amplification was done on the hBax/pcDNA3 template using the upper primer 5′ gacccaagctggccgacagctcggacactc 3′ and lower primer 5′ tgcgtgcctgagatccctcctccagaagagccgagac 3′ and subcloned into the HindIII and XhoI sites of hBax/pcDNA3. For Bax(P168E)/pcDNA3, recombinant PCR was carried out by doing PCR amplification on the hBax/pcDNA3 template using the upper primer CB15 and the lower primer 5′ ggtctgcaagttcggcttccaaattagagagggc 3′ and the upper primer 5′ tctatcttgacagctcggagactcgcagcc 3′ and the lower primer CB16. The PCR fragments were purified and mixed at 1:1 ratios. Recombinant PCR amplification was carried out on the annealed PCR fragments using primers CB15 and CB16 and subcloned into the Blpl and XhoI sites of hBax/pcDNA3. For Bax(S184A)/pcDNA3, PCR amplification was done on the hBax/pcDNA3 template using the upper primer 5′ gacccaagcttgaccgtgaccatctttgtggcgggagtg 3′ and lower primer CB16. The PCR fragments were purified and mixed at 1:1 ratios. Recombinant PCR amplification was performed on the hBax/pcDNA3 template using the upper primer 5′ gacccaagctggccgacagctcggacactc 3′ and lower primer 5′ tgcgtgcctgagatccctcctccagaagagccgagac 3′ and subcloned into the HindIII and XhoI sites of hBax/pcDNA3. For Flag-Bax(P168G)/pcDNA3, Bax(P168G)/pcDNA3 was digested with EcoRI/XbaI and subcloned sticky/blunt into EcoRI digested pLxIN. For His-Bak(P187E)/pcDNA3, recombinant PCR was carried out by PCR amplification on the His-Bak/pcDNA3 template using the upper primer 5′ ccggtggatccctcgaggcctgccgctag 3′ and complementary lower oligonucleotides, cleaved with and subcloned into the PmlI and HindIII sites of pEGFP-C2-XC. For EGFP-C2-X-TMB(Bax), PCR amplification was done on the hBax/pcDNA3 template using the upper primer 5′ gacccaagctggccgacagctcggacactc 3′ and the lower primer 5′ ccggtggatccctcgaggcctgccgctag 3′ and subcloned into the HindIII and BamHI sites of EGFP-C2. For EGFP-21aa-C(Bax), PCR amplification was used on the hBax/pcDNA3 template using the upper primer 5′ gacccaagctggccgacagctcggacactc 3′ and lower primer 5′ cccggatctgctagccagactcctcggc 3′ and subcloned into the HindIII and BamHI sites of EGFP-C2. For EGFP-23aa-C(Bax), PCR amplification was used on the hBax/pcDNA3 template using the upper primer 5′ gacccaagctggccgacagctcggacactc 3′ and lower primer 5′ cccggatctgctagccagactcctcggc 3′ and subcloned into the HindIII and BamHI sites of EGFP-C2. For EGFP-TM-SKMG, PCR amplification was used on the hBax/pcDNA3 template using the upper primer 5′ gacccaagctggccgacagctcggacactc 3′ and lower primer 5′ cccggatctgctagccagactcctcggc 3′ and subcloned into the HindIII and BamHI sites of EGFP-C2.
Figure S1. Conformation, intracellular targeting, and membrane association of Bax and Bak. (A) Anti-N-Bax and anti-N-Bak Western blots of subcellular fractions from HEK293 cells. Purity of the fractions was checked with anti-grp78/Bip and anti-KDEL (microsomes) and anti-COX-I (mitochondria) antibodies. (B) Anti-N-Bax Western blots of mitochondrial matrix, inner (MIM) and outer membrane fractions (MOM) of HEK293 cells and HEK293 cells transiently overexpressing Bax (HEK-Bax). (C) Anti-N-Bax, anti-N-Bak, and anti-COX-I Western blots of mitochondria from HEK293 cells, first treated with sodium carbonate (pH 12; Att), and then extracted with detergent (Ins). (D) Confocal microscopy of HeLa cells using anti-N-Bax and an antibody against a mitochondrial protein (Mito Marker).

Figure S2. Pro168 mutants of Bax reside in the cytoplasm of HEK293 cells and have their NH₂ termini exposed. Anti-N-Bax and mito marker coimmunofluorescence analysis of HEK293 cells transfected with Pro168 mutants of Bax. While Bax-expressing cells are rounded up and show punctate Bax fluorescence colocalizing with mitochondria, cells expressing BaxΔP168, Bax(P168A), Bax(P168G), or Bax(P168E) are stretched-out and display the mutant Bax proteins in the cytoplasm with their NH₂ termini exposed.
**Figure S3.** Bax and EGFP-Bax lacking the TMB have lost proapoptotic activity. Percentage of EGFP-positive/PI-negative cells, determined by FACS®, after cotransfection of HEK293 cells with EGFP and either pcDNA3, Bax, or BaxΔTMB (right) or after transfection with EGFP, EGFP-Bax, or EGFP-BaxΔTMB (left). The values were normalized to EGFP or pcDNA3/EGFP (cotransfections where maximal survival (100%) is seen. Values are the mean of five independent experiments ± SEM.

**Figure S4.** Mitochondrial targeting of Bax requires basic residues at the COOH terminus. EGFP (green) and anti-mito marker (red) cofluorescence analysis of HeLa cells transiently transfected with EGFP fused to the TMB of Bax carrying point mutations in the end-standing basic residues. To determine the localization of EGFP-TM-SSMG(Bax), human Bcl-2 was cotransfected and cells were costained with anti-hBcl-2, clone 124.
Figure S5. **FLAG-Bax(P168A) does not form aggregates in the cytosol and oligomerizes like Bax in the presence of NP-40.** Cytosolic extracts of parental HeLa cells and cells stably expressing FLAG-Bax or FLAG-Bax(P168A) were fractionated on an AKTA explorer Superose 6 gel filtration column in the absence (A) or presence (B) of 0.2% NP-40. 1-ml pooled fractions were analyzed by anti–N-Bax or anti-FLAG Western blotting. A control total extract is run on the right. Elution profiles of standard proteins (29–669 kD) are marked. Note that endogenous Bax, FLAG-Bax, and FLAG-Bax(P168A) all elute in the range of 30 kD (monomeric forms) in the absence of NP-40 but in a range up to 700 kD (oligomers) in the presence of NP-40.

Figure S6. **Pro168 mutants of EGFP-Bax are stable proteins.** (A) Anti-GFP Western blots of total extracts of HEK293 cells transfected with EGFP-Bax, EGFP-Bax(P168A), or EGFP-Bax(P168G) for 24 and 48 h. (B) The same as in A but treated at 24 h posttransfection with 10 μg/ml cycloheximide (CHX) for 1–4 h. The same extracts were probed with anti–14-3-3 as a control of equal loading. Note that EGFP, EGFP-Bax(P168A), and EGFP-Bax(P168G) all remain highly expressed throughout the transfection period, even when protein synthesis is inhibited, whereas EGFP-Bax expression decreases.