Substitutions of Potentially Phosphorylatable Serine Residues of Bax Reveal How They May Regulate Its Interaction with Mitochondria

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During apoptosis, the pro-apoptotic protein Bax relocalizes from the cytosol to the mitochondrial outer membrane. This relocalization is associated to major conformational changes, namely at the N- and C-terminal ends of the protein. Substitution of residues located at critical positions within the protein potentially stimulates or inhibits this process. In the present study, we investigated the hypothesis that phosphorylation of serine residues might trigger these conformational changes, with a focus on Ser 163 and Ser 184, which have been shown to be phosphorylatable by protein kinases GSK3β and Akt/PKB, respectively, and on Ser 60, which is located in a consensus target sequence for PKA. Substitutions of these serine residues by alanine or aspartate were done in wild type or previously characterized Bax mutants, and the capacity of the resulting proteins to interact with mitochondria and to release cytochrome c was assayed in yeast, which provides a tool to study the function of Bax, independently of the rest of the apoptotic network. We conclude that sequential phosphorylation of these serine residues might participate in the triggering of the different conformational changes associated with Bax activation during apoptosis.

The crucial function of Bcl-2 family proteins in the regulation of apoptosis is now well established (1). Among these proteins, membrane multidomain proteins Bax and Bak are essential for the completion of apoptosis because their double knock-out fully impairs this process (2). Their main function is the permeabilization of the outer mitochondrial membrane, leading to the relocalization of apoptogenic factors, such as cytochrome c, smac/diablo, omi/HtrA2, endonuclease G, and apoptosis-inducing factor (3–5).

Bax function in mitochondria permeabilization relies on its relocalization from the cytosol to the outer mitochondrial membrane (6–9). Membrane-associated Bax oligomerization (10) is further involved in the formation of the mitochondrial apoptosis-induced channel, a putative channel that allows relocalizing cytochrome c from the intermembrane space to the cytosol (11, 12). Bax may also participate in the opening of the permeability transition pore, leading to the rupture of the outer mitochondrial membrane and to the release of bigger proteins such as apoptosis-inducing factor (13), although the actual participation of this pore to the apoptotic process has been questioned (14). Bigger sized Bax channels might also participate in the unselective release of intermembrane space proteins (15).

The cascade of events driving the conversion of inactive, cytosolic Bax to mitochondrial, active Bax requires further clarification. It has been suggested that the N-terminal end of Bax controls its localization (16) because removal of the first 19 N-terminal residues of Bax strongly stimulates Bax relocalization and membrane insertion on isolated mitochondria (16, 17) in mammalian cells (17) and after heterologous expression in yeast cells (18). Increasing the mobility of this N-terminal end, termed ART (apoptosis regulation of targeting) had a similar stimulating effect both in mammalian and yeast cells (19, 20). The main consequence of ART movement is thought to be the exposure of the α1 helix, which can behave as a mitochondria-addressing sequence (17, 18) by interacting with the mitochondrial receptor Tom22, a component of the mitochondrial outer membrane protein translocation machinery (21). A second consequence of this movement would be the rupture of the interaction between residues in α1 and α2 helices, making these helices available for further interactions with other pro-apoptotic partners (22).

The NMR structure of full-length soluble Bax (8) has provided a powerful tool to predict, at a molecular level, the conformational changes that occur during the activation of Bax. The movements of the ART region and α1 helix were predicted from the observation that the 6A7 epitope, which covers the end of the ART region and the beginning of α1 helix, was masked in inactive Bax and became unmasked in active Bax (16). A recently published work confirmed this prediction, by showing that the exposure of this epitope required a movement of nearly 10 Å (23).
A great uncertainty still remains as to how Bax is inserted in the outer mitochondrial membrane. Based on the demonstration that the C-terminal hydrophobic domains of anti-apoptotic proteins Bcl-2 and Bcl-xL are required for their membrane addressing and function (24, 25), a similar property has been proposed for the C-terminal (a9) helix of Bax. Indeed, deletion or substitutions of the Ser\(^{184}\) residue within this helix resulted in constitutively green fluorescent protein-Bax addressing to mitochondria (7). Moreover, a peptide corresponding to Bax-a9 was shown to insert into artificial membranes, a property that was enhanced by the deletion of Ser\(^{184}\) (26). In addition to the a9 helix, the a5 and a6 helices have been suggested to form a hairpin structure within the membrane, because their predicted structure is close to that of bacterial toxins (27). Biochemical data showed that these helices could be inserted together (28) and are required for the pore activity of Bax (29).

However, if there are few doubts about the fact that a9 helix is a transmembrane domain, its actual role in Bax insertion has been questioned. The deletion of a9 fully inhibited the addressing of a green fluorescent protein-Bax fusion in cells containing endogenous Bax (6) but stimulated the insertion of in vitro-translated Bax added to isolated liver mitochondria (30) or expressed in cells lacking endogenous Bax, such as Bax-deficient glioblastoma (19) or yeast cells (18, 31). The putative conformational changes of a9 have been stimulated by substituting Ala or Val for Pro\(^{168}\), located in the loop between a8 and a9 helices; like the full deletion of a9, these substitutions prevented Bax addressing in cells expressing endogenous intact Bax (32) but stimulated the addressing of added Bax in Bax-deficient glioblastoma (9) and in yeast (20).

Conversely, the substitution of Bcl-xL helix, a true C-terminal membrane insertion domain, for a9 helix forced the mitochondrial localization of Bax but prevented its activation, suggesting that the insertion of Bax driven by its C-terminal end could not lead to an active conformation of the chimeric protein (33). Similarly, the addressing/insertion process of the variant carrying the Ser\(^{184}\) deletion was independent of the interaction with Tom22 (21), suggesting that this mutant, although inserted, did not display the accurate conformation. Moreover, a C-terminal c-myc-tagged Bax expressed in yeast was spontaneously inserted (31), suggesting that the addition of a short hydrophilic C-terminal tag to Bax suffices to stimulate its insertion, independently of the interaction with Tom22. Interestingly, a recent study carried out with this type of tagged constructions (34) did not reproduce the data showing the role of Tom22 that was previously evidenced with full-length, native proteins (21, 35).

These previous studies supported the view that slight movements in strategic positions might suffice to initiate profound conformational changes that allowed the shift from inactive soluble to active/membrane-inserted Bax. Physiologically, such movements could result from the formation/disappearance of salt bridges via cycles of phosphorylation/dephosphorylation of specific residues. Indeed, correlations between the phosphorylation of Bax and its capacity to initiate apoptosis have been reported. Phosphorylation of Ser\(^{184}\) by Akt/PKB\(^{3}\) has been shown to promote cell survival by preventing Bax translocation in different models (36–38), and this effect was antagonized by protein phosphatase 2A-driven dephosphorylation (39). On the opposite, Ser\(^{165}\) phosphorylation, possibly driven by GSK3\(\beta\), was able to initiate Bax activation in neuronal cells (40).

Yeast happens to be a potent tool for these studies; even if some primitive form of programmed cell death has been observed in yeast (41), Bax expression was not able to trigger this process (42). Consequently, yeast allows assaying the interaction of Bax with mitochondria in a living cell, independently of the apoptotic network. When native full-length human Bax was expressed in yeast, the protein remained in the cytosol, like in healthy mammalian cells (18). The destabilization of the soluble conformation by mutating the N- or the C-terminal end or by substituting crucial residues was enough to initiate the translocation of the protein to the mitochondrial outer membrane followed by the relocation of cytochrome c (18, 20, 43). In addition, like in mammalian cells, this insertion is dependent on Tom22p (21). Electrophysiological studies demonstrated that, under these conditions, a large channel with properties similar to those of the mitochondrial apoptosis-induced channel was created in the mitochondrial outer membrane of yeast cells (11), showing that Bax function was retained. We have previously reported that numerous mutations in the ART, in the a1 helix, and within or around the a9 helix have the same consequences on Bax expressed in either yeast or Bax-deficient mammalian cells (17, 20, 22).

In the present study, we investigated further the effects of the introduction of point mutations at critical positions within Bax sequence, on Bax translocation, and on activity in yeast cells. This namely allows us to hypothesize that known and putative phosphorylations of serine residues could regulate Bax activation.

**EXPERIMENTAL PROCEDURES**

**Strains and Plasmids**—The wild-type haploid yeast strain W303-1B (mat a, ade2, his3, leu2, trp1, ura3) was used throughout this study. The gene encoding complete human Bax without tags was cloned between the EcoRI and PmeI sites of the pYES3/CT plasmid (Invitrogen). Site-directed mutageneses were done using the QuikChange method (Stratagene) with 27-mer nucleotides (MWG Biotech). The whole mutant Bax genes were entirely sequenced on both strands to verify the introduction of the desired substitutions and the absence of additional mutations.

**Cultures and Mitochondria Isolation**—Yeast cells were grown aerobically at 28 °C in a synthetic medium (0.17% yeast nitrogen base, 0.5% ammonium sulfate, 0.1% potassium phosphate, 0.2% Drop Mix, 0.01% auxotrophic requirements, 2% DL-lactate, pH 5.5). Because the pYES3/CT plasmid bears the TRP1 gene, tryptophan was omitted in the medium for a positive selection of the plasmid. The cells were grown to an optical density of 0.5 at 550 nm (5 × 10\(^6\) cells/ml). 0.5% galactose was added to the medium to trigger Bax expression, and the cells expressed in yeast was spontaneously inserted (31), suggesting that the addition of a short hydrophilic C-terminal tag to Bax suffices to stimulate its insertion, independently of the interaction with Tom22. Interestingly, a recent study carried out with this type of tagged constructions (34) did not reproduce the data showing the role of Tom22 that was previously evidenced with full-length, native proteins (21, 35).

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3 The abbreviations used are: Akt/PKB, protein kinase B; GSK3\(\beta\), glycogen synthase kinase 3\(\beta\); PKA, protein kinase A.
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**RESULTS**

Ionic Interactions Participate in the Stabilization of Bax under an Inactive Conformation—NMR data obtained on soluble Bax highlighted the mobility of the ART (8). Increasing further this mobility by substituting glycine for Pro13 sufficed to constitutively address Bax to mammalian mitochondria (19), and the double substitution of glycine for Pro8/Pro13 had a similar effect in yeast (20). It should be noted that the four residues located between the two prolines (Arg-Gly-Gly-Gly) are further this mobility by substituting glycine for Pro13 sufficed to form a stable ion pair (20). When Arg8 Ca moves with the ART region, these two charges were further grown for 14 h. Typically, the optical density of the cultures reached 4–6 units at 550 nm. The expression levels of the different Bax mutants were measured on whole cell extracts (20) and were comparable (Fig. 1), showing that the different substitutions did not strongly alter the stability of the protein in yeast. Mitochondria were isolated by differential centrifugation from zymolyase-treated cells, as described previously (20).

**SDS-PAGE and Western Blotting**—50 μg of mitochondrial proteins were loaded onto 12.5% SDS-PAGE gels. After electrophoresis, the proteins were transferred onto polyvinylidifluoride difluoride membranes (Problott; Applied Biosystems). The membranes were saturated with 5% milk in phosphate-buffered saline containing 0.02% Tween 20 and probed with a polyclonal anti-Bax antibody (N20; Santa Cruz Biotechnologies; 1/5,000) and a monoclonal anti-yeast porin antibody (Molecular Probes; 1/5,000). Horseradish peroxidase-coupled secondary antibodies were from Jackson and used at 1/10,000. Peroxidase activity was revealed by chemiluminescence (ECL+; Amersham Biosciences).

**Cytochrome Measurements**—Mitochondria suspensions (5 mg proteins/ml) were placed in the cuvettes of a double-beam/double wavelength spectrophotometer (Aminco DW2000). The content of the reference cuvette was oxidized with potassium ferricyanide, and that of the sample cuvette was reduced with sodium dithionite. The difference spectra were acquired between 500 and 650 nm. Cytochromes content was measured from the differences of absorbance 550 – 540, 561 – 575, and 603 – 630 for c, b, and aα3, respectively. Cytochrome b is a membrane protein that is not released and served as an internal standard. The results were then expressed as cytochrome c/cytochrome b concentration ratios (20).

**[^2P]Phosphate Labeling**—The cells were grown in a low phosphate synthetic medium (2 ml) until the culture reached a cell density of 5 × 10⁶ cells/ml (44). 0.5% galactose were added to induce Bax expression, and [^2P]phosphate (0.25mCi/ml) was added 2 h later. After a 2-h incubation, the cells were centrifuged, washed twice, and treated with zymolase (10 mg/ml) for 1 h. The cells were centrifuged, washed twice, and resuspended in 0.5 ml of lysis buffer (IP50 kit; Sigma) supplemented with a mixture of protease inhibitors (Complete; Roche Biochemicals), 10 mM sodium fluoride, and 1 mM sodium orthovanadate. 2 μg of monoclonal anti-Bax antibody (2D2; Sigma) was added, and the lysate was incubated overnight at 4 °C. Protein G-coupled agarose beads (IP50; Sigma) were added and incubated for 8 h. Washing and recuperation of the samples were done following the manufacturer’s instructions. Identical samples were loaded in parallel onto two SDS-PAGE gels and blotted. One was revealed by autoradiography, and the other was probed with the polyclonal anti-Bax antibody.

**FIGURE 1**. Bax mutants constructed in this study. Top, mutants were constructed from full-length human Bax α without tag. The N-terminal ART domain is in italic type. The α helices are underlined. The targeted residues are in bold type, and the different substitutions are indicated. Bottom, Western blots of Bax and Porin on whole cell extracts indicate that all of the mutants are expressed at approximately equivalent levels.
likely move away from each other at an average distance of 12.7 Å. In addition, the axes of the charges move from an angle of 111° under the tight conformation to an angle of 80° under the open conformation, which might further contribute to the loss of interaction between these charges.

To test this hypothesis, site-directed mutagenesis was carried out at these two positions. The single substitutions of Glu for Arg9 or of Lys for Asp154 triggered a massive addressing of Bax to mitochondria (Fig. 3A). Moreover, the addressed proteins were active because they triggered the translocation of cytochrome c (Fig. 3B) to the cytosol. On the opposite, the double-substitution Arg9 to Glu/Asp154 to Lys led to a protein that, like native Bax (Arg9/Asp154), was not translocated to mitochondria. These data support the hypothesis that the presence of opposite charges at positions 9 and 154 participates in the stabilization of ART tight conformation.

A second ionic interaction between Asp33, in α1 helix, and Lys64, in α2 helix, participating in the stabilization of α1 helix within the Bax structure had previously been identified (22). These charges potentially form an ion pair (distance is ∼4 Å; Fig. 4A) and substitutions of Ala for Asp33 or of Asp for Lys64 activate Bax in mammalian cells (22). A similar effect of these substitutions has been observed in yeast because the single mutants Ala33 and Asp64 were able to promote the release of cytochrome c (Fig. 4B). Interestingly, the single Lys33 substitution did not activate Bax, because that could have been expected from the repulsive effect of Lys33/Lys64. This suggested that, in this mutant, a neighboring negative charge might interact with Lys33 and favor the maintenance of α1 helix in the “closed” conformation. The observation of Bax structure did not reveal the presence of any negative charges close enough to explain this inhibitory effect. However, a serine residue in posi-
tion 60 lies in a consensus PKA phosphorylation site (Lys-Lys-Leu-Ser) and at less than 4 Å from Asp33 (Fig. 4A). The possibility that a phosphorylation of this residue might participate in Bax activation was then investigated.

Putative Phosphorylation of Serine 60 Might Regulate the Interaction between α1 and α2—The substitution of Ala for Ser60 was built. Because we predicted that the absence of a phosphorylation of Ser60 would prevent Bax activation, this substitution was introduced in an active Bax variant. As reported previously, the single substitution of Ala for Pro168 was able to activate Bax by increasing the mobility of α9-helix both in yeast (Ref. 20, and see Fig. 3 above) and in mammalian cells (9). The substitution of Ala for Ser60 in the variant previously activated by the substitution Ala168 strongly inhibited both Bax translocation and cytochrome c release (Fig. 5). This supported the view that Ser60 had a stimulating function in Bax translocation that could not be achieved by Ala60.

The Ser60 to Asp substitution mutant was then built to mimic the negative charge of a phosphorylated serine residue and introduced into the wild-type Bax protein. As predicted, the translocation of the protein was strongly stimulated by this substitution (Fig. 5A). However, this stimulation of the translocation was not followed by a stimulation of the release of cytochrome c (Fig. 5B). This suggested that the moderate destabilization of the interaction between Asp33 and Lys64 initiated by the introduction of a negative charge in position 60 was sufficient to stimulate Bax translocation but not to confer its ultimate active conformation. This absence of activity was not caused by the Asp60 mutation itself. Indeed, this mutation was introduced in the moderately active mutant Gly8/Gly13, whose activation is triggered by the increase of the movements of ART (17, 19, 20); the triple mutant Gly8/Gly13/Asp60 was more inserted and exhibited a higher capacity to release cytochrome c than the double mutant Gly8/Gly13 (Fig. 6), whereas the Ala60 substitution was, as expected, without effect.

These data support the hypothesis that the introduction of a negative charge at position 60 has a further stimulating effect on Bax insertion and activation on a moderately activated protein (see also below). However, it was not sufficient, by itself, to provide a full activation of the protein. The destabilizing effect of the introduced negative charge of Asp60 was obviously less dramatic than the complete suppression of the ion pair in mutants Ala33 or Asp64 and probably did not confer enough structural changes to reach the fully active conformation. This suggests that stepped conformational changes corresponding to different functional states of Bax may occur, in accordance to the previously formulated hypothesis that distinct Bax domains are involved in translocation, insertion, and activation (9).

Stimulation of Bax Activation by Substitution at Position 163—Ser163 has been shown to be phosphorylated in neuronal cells, probably by GSK3β (40). Interestingly, this residue is located in the loop between helices α8 and α9 and in the vicinity of residues Arg9 and Asp154 when the ART region is in the closed conformation. We evaluated the hypothesis that the phosphorylation of Ser163 might actually participate in conformational changes associated to Bax activation.

Substitution of Asp for Ser163 in wild-type Bax marginally stimulated both Bax translocation and Bax-induced release of cytochrome c (Fig. 7), suggesting that the introduction alone of a negative charge at this position had some activating effect but was not sufficient to promote enough destabilization to move ART and fully promote Bax activation. We reasoned that a movement of α9 should also be required. The Ser163 to Asp substitution was then introduced in the inactive double mutant.

FIGURE 5. Mutagenesis of Ser60. Mitochondria were isolated from yeast cells expressing different Bax mutants, as described under “Experimental Procedures.” A, Western blot analysis of the mitochondrial steady-state level of Bax, with Porin as a standard. Bax α stands for the full-length human protein, without mutations. B, cytochrome c/cytochrome b ratio in the different mitochondria preparations. The values are the averages (±S.D.) from three to eight mitochondria preparations.

FIGURE 6. Mutagenesis of Ser60 in the ART mutant. Mitochondria were isolated from yeast cells expressing different Bax mutants, as described under “Experimental Procedures.” A, Western blot analysis of the mitochondrial steady-state level of Bax, with Porin as a standard. Bax α stands for the full-length human protein, without mutations. B, cytochrome c/cytochrome b ratio in the different mitochondria preparations. The values are the averages (±S.D.) from three to five mitochondria preparations.
The substitution of Asp for Ser184, which introduced a negative charge mimicking the phosphorylation, did not have a significant stimulating effect on translocation (Fig. 8B), suggesting that the mutant protein was more active than the wild type. Further, the reverse substitution of Lys for Ser184 produced a protein that, like in mammalian cells, was completely absent from mitochondria, possibly indicating the formation of an alternative stabilizing interaction between Lys184 and Asp94. These results were in accordance with those of Nechushtan et al. (7) on green fluorescent protein-Bax constructs, showing that the behavior of Bax mutants at this position was fully conserved between yeast and mammalian cells. In accordance to the reports indicating that this residue could be phosphorylated and that this phosphorylation negatively regulated Bax translocation (37, 38), we wondered whether wild-type human Bax expressed in yeast also supported an inactivating phosphorylation at this position. Bax-expressing cells were labeled and Bax was immunoprecipitated. In vivo [32P]phosphate labeling of Bax showed that the wild-type protein, as well as the single mutant carrying Ser to Asp substitution at position 60 were strongly phosphorylated. The substitution Ser to Asp at position 168 completely absent from mitochondria, possibly indicating the formation of an alternative stabilizing interaction between Lys184 and Asp94. This suggested that a yeast protein kinase actually phosphorylated wild-type Bax on Ser184, thus participating in the absence of mitochondrial translocation of the wild-type protein.

**DISCUSSION**

Modulation of Bax Function by Phosphorylation—In the recent years, a lot of evidence supported the hypothesis that Bcl-2 family members could be phosphorylated and that this phosphorylation might play a role in the regulation of their functions. Different reports have suggested that the phosphorylation of anti-apoptotic protein Bcl-2 and Bcl-xL regulated their stability (43, 45, 46) and, possibly, their addressing to mitochondria preparations.
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A reciprocal observation could be done for Ser\(^{163}\) (40). This residue is likely to be phosphorylated by GSK3\(\beta\), and this phosphorylation would participate in the activation of Bax. Because GSK3\(\beta\) can itself be phosphorylated, hence inactivated, by Akt/PKB (49), these two phosphorylatable residues would provide an interesting double-switch in the regulation of Bax activation because Akt/PKB would prevent Bax activation directly by phosphorylating Ser\(^{163}\) and indirectly by preventing the phosphorylation of Ser\(^{163}\).

The aim of the present work was to investigate how these phosphorylations could be integrated within the general scheme of Bax conformational changes that occur during its addressing, translocation, insertion, and activation within the mitochondrial outer membrane. For this reason, we included studies of Ser\(^{60}\), although the phosphorylation of this residue has not been reported to date. However, its position in a conserved consensus sequence for PKA-dependent phosphorylation, as well as its proximity with the two residues Asp\(^{33}\)/Lys\(^{64}\), the function of which has been demonstrated in the maintenance of the inactive conformation of Bax (22), made it an interesting target for our studies.

Regulations of Bax Function by ART Movement—It is now widely accepted that the movement of the N-terminal end (ART) of Bax is a crucial step toward the activation of the protein (see Ref. 50 for review). Not only does this movement allow for exposing the \(\alpha_1\) helix, the potential mitochondrial addressing sequence (17, 21, 23), but it also favors the liberation of Asp\(^{33}\) for the interaction with pro-apoptotic BH3-only proteins tBid and PUMA (22). Changes in the conformation of the peptide bonds around Pro\(^{13}\) have been shown to provide enough amplitude to trigger the whole movement of the N-terminal end required for Bax addressing, insertion, and activation (17, 19, 20). From these observations, the question regarding the stabilization of the ART region in its closed conformation then arose. The observation of the NMR structure revealed the existence of some proximity between the Arg\(^{9}\) and the Asp\(^{154}\). The results reported in the present paper clearly showed that the presence of two similar charges at these positions triggered Bax activation, whereas the presence of two opposite charges promoted the inactive conformation. These charges are relatively far from each other (7.3Å), not close enough to create a stable salt bridge. However, this proximity might be sufficient to slow down the movements of ART and favor the stabilization of the tight conformation.

This further supports the crucial role of ART mobility in the whole process of Bax activation. However, this does not provide a clue for the trigger(s) that are required for this activation. From the present study, the three serine residues 60, 163, and 184 have this capacity.

Correlation between Phosphorylation and Conformational Changes—The putative phosphorylation of Ser\(^{60}\) likely destabilizes the salt bridge between Asp\(^{33}\) and Lys\(^{64}\). Our study shows that the substitution by a nonphosphorylatable residue (Ala\(^{60}\)) prevented the activation of Bax caused by a movement of \(\alpha_9\) (Pro\(^{168}\) to Ala). On the other hand, the substitution of Ser\(^{60}\) by Asp, introducing a negative charge at this position, is not sufficient to trigger the full activation of wild-type Bax. Keeping in mind that an aspartate residue might not have strictly the same consequences as a phosphoserine residue on Bax structure (in terms of both size and charge), the lack of full activation in mutant Asp\(^{60}\) might indicate that phosphorylation of Ser\(^{60}\), if it occurs, is not the primary event triggering Bax conformational change but might be required at a later step, to favor the disruption of the interaction between \(\alpha_1\) and \(\alpha_2\), making these domains accessible for interactions with other partners.

It is noteworthy that, in our study, there is not an absolute correlation between the amount of mitochondrial Bax and the extent of cytochrome c release. For example, in Fig. 3, the higher mitochondrial amount of the Ala\(^{60}\) mutant, as compared with the Glu\(^7\) mutant, is associated to a slightly lower capacity to release cytochrome c. Previous reports in the literature showed examples where Bax, although mitochondria-localized, was inactive (51, 52). Our data further support the view that mitochondrial Bax may exist under different stable conformations that may exhibit different activities, possibly depending on interactions with other partners or on oligomerization status. Precise measurements of single-channel activities of Bax...
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![Diagram of Bax Phosphorylation](image)

**FIGURE 10. Hypothesis of regulation of Bax by phosphorylation.** The hypothesis is based on data reported in references (36–40, 49) and the present paper. In healthy cells, Bax-Ser184 is phosphorylated by AKT/PKB, which also phosphorylates and inactivates GSK3β, thus preventing the phosphorylation of Ser163. Ser60 is not accessible to phosphorylation by PKA. This provides a tight conformation that cannot interact with mitochondria. In apoptotic cells, AKT/PKB does not phosphorylate Bax-Ser184, allowing GSK3β phosphorylation of Ser163. Movements of ART and of the helix α9 allow opening of the protein, making Ser60 accessible to PKA-driven phosphorylation that may further contribute to the destabilization between α1 and α2 and their interaction with BOPs (22) and the mitochondrial receptor Tom22 (21). Cic (cytosol locked-in conformation) and Clac (cytotoxicity c-association conformation) refer to the conformations of Bax defined in Refs. 9 and 50.

mutants, by means of electrophysiological methods (11, 12), could potentially provide a direct correlation between Bax conformation and activity.

The phosphorylation of Ser184 has already been largely documented by works in mammalian cells. The fact that this residue is phosphorylated when expressed in yeast supports the hypothesis that it is easily accessible to phosphorylation even by a nonmammalian protein kinase. This probably contributes to the fact that, like in healthy human cells, Bax remains in the cytosol of yeast cells. This points to an important aspect of the regulation of Bax addressing: although it has been suggested that Bax was retained in the cytosol through interactions with proteins such as Ku70, survivin or 14-3-3 (54, 55), phosphorylation of Ser184 is sufficient to maintain Bax in a cytosolic conformation in yeast, which does not contain these putative partners. This supports the view that the closed conformation of Bax is by itself sufficient to prevent its interaction with mitochondria.

Although less documented than the phosphorylation of Ser184, phosphorylation of Ser163 appears to be required for the activation of Bax, at least in some model systems (40). It is particularly interesting to note that, if the introduction of a negative charge at position 163 cannot, alone, stimulate Bax, it is able to relieve the inhibitory effect of Ala60 substitution. Given that Ser163 is a potential target of GSK3β, which is itself a target of Akt/PKB, it is tempting to speculate that the inhibition of Akt/PKB, which is expected to result in the dephosphorylation of Ser184 and the phosphorylation of Ser163, would induce structural changes sufficient to allow the accessibility of Ser60 to another kinase (PKA).

A model of regulation of Bax phosphorylation by the three protein kinases Akt/PKB, GSK3β and PKA can be proposed as a working hypothesis (Fig. 10). This hypothesis takes into account the respective effects of Akt/PKB-dependent phosphorylation of Ser184 and GSK3β-dependent phosphorylation of Ser163. The putative PKA-dependent phosphorylation of Ser60 was included to take the behavior of substituted mutants at this position into account. Heterologous co-expression of different Bax mutants with these different protein kinases will now be achieved in yeast to validate and refine this hypothesis.

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