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Conformation of the Bax C-terminus regulates subcellular location and cell death

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Bax, a pro-apoptotic member of the Bcl-2 family, translocates from the cytosol to the mitochondria during programmed cell death. We report here that both gain-of-function and loss-of-function mutations can be achieved by altering a single amino acid in the Bax hydrophobic C-terminus. The properly mutated C-terminus of Bax can target a non-relevant protein to the mitochondria, showing that specific conformations of this domain alone allow mitochondrial docking. These data along with N-terminus epitope exposure experiments suggest that the C- and the N-termini interact and that upon triggering of apoptosis, Bax changes conformation, exposing these two domains to insert into the mitochondria and regulate the cell death machinery.

Keywords: apoptosis/Bcl-2/GFP/mitochondria/point mutations

Introduction

Apoptosis, or programmed cell death, is a fundamental process controlling normal tissue homeostasis by regulating a balance between cell proliferation and death (Vaux *et al*., 1994; Jacobson *et al*., 1997). This process, which entails the autolytic degradation of cellular components, is characterized by blebbing of cell membranes, shrinkage of cell volumes and condensation of nuclei (Kerr *et al*., 1972). Among the various apoptotic factors identified so far, members of the Bcl-2 family represent some of the most well-defined regulators of this death pathway. Some members of the Bcl-2 family, including Bcl-2, Bcl-X_L, Ced-9, Bcl-w and Mcl-1 promote cell survival, while other members including Bax, Bcl-X_S, Bad, Bak, Bid, Bik and Bim have been shown to potentiate apoptosis (for a review, see Adams and Cory, 1998). A number of diverse hypotheses have been proposed so far regarding the possible biological functions of the Bcl-2 family members. These include dimer formation (Oltvai *et al*., 1993), protease activation (Chinnaiyan *et al*., 1996), mitochondrial membrane depolarization (Zamzami *et al*., 1996), generation of reactive oxygen intermediates (Hockenbery *et al*., 1993), regulation of calcium flux (Lam *et al*., 1994; Huiling *et al*., 1997) and pore formation (Antonsson *et al*., 1997; Marzo *et al*., 1998).

Bax, a 21 kDa death-promoting member of the Bcl-2 family, was first identified by virtue of its association with Bcl-2 (Oltvai *et al*., 1993). Overexpression of Bax accelerates cell death in response to a wide range of cytotoxic insults. Bax-deficient mice have increased numbers of neurons and the males are sterile (Knudson *et al*., 1995). Bax and Bcl-2 have three conserved regions known as the BH1, BH2 and BH3 domains (Oltvai *et al*., 1993), and hydropathy analysis of the sequences of these proteins indicates the presence of a hydrophobic transmembrane segment at their C-terminal ends. Bcl-2 has been localized to membranes of various organelles including mitochondria, the nucleus and the endoplasmic reticulum (Krajewski *et al*., 1993; Nguyen *et al*., 1993). Recently, Bax was reported to be a cytosolic protein in healthy living cells that, upon induction of apoptosis, translocates into mitochondria (Hsu *et al*., 1997; Wolter *et al*., 1997; Gross *et al*., 1998). This translocation process is rapid and occurs at an early stage of apoptosis (Wolter *et al*., 1997).

The 21 amino acid C-terminal hydrophobic segment of Bax appears to play a role in targeting Bax to mitochondria during apoptosis, as deletion of this segment in Bax abrogates its ability to insert into mitochondria during apoptosis (Wolter *et al*., 1997). In this study, we have examined a series of mutations to investigate the role of this hydrophobic segment in the targeting specificity of Bax from the cytosol to mitochondria and the importance of mitochondrial targeting to Bax bioactivity.

Results

Point mutations in the Bax C-terminus regulate subcellular localization

To explore the mechanism of the dramatic translocation of Bax from the cytosol to mitochondria during apoptosis, we focused on the hydrophobic C-terminal tail. As shown in a previous report (Wolter *et al*., 1997), the full-length cDNA of human Bax fused to the green fluorescent protein (GFP) is diffuse in the cytosol in healthy cells, and becomes punctate and mitochondria-bound in cells undergoing apoptosis. On the other hand, Bax lacking its 21 amino acid hydrophobic tail was diffuse in the cytosol of healthy cells as well as in cells treated with staurosporine, a kinase inhibitor used extensively to trigger apoptosis (Weil *et al*., 1996). Searching for the shortest C-terminus deletion we could create and still maintain the ability of Bax to translocate during apoptosis, we found that removing 10 or even only five amino acids could inhibit membrane insertion of GFP–Bax in Cos-7 cells (Figure 1A) and abrogate the death promotion ability (data not shown). By changing or deleting nearly each one of the 10 amino acids comprising the C-terminus of Bax, we found that Ser184 is most important in the regulation of Bax subcellular localization. Mutation of Ser184 (Figure 1B) can freeze

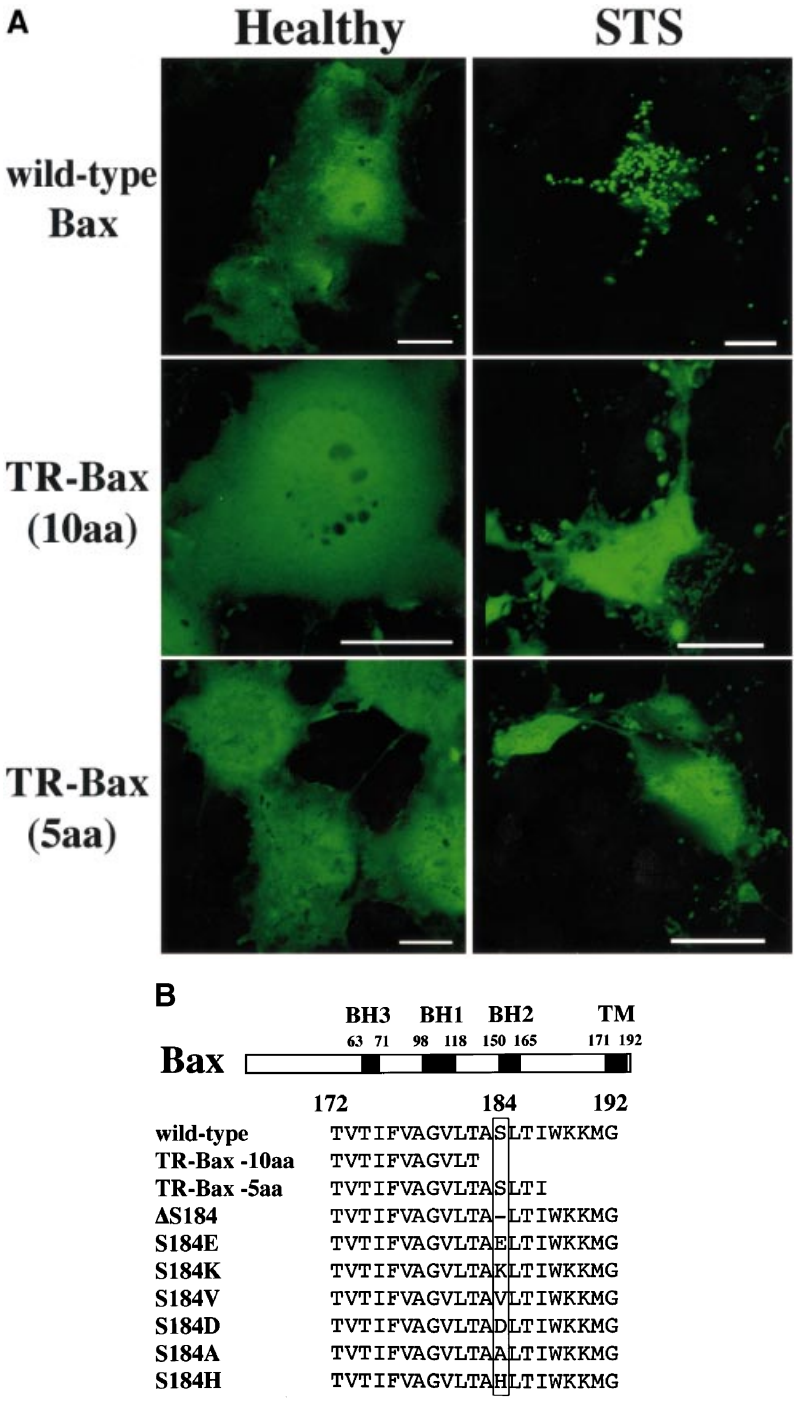


Fig. 1. C-terminal truncations and mutations of Bax. (**A**) Subcellular localization of GFP–Bax and GFP–Bax truncations before and after induction of apoptosis. Twenty hours after transfection, cells in the right panels were treated with 1 μM staurosporine (STS). After 5 h, STS-treated and untreated cells were examined by confocal microscopy. Both five and 10 amino acid C-terminal truncations [TR-Bax (5aa) and TR-Bax (10aa), respectively] were diffuse before and after treatment, while the wild-type Bax was punctate after STS treatment. Bar, 25 μm. (**B**) Schematic representation of the different C-terminal mutations used in the construction of Bax expression vectors, either GFP linked or as intact protein.

Bax into one of two subcellular states depending upon the amino acid substitution. Changing Ser184 to valine or deleting the serine entirely causes Bax to localize with mitochondria in healthy cells, whereas changing Ser184 to histidine, glutamic acid, lysine or aspartic acid results in a cytosolic localization in healthy cells as for the wild-type protein (Figure 2). The Ser184 to alanine mutant had an intermediate distribution, with the protein partially diffuse and partially co-localized with mitochondria (Figure 2). All those Bax mutants that localized to mitochondria upon expression in healthy cells (ΔS-184, S184A and S184V) remained mitochondria-bound and punctate after induction of apoptosis. S184H was cytosolic before apoptosis and moved to co-localize with mitochondria after apoptosis similarly to wild-type Bax. However, changing Ser184 to a charged amino acid (S184K, S184E and S184D) resulted in a diffuse, cytoplasmic localization even after the cells had undergone apoptosis (S184K, in

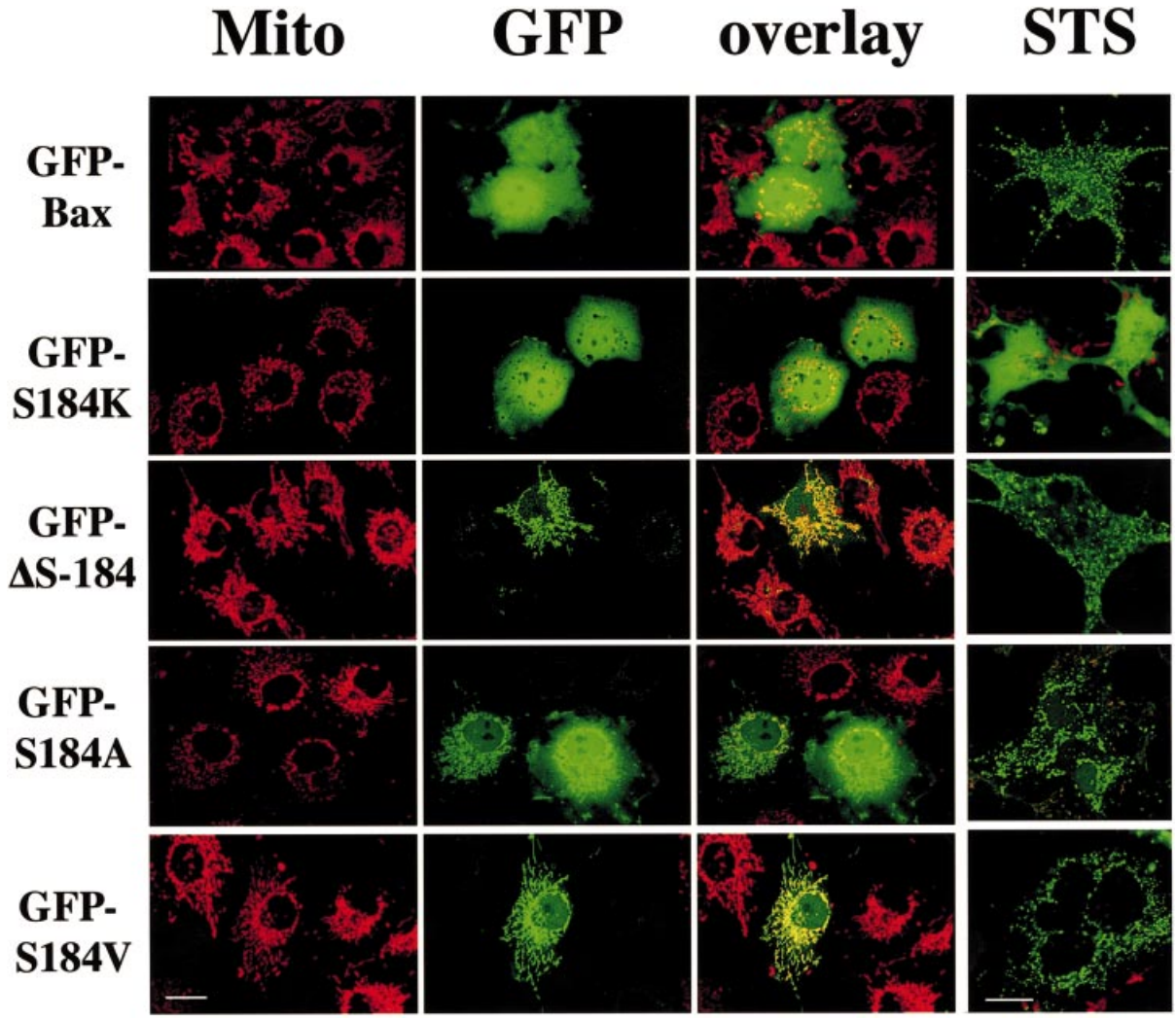


Fig. 2. Subcellular localization of GFP–Bax and GFP–Bax mutants before and after induction of apoptosis. Healthy and apoptotic Cos-7 cells, expressing GFP fusion proteins, were treated with 20 ng/ml Mitotracker red CMXRos to stain for mitochondria and then examined by laser fluorescence confocal microscopy. The fields shown were visualized independently by laser fluorescence confocal microscopy at the appropriate wavelength for Mitotracker red CMXRos (Mito) and GFP, and then the two images were overlaid (overlay). To induce apoptosis, 1 μM staurosporine was applied for 5 h (STS). Bar, 25 μm.

Figure 2). Thus, three different patterns of subcellular localization could be identified by different amino acid usage at position 184 in the C-terminal tail; a constitutive cytosolic localization, a constitutive mitochondrial localization and a migratory form that shifts from cytosol to mitochondria during apoptosis as does the wild-type form. Thus, the conformation of the C-terminus appears capable of regulating Bax localization.

Bax mutants overexpressed in Bax$^{-/-}$ cells display the same subcellular localization as in wild-type cells

We examined whether the subcellular distribution of either wild-type or mutant Bax is altered due to the presence of the endogenous Bax by using a primary culture of fibroblasts generated from the Bax knockout mouse embryo (Knudson *et al*., 1995). The full-length cDNA and various mutants of the human Bax fused to GFP were transfected into *Bax*$^{-/-}$ fibroblasts (Figure 3A). The transiently expressed GFP–Bax was fully diffuse throughout the cell cytoplasm. Upon exposure of *Bax*$^{-/-}$ cells to staurosporine, Bax becomes punctate and co-localizes with mitochondria (Figure 3A). Following the GFP–Bax in these cells reveals that a very dramatic subcellular change occurs in 10–15 min (for a movie, see http://intra.ninds.nih.gov/snb/bs/bax.mov). All GFP–Bax mutants displayed the same pattern of distribution as observed when expressing them in endogenous Bax-expressing cells (*Bax*$^{+/+}$). For example, deletion of Ser184 results in a complete localization with mitochondria, whereas S184A shows a partially diffuse and partially mitochondrial co-localization in Cos-7 cells (Figure 2).

The Bax hydrophobic tail alone is sufficient to target mitochondria

The surprisingly contrasting subcellular localization of Bax mutants with a single amino acid alteration in the C-terminal tail region led us to examine whether the tail of Bax alone was sufficient to target GFP to mitochondria. We constructed two plasmids encoding GFP fusion proteins: one had a 21 amino acid peptide homologous to the last 21 amino acids of Bax (GFP–21) and the second had

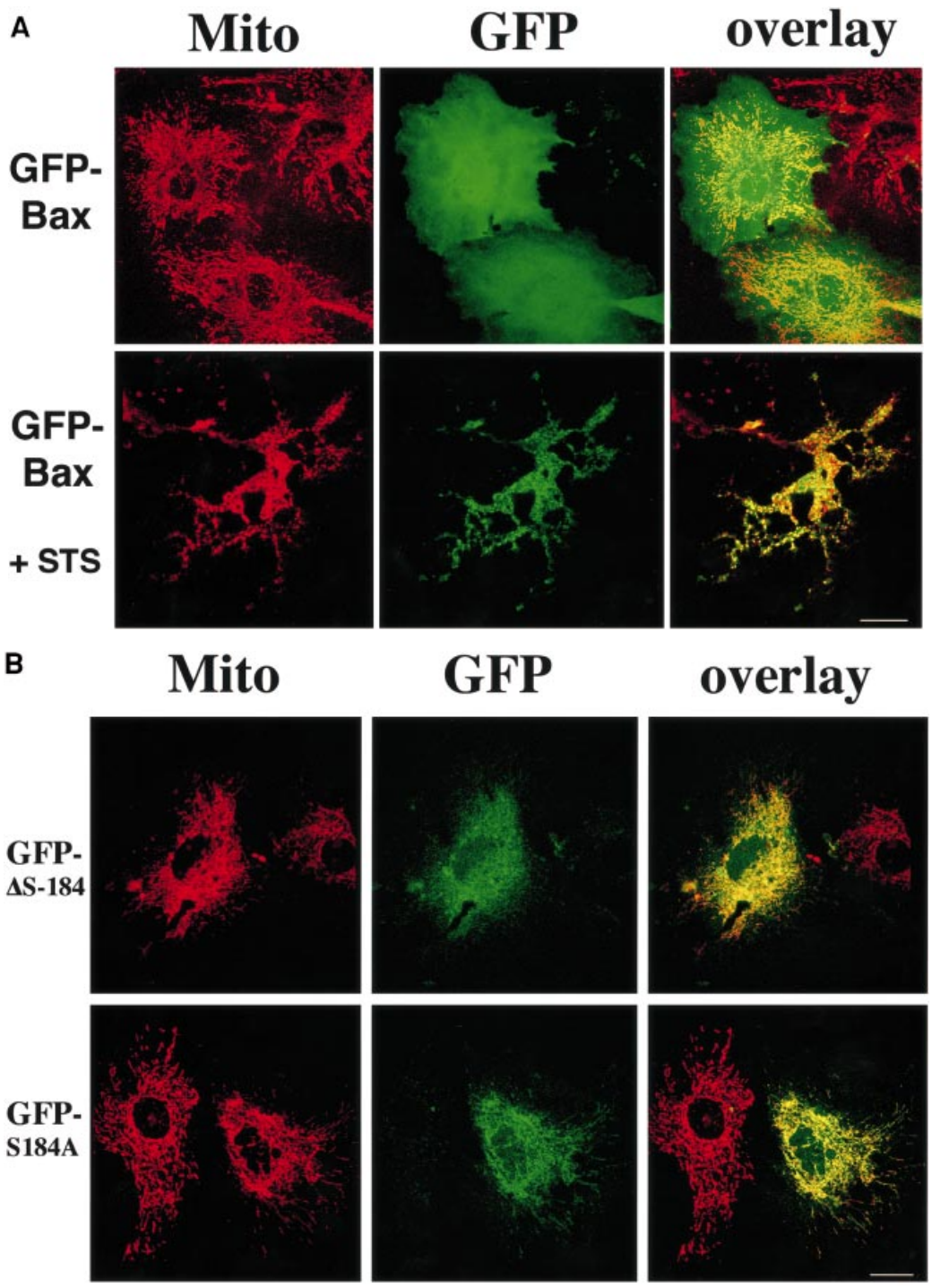


Fig. 3. GFP–Bax and GFP–Bax mutant distribution in Bax knockout cells (*Bax*$^{-/-}$) before and after induction of apoptosis. (**A**) Fibroblast cultures from the *Bax*$^{-/-}$ mouse, transiently expressing GFP–Bax, were treated with 20 ng/ml Mitotracker red CMXRos to stain for mitochondria and 1 μM staurosporine for 5 h to induce apoptosis. Each field was visualized independently by laser fluorescence confocal microscopy at the appropriate wavelength for Mitotracker red CMXRos (Mito), GFP and then for the two images overlaid (overlay). Bar, 25 μm. (**B**) Co-localization with the mitochondria of the mutants GFP–ΔS-184 and GFP–S184A transiently expressed in *Bax*$^{-/-}$ cells. Examination by laser fluorescence confocal microscopy reveals that without any apoptosis induction, the majority of the GFP fusion proteins localize to the mitochondria. Bar, 25 μm.

the same tail excluding Ser184 (GFP–20) fused to the C-terminus of the GFP, the same mutation as the constitutively mitochondria-bound Bax mutant (Figure 4A). Expressing the GFP–tail fusion proteins in either monkey kidney Cos-7 cells (Figure 4B) or Bax knockout mouse embryo fibroblasts (MEFs; data not shown) resulted in the same pattern of protein distribution as seen with the respective parent Bax (Figures 2 and 3). The wild-type

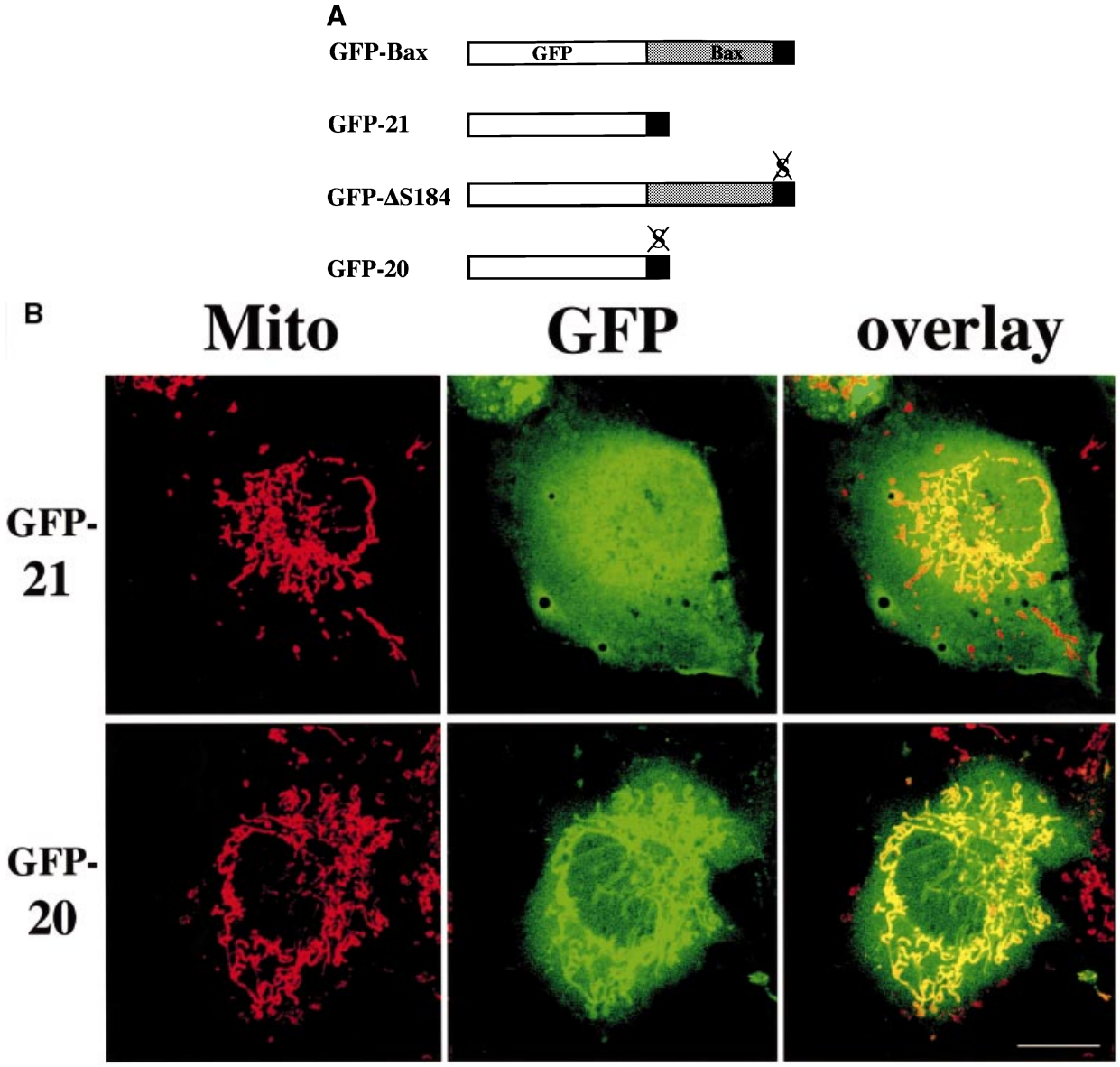


Fig. 4. The mutant hydrophobic tail of Bax alone targets GFP to mitochondria. (**A**) Schematic representation of the construction of GFP linked to the last 21 amino acids of wild-type Bax (GFP–21), and the last 20 amino acids of the ΔS-184 mutant (GFP–20). (**B**) Cos-7 cells transiently expressing GFP linked to Bax tails examined by confocal microscopy display a subcellular distribution similar to that of the respective version of full-length Bax. GFP–21 displays a diffuse cytosolic distribution, whereas GFP–20 co-localizes with the mitochondria. Cells were treated with 20 ng/ml Mitotracker red CMXRos. Each field was visualized independently by laser fluorescence confocal microscopy at the appropriate wavelength for Mitotracker red CMXRos (Mito), for GFP and then for the two images overlaid (overlay). Bar, 25 μm.

fusion (GFP–21) remained cytosolic and diffuse, while the mutated form (GFP–20) co-localized with the mitochondrial marker without any apoptotic stimuli (Figure 4B). Thus, the mutant tail of Bax is sufficient to target a cytosolic protein (GFP) to mitochondria. Upon induction of apoptosis, GFP–21 remained cytosolic and did not move to mitochondria, as did GFP–Bax. GFP–20 remained localized to the mitochondria after apoptosis induction, as did GFP–ΔS-184.

Different Bax mutants display distinct pro-apoptotic activities

We examined the bioactivity of the Bax mutants having different subcellular distributions. The assay measuring cell viability was based on the reduction in expression of a luciferase reporter gene co-expressed with Bax or Bax mutants, either untagged or as GFP fusion proteins. These Bax constructs were assayed for the ability to promote death in the presence and absence of endogenous Bax. Overexpressing GFP–Bax resulted in a reduction of ~60% in luciferase production in Cos-7 and L929 cells (Figure 5A), and of ~90% in MEF *Bax*$^{-/-}$ cells (Figure 5B). Measuring the toxicity of the untagged proteins yielded the same results as the corresponding GFP fusion proteins (data not shown). Interestingly, the mutant GFP–ΔS-184 was much more toxic than wild-type Bax in all three cell types; luciferase activity was 40-fold lower than wild-type Bax in L929 cells (Figure 5A). Another mutant, GFP–S184A, also showed a remarkably high cytotoxicity, whereas GFP–S184H and GFP–S184V were similar in potency to wild-type Bax. Surprisingly, the mutants GFP–S184K, GFP–S184E and GFP–S184D not only did not

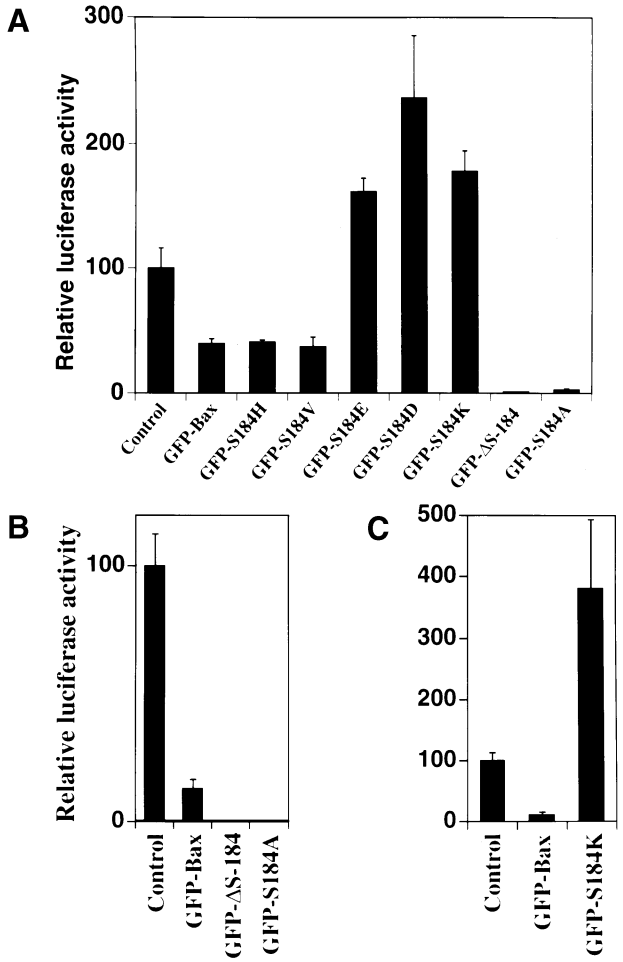


Fig. 5. Different GFP–Bax mutants display opposite effects on cell viability. (**A**) L929 mouse fibroblasts, and (**B**) and (**C**) *Bax*$^{-/-}$ cells were transiently co-transfected with the pGL3 luciferase reporter construct (0.25 μg) and different GFP fusion constructs of either wild-type or mutated Bax (1 μg). The C3-EGFP expression vector was transfected as a control. After 40 h of incubation, cells were harvested and processed for luciferase assays. Results were quantitated with a scintillation counter and displayed as a percentage of the control. The error bars show a mean standard error determined from at least three independent measurements.

harm the cells but also seemed to protect cells from transfection-induced cell death (Figure 5A and C). Thus, two mutants with constitutive mitochondrial association had greater toxicity than normal Bax, and three mutants with no mitochondrial association had no toxicity. These data strongly suggest that mitochondrial binding of Bax is an important step in cell death induction.

Bcl-X_L and Bcl-2 block the pro-apoptotic activity of Bax mutants but do not prevent constitutive mitochondrial localization

We have identified forms of Bax that bind mitochondria more readily and are more potent in promoting apoptosis than the wild-type protein. How the Bax homologs Bcl-2 and Bcl-X_L block apoptosis is not known, although both localize to mitochondria to a greater extent than normal Bax. We tested the potency of Bcl-X_L and Bcl-2 in countering the ability of the various Bax mutants to promote death. Co-transfection with Bcl-X_L can significantly overcome the apoptosis activity of Bax and the more toxic Bax mutants (Figure 6A). The fold protection of Bcl-X_L was similar regardless of the potency of the Bax variant. We asked whether this cell protection was due to competition between Bax and Bcl-X_L for binding to the mitochondria. A 5-fold excess of Bcl-X_L co-expressed with the constitutive mitochondrial-docking mutants, GFP–S184A, GFP–S184V and with GFP fused to the Bax mutant tail (GFP–20), showed no influence on the mitochondrial co-localization (Figure 6B). A reciprocal experiment of co-expression of GFP–Bcl-X_L, which is mostly membrane-bound (lowest panel Figure 6B, and Wolter *et al.*, 1997), with a 5-fold excess of the untagged Bax mutant ΔS-184 showed no effect on Bcl-X_L distribution (data not shown). Bcl-X_L remained localized mostly to the mitochondria. The same results were obtained with competition for GFP–Bcl-2 (data not shown).

Our initial observations showing that Bax translocates entirely to the mitochondria with no trace of overexpressed protein left in the cytosol (Figures 1–3) also suggest that the number of Bax-binding sites on the mitochondria is not saturable. Furthermore, a cytotoxicity assay in which luciferase activity was measured in cells co-expressing Bax with the non-toxic, constitutive docking GFP–20 show that GFP–20 did not inhibit or compete for Bax toxicity (Figure 6C). Thus, no evidence of saturable or competable binding of Bax to the mitochondria was found.

Conformational changes in Bax mutants

To determine if the differences in toxicity and subcellular localization of various Bax mutants resulted from altered three-dimensional structure, we explored the epitope exposure of several of the Bax mutants. A series of immunoprecipitations was performed on L929 cell extracts overexpressing Bax, S184K mutant and Bax lacking the C-terminal 21 amino acids (Wolter *et al.*, 1997). We used two monoclonal antibodies capable of recognizing an exposed (1F6) and a normally hidden (6A7) epitope in the human Bax N-terminus (Hsu and Youle, 1997). As can be seen in Figure 7A, wild-type Bax, S184K and C-terminal truncated Bax all bind 1F6 antibody; however, large differences can be seen in the efficiency of binding to 6A7. Only the truncated form of Bax lacking the hydrophobic tail could bind 6A7, while the two forms of Bax having a full-length tail did not expose that specific epitope. The highly toxic Bax mutants ΔS-184, S184A and S184V could not be used in this type of experiment due to the fact that these constitutive mitochondrial-docking proteins could not be extracted from the cells' mitochondria without the use of detergents, and detergents cause spontaneous conformational changes in Bax (Hsu and Youle, 1998). We examined the conformation of these constitutive mitochondrial-localizing mutants using immunofluorescent labeling performed on fixed Cos-7 cells. The monoclonal antibody 1F6 binds to Bax in both the diffuse state before apoptosis (Figure 7B, upper left) and in the punctate, mitochondria-bound state after apoptosis (Figure 7B, upper right). In contrast, the antibody 6A7 does not bind native Bax in the soluble state (Figure 7B, upper left) but does bind the mitochondria-bound form of native Bax after apoptosis (Figure 7B, upper right). We examined the conformation of two mutant forms of Bax

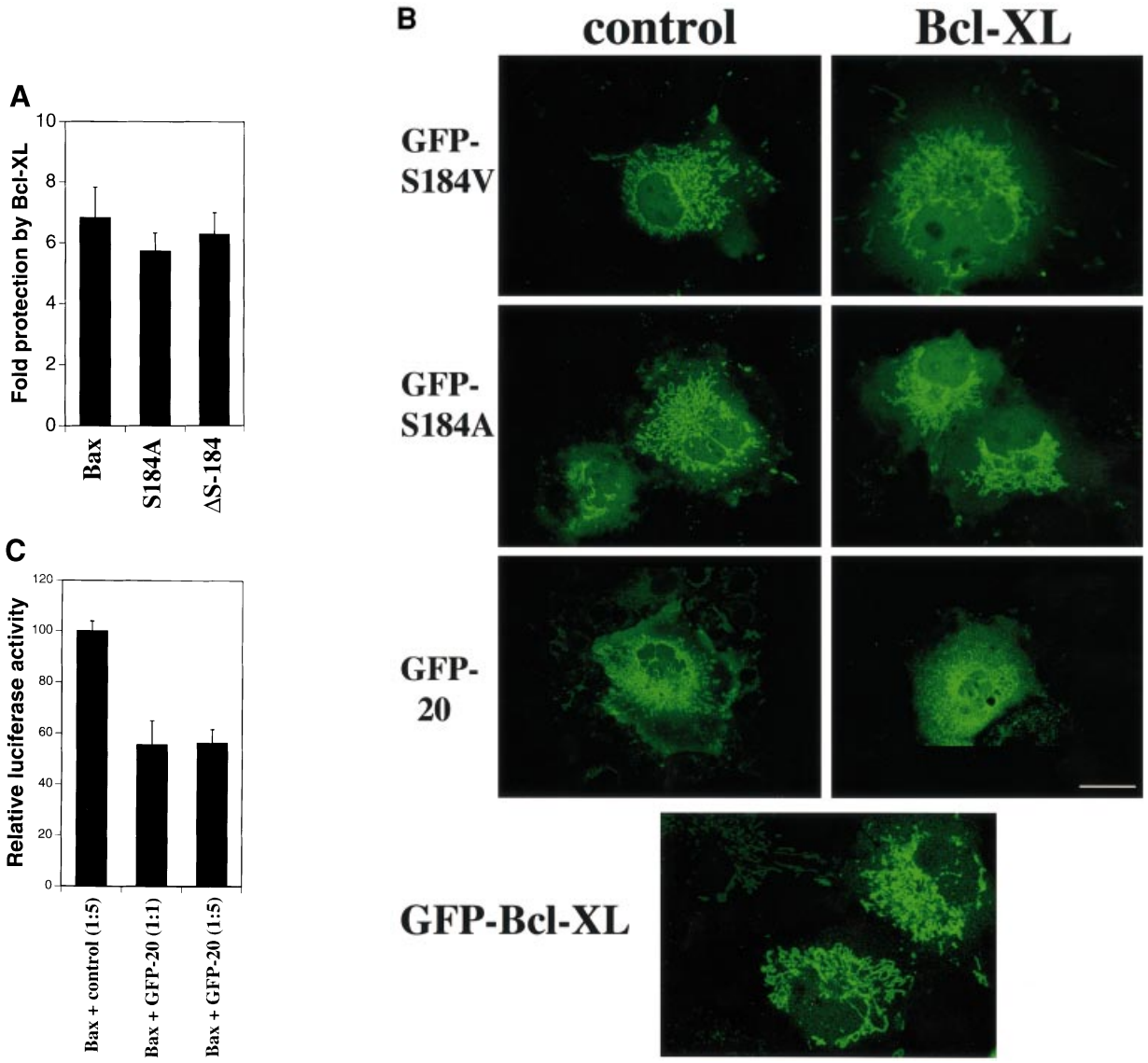


Fig. 6. Competition for Bax-binding sites. Bcl-X_L blocks Bax and more highly toxic Bax mutants to a similar extent. (**A**) L929 cells were transiently co-transfected with pGL3 luciferase reporter construct (0.1 μg), Bcl-X_L (2.5 μg) and either GFP–Bax, GFP–ΔS-184 or GFP–S184A (0.5 μg). C3-EGFP expression vector was used as a control. After 40 h of incubation, cells were harvested and processed for luciferase assay. Results were quantitated with a scintillation counter and displayed as fold increase in luciferase expression in relation to the vector control. The error bars show a mean standard error determined from at least three independent measurements. (**B**) No competition for mitochondrial binding by Bcl-X_L. Cos-7 cells were transiently co-transfected with GFP–S184V, GFP–S184A and GFP–20 (0.5 μg) either with pcDNA 3.1 control or with Bcl-X_L (2.5 μg). Upon examination by confocal microscopy, no significant effect could be seen in the distribution of GFP-linked proteins due to Bcl-X_L. Cells transfected with GFP–Bcl-X_L alone show a predominantly mitochondrial localization. Bar, 25 μm. (**C**) No competition for Bax toxicity by the mutated Bax tail–GFP. L929 cells were transiently co-transfected with pGL3 luciferase reporter construct (0.1 μg), GFP–Bax and GFP–20 in a 1:1 and 1:5 ratio. The C3-EGFP expression vector was used as a control. After 40 h of incubation, cells were harvested and processed for luciferase assay. Results were quantitated with a scintillation counter and displayed as a percentage of the control. The error bars show a mean standard error determined from at least three independent measurements.

that constitutively insert into mitochondria. Although both ΔS-184 and S184V bind 1F6 (Figure 7B, lower left and right), neither binds to 6A7 before the induction of apoptosis. Interestingly, after the induction of apoptosis, both the ΔS-184 and S184V bind 6A7. Thus, the conformational changes that regulate membrane insertion in the mutant forms of Bax, either to keep Bax in the cytosol (Figure 7A) or to keep Bax in the mitochondria (Figure 7B), do not cause exposure of the 6A7 epitope.

Discussion

Bax moves rapidly from the cytosol to the mitochondria after initiation of apoptosis (Hsu *et al*., 1997; Wolter *et al*., 1997; Gross *et al*., 1998). We have explored the molecular mechanism of this movement and the relationship between intracellular distribution and bioactivity of Bax, by analyzing a large series of point mutations in the C-terminal tail of Bax. Our observations show that the C-terminus plays a key role in determining Bax subcellular localization and

demonstrate that insertion into the mitochondria is essential for pro-apoptotic activity. The effects of single amino acid substitutions and deletions suggest that Bax undergoes a specific conformational change involving the C-terminal 10 amino acids that enables it to bind the mitochondria and promote death.

Binding to mitochondria appears to be an essential although not sufficient requirement for cell death, based in part on previous experiments and more substantially on the results presented here. If the C-terminus is eliminated, Bax no longer binds to mitochondria and is no longer active (Wolter *et al*., 1997). The point mutations in the C-terminus, S184D, S184E and S184K, that block binding to mitochondria eliminate Bax pro-apoptotic activity. On the other hand, point mutations that increase binding to mitochondria (S184A and ΔS-184) actually increase Bax toxicity. Recent work in another laboratory (Gross *et al*., 1998) also shows that triggering mitochondrial association by Bax dimer formation triggers toxicity. Thus, several lines of evidence are now consistent with the theory that Bax insertion into mitochondria represents an essential step in its bioactivity.

It was not known previously if the cause of Bax insertion into mitochondria during apoptosis was due to alterations in the mitochondria, alterations in Bax or due to the action of a mediator molecule. Our findings show that the C-terminal tail of Bax is a key determinant of Bax subcellular localization. GFP is cytosolic when it is not fused to another protein and remains cytosolic when fused to the wild-type Bax tail. However, GFP fused to the Bax tail lacking Ser184 inserts into mitochondria (Figure 4), showing that the same tail mutation that targets Bax to mitochondria is sufficient to target other proteins to the mitochondria.

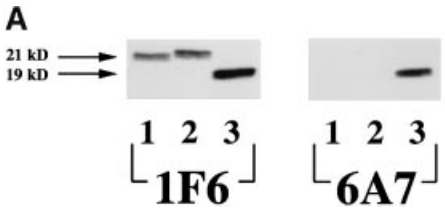
A plausible explanation for these observations is that the subcellular localization of Bax is determined by the conformation of the C-terminal tail. The C-terminus may be able to fold onto itself so that the wild-type tail is unable to bind mitochondria whereas the Ser184 deletion exposes the tail allowing insertion into mitochondria. Interestingly, the GFP–wild-type tail (GFP–21) does not insert into mitochondria during apoptosis as GFP–Bax does, suggesting that other domains in Bax, perhaps in the N-terminus or BH3 domain, regulate the conformation of the C-terminus during cell death.

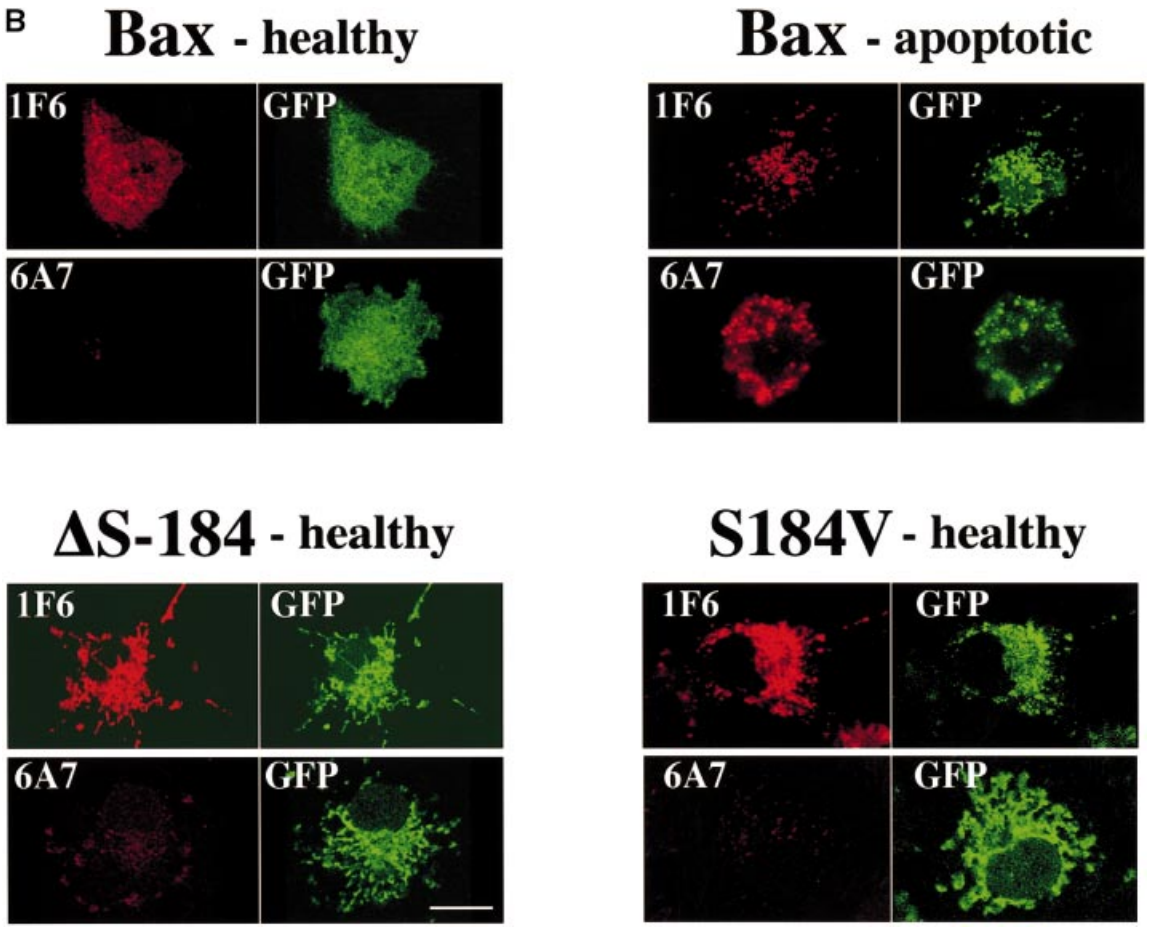
The three-dimensional structure of Bax has not been reported. Although the structure of the Bax homolog Bcl-X_L has been partially solved, the C-terminal tail was excluded from the recombinant Bcl-X_L protein. Thus, no information currently exists on the structure of the C-terminal hydrophobic tail of any member of the Bcl-2 family or of how the tail may interact with the rest of the molecule. The monoclonal antibody 6A7, raised against the peptide amino acids 13–19 in the N-terminus of Bax, does not bind the soluble form of Bax in healthy cells (Hsu *et al*., 1997) but does recognize Bax after the conformational change associated with membrane insertion occurs in apoptotic cells (Hsu and Youle, 1998). We found that deletion of the C-terminal 21 amino acids results in exposure of the 6A7 epitope at the N-terminus (Figure 7A). Examining the constitutive mitochondrial localizing mutants, we found no 6A7 exposure before cells undergo apoptosis (Figure 7B), indicating that the C-terminal tail of the soluble, cytoplasmic form of Bax interacts with the N-terminal domain, amino acids 13–19. Our study suggests that wild-type Bax undergoes a conformational change as an essential early step in apoptosis where the C-terminus disengages its association with the N-terminal amino acids. This model is consistent with recent data showing that deletion of the N-terminus of Bax resulted in auto-insertion of the molecule into the mitochondrial membrane (Goping *et al*., 1998).

How this C-terminus fits into the N-terminus remains unknown, but the crystal structure of Bcl-X_L may shed some light on the interaction (Muchmore *et al*., 1996). As can be seen in Figure 7C, the N-terminus and the BH3 domain are adjacent to the C-terminal amino acid 197, beyond which the structure remains unknown (Muchmore *et al*., 1996). Our results suggest that the Bax C-terminal tail lies across the colored (blue and green, Figure 7C) regions and inhibits access of specific antibodies to the BH3 domain and a portion of the N-terminus.

Furthermore, other important functions of Bax could also be controlled by this conformational change. The BH3 domain is the one region known to be required for Bax induction of cell death (Zha *et al*., 1996a). It is intriguing to consider how regulated exposure of the BH3 domain during the induction of apoptosis may control cell viability. Interestingly, if the BH3 domain is eliminated, GFP–Bax constitutively inserts into mitochondria although the protein no longer stimulates apoptosis (unpublished data), and point mutations in the BH3 domain cause mitochondrial binding of Bax with no initial induction of apoptosis (Wang *et al*., 1998). This localization of BH3 mutants is similar to that of some of the tail mutants described here, showing further evidence of an interaction of the BH3 domain with the C-terminal tail.

How S184D, S184E and S184K block apoptosis remains to be studied. The fact that the three mutants that cannot insert into mitochondria following apoptosis induction actually inhibit cell death is intriguing. A single amino acid change can shift Bax from becoming more toxic to being cell death protective. Co-expressing wild-type Bax with a 5-fold excess of the protective mutants did not result in a significant decrease in cell death but did increase the amount of the wild-type protein left in the cytosol and the nucleus. Although most of the wild-type Bax co-localized with the mitochondria and cells died, our results indicate a possible interference of the protective mutants with the wild-type translocation. In addition, a series of immunoprecipitations using S184K mutant and wild-type Bax showed no difference between them in binding to Bcl-X_L (data not shown). Solving this mystery may help explain how Bcl-2 and Bcl-X_L block cell death. The three charged amino acid substitutions, Asp, Glu and Lys, may block hydrophobic insertion into the membrane and it is possible that they could mimic phosphorylation of serine. Phosphorylation has been implicated in the regulation of both Bad and Bcl-2 (May *et al*., 1994; Haldar *et al*., 1995; Zha *et al*., 1996b; Ling *et al*., 1998). Bad is a pro-apoptotic member of the Bcl-2 family, sharing weak homology with the other members mainly within the BH3 domain. In spite of the fact that Bad lacks a hydrophobic C-terminus, it was shown to shift from being cytosolic to membrane bound (Zha *et al*., 1997), similarly to Bax. It has also been suggested that the phosphorylation enables Bad to


A
21 kD
19 kD
1 2 3
1F6
1 2 3
6A7


B
Bax - healthy
1F6
GFP
6A7
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Bax - apoptotic
1F6
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ΔS-184 - healthy
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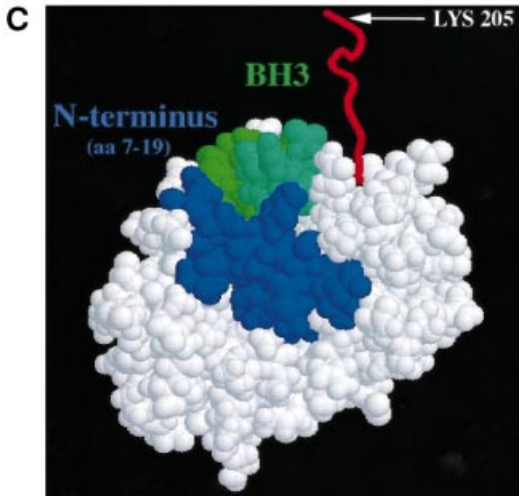
C
LYS 205
BH3
N-terminus
(aa 7-19)

bind 14-3-3 and prevent it from targeting membrane-bound Bcl-X_L to enhance survival (Zha *et al*., 1996b). Although Ser184 appears important in the control of Bax localization and toxicity, we have no evidence that phosphorylation is involved. In experiments we conducted, no phosphorylation of Bax was found before or after cell death (data not shown). At least one amino acid, histidine, can substitute for Ser184, yielding the same regulated insertion into membranes and promotion of cell death.

One of the important pieces missing from the current models describing the mode of action and regulation of Bax is the nature of the specific binding sites on the mitochondria that allow its shift in subcellular localization. As shown herein (Figures 2 and 3) and also by Wolter *et al*. (1997), overexpressed GFP–Bax can translocate completely to the mitochondria. Furthermore, in healthy cells, the mitochondria also bind the highly overexpressed constitutive docking mutants (Figures 2A and 6B). We see no influence on Bax promotion of cell death with an excess of the non-toxic, auto-inserting GFP–20 (Figure 6C), suggesting that there is a large excess of receptors/binding sites for the endogenous Bax. The ability of Bcl-X_L (Figure 6) and Bcl-2 (data not shown) to protect cells from apoptosis (Figure 6A) does not appear to result from their competition for Bax docking onto the mitochondria.

Changing Ser184 of the Bax C-terminus to a valine (S184V) resulted in an interesting new phenotype. This mutant constitutively localized with the mitochondria (Figure 2B) yet exhibited pro-apoptotic activity equal to that of GFP–Bax (Figure 5A). This differs from the other two constitutive mitochondrial-bound mutants, GFP–ΔS-184 and GFP–S184A, that were significantly more toxic than Bax. All three proteins have an N-terminus epitope (6A7) hidden that becomes exposed once cells start dying. These results may indicate an additional step in toxicity, such as formation of dimers (Gross *et al*., 1998) or binding a particular mediator, which regulates the bioactivity of Bax. Both ΔS-184 and S184A mutants may be more susceptible to the apoptotic trigger or spontaneously undergo triggering, while S184V with a slight conformational difference requires the next step as does the wild-type Bax. Whatever physiologically triggers Bax to bind mitochondria during apoptosis, it probably imparts a conformational change on Bax similar to the state of the auto-inserting mutants reported here.

Materials and methods

Restriction and modifying enzymes and oligonucleotide primers were obtained from Gibco-BRL (Gaithersburg, MD). DNA sequencing was performed with a Sequenase II kit (US Biochemical Corp., Cleveland, OH). All media and antibiotics were obtained from Biofluid Inc. (Rockville, MD). Except where noted, reagents were purchased from Sigma Chemical Co. (St Louis, MO).

Generation of expression constructs

Wild-type full-length human Bax and its various mutants were synthesized by a standard PCR using the cDNA for human *Bax* (a gift of S.J.Korsmeyer, HHMI and Washington University, St Louis, MO) as a template. The following primers were used in the amplification reactions introducing a *Hin*dIII site for the N-terminus (5′-CCAGTGAAGCTTATGG ACGGGTCCGGGGAGC) and an *Eco*RI site for the C-terminus of the wild-type (5′-GCGTCAGAATTCTCAGCCCATCTTCTTCCAGAT) and the different mutations: ΔS-184 (5′-GCGTCAGAATTCTCAGCCCATCTTCTTCCAGATGGTGAGGGCGGTGAG); S184A (5′-GCGTCAGAATTCTCAGCCCATCTTCTTCCAGATGGTGAGCGCGGCGGTGAG); S184K (5′-GCGTCAGAATTCTCAGCCCATCTTCTTCCAGATGGTGAGCTTGGCGGTGAG); S184V (5′-GCGTCAGAATTCTCAGCCCATCTTCTTCCAGATGGTGAGCACGGCGGTGAG); S184H (5′-GCGTCAGAATTCTCAGCCCATCTTCTTCCAGATGGTGAGGTGGGCGGTGAG); S184D (5′-GCGTCAGAATTCTCAGCCCATCTTCTTCCAGATGGTGAGATCGGCGGTGAG); and S184E (5′-GCGTCAGAATTCTCAGCCCATCTTCTTCCAGATGGTGAGCTCGGCGGTGAG). All PCRs were done by 30 cycles of 1 min at 94°C, 2 min at 60°C and 3 min at 72°C, using *Pfu* polymerase (Stratagene, La Jolla, CA). The resulting PCR products were digested with *Hin*dIII and *Eco*RI and cloned into the mammalian expression vectors, pcDNA-3.1 (Invitrogen, Carlsbad, CA) and C3-EGFP (Clontech Laboratories, Inc., Palo Alto, CA). To generate the GFP fusion to the wild-type C-terminus of Bax (GFP–21) and a mutated form lacking Ser184 (GFP–20), the following pairs of oligonucleotides, encoding 21 and 20 amino acids flanked by *Hin*dIII and *Eco*RI sites, were synthesized: (forward) 5′-AGCTTACCATCTTTGTGGCGGGAGTGCTCACCGCCTCGCTCACCATCTGGAAGAAGATGGGCTGAG, (reverse) 5′-AATTCTCAGCCCATCTTCTTCCAGATGGTGAGCGAGGCGGTGAGCACTCCCGCCACAAAGATGGTA, and (forward) 5′-AGCTTACCATCTTTGTGGCGGGAGTGCTCACCGCCCTCACCATCTGGAAGAAGATGGGCTGAG, (reverse) 5′-AATTCTCAGCCCATCTTCTTCCAGATGGTGAGGGCGGTGAGCACTCCCGCCACAAAGATGGTA. The oligonucleotides were annealed and ligated to the *Hin*dIII–*Eco*RI-digested C3-EGFP expression vector. The resulting mutants and GFP fusion constructs were all confirmed by restriction endonuclease digestion and DNA sequence analysis. Equal levels of protein expression were confirmed in a cell-free system by expressing the different mutants in the TNT® Quick Coupled Transcription/Translation System (Promega) as described by the manufacturer. *Escherichia coli* strain DH5α (Stratagene, La Jolla, CA) was used for all plasmid transformations and propagations.

Cell culture

L929 murine fibrosarcoma and Cos-7 green monkey renal epithelia cell lines (American Type Culture Collection, Rockville, MD) were grown in Earle's minimum essential medium and Dulbecco's modified Eagle's medium (DMEM), respectively, each supplemented with 10% heat-treated fetal calf serum (FCS), 2 mM glutamine, non-essential amino acids, 2.5 mM sodium pyruvate, 100 U/ml penicillin and 100 μg/ml streptomycin. MEFs generated from either normal or Bax knockout mouse embryos (Knudson *et al*., 1995) were kindly provided by S.J.Korsmeyer. These primary cultures passage 4–6 of $Bax^{-/-}$ and $Bax^{-/+}$ were grown in DMEM supplemented with 10% heat-treated FCS, 2 mM glutamine, 0.1 mM β-mercaptoethanol, 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were cultured at 37°C in 5% CO_2.

Fig. 7. Bax C-terminus conformational changes induce N-terminus exposure. (**A**) Immunoprecipitation of the human Bax from the soluble protein extracts of Bax-transfected mouse L929 cells. Human Bax 1F6 and universal Bax 6A7 monoclonal antibodies (raised against amino acids 8–14 and 13–19, respectively) coupled to Sepharose beads were used to immunoprecipitate full-length wild-type Bax (lane 1), Bax mutant S184K (lane 2) and Bax with the 21 C-terminal amino acid truncated (lane 3). (**B**) Immunofluorescent labeling of Cos-7 cells transfected with GFP–Bax and GFP–Bax mutants. Following transfection, cells were fixed and permeabilized as described in Materials and methods. Two anti-Bax monoclonal antibodies (1F6 and 6A7) were used for primary labeling and Texas red-conjugated anti-mouse IgG served as the secondary antibody. The fields shown were visualized independently by laser fluorescence confocal microscopy at the appropriate wavelength. The two upper panels show GFP–Bax in healthy and apoptotic cells, while the lower two panels show the mutants GFP–ΔS-184 and GFP–S184V in healthy cells. Apoptotic cells expressing these mutants expose the same epitopes as the wild-type Bax shown in the upper right panel. Bar, 25 μm. (**C**) Space-filling representation of the crystal structure of human Bcl-X_L (Muchmore *et al*., 1996) shows a close proximity between the N-terminus (blue), BH3 domain (green) and the last amino acids to be resolved (amino acids 197–233 were not determined by the X-ray solution) (red). The figure was prepared with the program RasMol (Sayle and Milner-White, 1995).

Transfection and immunoprecipitation

For measurement of cell death, 2×10^5 cells per well in 6-well tissue culture plates (Becton Dickinson Labware, Bedford, MA) were plated in preparation for transfection, and were transfected 40 h later using the cationic lipid LipofectAmine (Gibco-BRL) as described by the manufacturer. A total of 2.5 μg of plasmid DNA was used, and either 8 (L929) or 4 μl (Cos-7 and MEF) of LipofectAmine per well. For the confocal microscopy, 1×10^4 cells were plated on a 2-well Lab-Tek chambered coverglass (Nalge Nunc, Naperville, IL) and transfected 24 h later using 1 μg of plasmid DNA and 4 μl of LipofectAmine per chamber. For the competition studies using three different plasmids, the amount of LipofectAmine was the same as above, and the total DNA did not exceed 3 μg. For immunoprecipitation, L929 cells in a 10 cm plate were transfected with 10 μg of plasmid DNA using 50 μl of LipofectAmine. At 40 h following transfection, cells were collected, washed, resuspended in 10 mM HEPES, pH 7.4, 125 mM NaCl, 2 μg/ml aprotinin, 2 μg/ml leupeptin and 25 μg/ml phenylmethylsulfonyl fluoride (PMSF), and disrupted by 3–4 cycles of freeze and thaw (dry ice/ethanol and 37°C, respectively). Soluble protein was obtained by a high speed centrifugation (200 000 *g*) and the soluble extracts were brought to 1 ml with 10 ml of 10 mM HEPES, pH 7.4, and 150 mM NaCl. The soluble extracts were mixed with 40 μl of CNBr-activated Sepharose 4B coupled with anti-universal Bax 6A7 and the anti-human 1F6 monoclonal antibodies (Hsu and Youle, 1998). After 2 h incubation at 4°C with constant mixing, the reactions were spun briefly and the pellets were washed six times with 1 ml of 10 mM HEPES pH 7.4, 125 mM NaCl. The resulting binding proteins were eluted with 80 μl of 1% SDS in phosphate-buffered saline (PBS) and kept at –20°C until Western blot analysis.

Measurement of cell viability

To evaluate the effects of overexpression of Bax or Bax mutants on cell viability in culture, the mammalian expression vector pGL3 (Promega) carrying the firefly luciferase (Luc) structural gene was transfected along with either Bax, Bax mutants or control DNA plasmids. A 1:4 (Luc:tested gene) molar ratio was used to co-transfect L929, $Bax^{-/-}$ MEFs and Cos-7 cells in 6-well tissue culture plates as described above. At 40 h following transfection, the cells were washed with PBS and harvested using the luciferase assay system (Promega, Madison, WI). Luciferase activity was measured by liquid scintillation counting (Turku, Finland) using 20 μl of the cellular extract. In every experiment, each construct was tested in quadruplicate, and experiments were repeated three times. Cell viability is shown as the relative luciferase activity of the tested construct compared with the specified control plasmid.

Confocal microscopy

Either live or fixed and permeabilized Cos-7 cells were used for confocal microscopy. Cells were grown and transfected as described above on a one thickness 2-well Lab-Tek chambered coverglass (Nalge Nunc). After 24–48 h, confocal microscopy of live cells was performed by incubation with 20 ng/ml of a mitochondrion-specific dye (Mitotracker red CMXRos; Molecular Probes Inc., Eugene, OR) and/or 1–3 μM staurosporine as indicated in individual experiments. Images were collected on an LSM 410 microscope with a 40×1.2 NA Apochromat objective (Carl Zeiss, Thornwood, NY). The 488 and 568 nm lines of a krypton/argon laser were used for fluorescence excitation of GFP and Mitotracker red CMXRos, respectively. The temperature of the specimen was maintained between 35 and 37°C with an air stream incubator. For immunofluorescent labeling, the cells were washed with PBS followed by fixation with 2% paraformaldehyde and permeabilization with 0.04% saponin. The fixed cells were blocked with normal goat serum, probed with mouse anti-Bax monoclonal antibodies (1F6 and 6A7) and stained with Texas red-conjugated goat anti-mouse IgG antibodies (KPL, Gaithersburg MD). The coverglasses finally were washed, mounted and examined using the confocal laser microscope as described above.

Western blot analysis

For Western blotting, samples were separated by electrophoresis using 14% SDS–polyacrylamide gels. Gels were electrotransferred onto Immobilon-P membranes (Millipore Corp., Waters Chromatography, Milford, MA). The blots were blocked in PBS/0.05% Tween-20 containing 5% fetal bovine serum and incubated with an anti-human Bax polyclonal antibody. Primary antibody binding was detected by blotting with sheep anti-rabbit F(ab′) linked to horseradish peroxidase (Amersham Corp., Arlington Heights, IL), followed by band visualization using enhanced chemiluminescence (ECL) as described by the manufacturer (Amersham Corp.).

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