The Phosphatidylinositol 3-Kinase (PI3K)-Akt Pathway Suppresses Bax Translocation to Mitochondria*

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Bax, a proapoptotic member of the Bcl-2 family, localizes largely in the cytoplasm but redistributes to mitochondria in response to apoptotic stimuli, where it induces cytochrome c release. In this study, we show that the phosphatidylinositol 3-OH kinase (PI3K)-Akt pathway plays an important role in the regulation of Bax subcellular localization. We found that LY294002, a PI3K inhibitor, blocked the effects of serum to prevent Bax translocation to mitochondria and that expression of an active form of PI3K suppressed staurosporine-induced Bax translocation, suggesting that PI3K activity is essential for retaining Bax in the cytoplasm. In contrast, both U0126, a MEK inhibitor, and active MEK had little effect on Bax localization. In respect to downstream effectors of PI3K, we found that expression of active Akt, but not serum and glucocorticoid-induced protein kinase (SGK), suppressed staurosporine-induced translocation of Bax, whereas dominant negative Akt modestly promoted Bax translocation. Expression of Akt did not alter the levels of Bax, Bcl-2, Bcl-XL, or phosphorylated JNK under the conditions used, suggesting that there were alternative mechanisms for Akt in the suppression of Bax translocation. Collectively, these results suggest that the PI3K-Akt pathway inhibits Bax translocation from cytoplasm to mitochondria and have revealed a novel mechanism by which the PI3K-Akt pathway promotes survival.

Apoptosis plays a critical role in the normal development and maintenance of tissue homeostasis (1, 2). The regulation of mitochondrial membrane integrity and the release of cytochrome c from mitochondria are important processes during apoptosis (3, 4) and are controlled by the Bcl-2 family (5, 6). The Bcl-2 family includes both pro- and antiapoptotic members that possess up to four conserved Bcl-2 homology domains designated BH1, BH2, BH3, and BH4 (5, 6). Many of the antiapoptotic members, including Bcl-2 and Bcl-XL, contain all four domains, whereas the proapoptotic members, including Bax and Bak, lack the BH4 domain, and the other proapoptotic members, so-called “BH3 domain only proteins,” including Bid, Bim and Bad, contain only the BH3 domain. One of the intriguing aspects of the Bcl-2 family is their subcellular localization and translocation. For example, antiapoptotic members such as Bcl-2 and Bcl-XL and proapoaptotic members such as Bak localize predominantly at mitochondria and regulate the mitochondrial membrane integrity and cytochrome c release. On the other hand, other proapoptotic members such as Bax, Bid, and Bad reside in cytoplasm in healthy cells (5). In response to apoptotic stimuli, these cytosolic proapoptotic members redistribute to mitochondria and promote cytochrome c release. For example, active caspase-8 cleaves p22 Bid into p15 Bid, which translocates to mitochondria (7, 8). For Bad, the localization is regulated by phosphorylation; survival signals induce phosphorylation of Bad, resulting in 14-3-3 binding and sequestration from its mitochondrial target, Bcl-XL (9, 10). Therefore, the loss of survival signals translocates Bad to mitochondria through dephosphorylation (11, 12).

After translocation to mitochondria, Bax induces cytochrome c release either by forming a pore through oligomerization or by opening a channel called voltage-dependent anion channel (VDAC)1 via direct interaction (13, 14). Bax translocation to mitochondria may serve as a key integration point for various apoptosis signals because Bax translocation takes place in response to a wide variety of apoptotic stimuli such as staurosporine, dexamethasone, etoposide, nitric oxide, Fas, cell detachment, and γ-irradiation (15–20) and because the essential roles of Bax in inducing apoptosis have been revealed by gene disruption of Bax alone (21, 22) and of both Bax and Bak (23–25). Recent studies have shown that NGF treatment of sympathetic neurons (26) and IL-7 treatment of thymocytes (27) inhibit Bax translocation to mitochondria, suggesting that some survival signals also regulate Bax translocation. However, how survival signals regulate Bax localization has yet to be clarified.

It has been shown that the phosphatidylinositol 3-OH kinase (PI3K) pathway and the mitogen-activated protein kinase (MAPK) pathway play critical roles in promotion of cell survival (28–31). PI3K phosphorylates the 3-OH position of the inositol ring in phosphatidylinositol (PtdIns), generating PtdIns(3,4)P2 and PtdIns(3,4,5)P3. Both PtdIns(3,4)P2 and PtdIns(3,4,5)P3 bind to the pleckstrin homology domain of PDK1, which in turn activates downstream targets such as Akt and SGK (32, 33). Akt inhibits apoptosis by inactivating proapoptotic proteins such as Bad, caspase-9, forkhead, and Nur77 and by activating antiapoptotic proteins such as NF-κB and cAMP-response element-binding protein (9, 10, 34–40). Recent studies have suggested that Akt inhibits apoptosis at a premi-

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1 The abbreviations used are: VDAC, voltage-dependent anion channel; NGF, nerve growth factor; IL, interleukin; PI3K, phosphatidylinositol 3-OH kinase; MAPK, mitogen-activated protein kinase; PtdIns, phosphatidylinositol; SGK, serum and glucocorticoid-induced protein kinase; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; PBS, phosphate-buffered saline; DTT, dithiothreitol; GFP, green fluorescent protein; JNK, c-Jun N-terminal kinase; PDK, 3-phosphoinositide-dependent kinase.
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RESULTS AND DISCUSSION

Recent studies have suggested that some survival-promoting conditions can suppress Bax translocation to mitochondria (20, 26). We first examined whether serum treatment can also suppress Bax translocation in HeLa cells because serum is known to promote survival. To examine this, intracellular localization of endogenous Bax was detected by immunocytochemistry. As shown in Fig. 1, Bax localized diffusely in the cytoplasm in the presence of serum. In contrast, serum deprivation induced Bax translocation from cytoplasm to mitochondria as revealed by localization of Bax at mitochondria visualized by a mitochondrial marker, MitoTracker CMXRos (Fig. 1, A and B). Addition of 50 μM Z-VAD-CH₉DCB, a wide spectrum caspase inhibitor, had little effect on Bax translocation induced by serum deprivation.
Fig. 2. PI3K activity is required for retaining endogenous Bax in the cytoplasm. A, HeLa cells were incubated without serum in the presence of 50 μM Z-VAD-CH₂DCB for 6 h, pretreated with 10 μM U0126 or 10 μM LY294002 for 30 min, and stimulated with 10% serum for either 10 min (phospho-MAPK) or 90 min (phospho-Akt). Cell extracts were subjected to immunoblotting with anti-phospho-Akt antibody, anti-Akt antibody, anti-phospho-MAPK antibody or anti-MAPK antibody. B, HeLa cells were incubated without serum in the presence of 50 μM Z-VAD-CH₂DCB for 6 h and then with or without serum for 48 h. The cells were stained with anti-Bax antibody and MitoTracker CMXRos, and the percentage of cells displaying punctate Bax was determined. Data represent the mean ± S.D. (n = 5) of three independent experiments. p values for significantly different pairwise comparisons are indicated. N.S., not significant. C, after incubation without serum for 6 h, HeLa cells were incubated with or without serum for 48 h in the absence of caspase inhibitor. Then the cells were fixed and stained with anti-Bax antibody and Hoechst33258. The percentage of cells displaying punctate Bax distribution (% mitochondrial Bax) and the percentage of cells with pyknotic nucleus (% apoptotic cells) are indicated in the left panel. In the right panel, the percentage of apoptotic cells to the population of cells with cytoplasmic Bax (white columns) and that with mitochondrial Bax (gray columns) are indicated. DMSO, dimethyl sulfoxide.
by serum deprivation (data not shown). These results demonstrated that serum is necessary for retaining Bax in the cytoplasm and that Bax translocation by serum deprivation is a caspase-independent event.

It has been shown that the PI3K-Akt pathway and the MAPK pathway play important roles in growth factor-promoted cell survival by inhibiting several steps of apoptosis signaling depending on cell types and contexts (28–31). The MAPK pathway has been shown to inhibit cytochrome c-induced caspase activation (31), but it is not known whether it also inhibits premitochondrial steps, including Bax translocation and cytochrome c release. On the other hand, active Akt has been shown to inhibit UV-induced cytochrome c release in Rat1 fibroblasts (41), whereas PI3K activity has been reported to be dispensable for NGF-mediated suppression of Bax translocation in sympathetic neurons (45). Thus it remains unclear so far which molecules are responsible for growth factor suppression of Bax translocation to mitochondria. To examine whether PI3K or MEK mediates serum suppression of Bax translocation, HeLa cells were treated with LY294002, a PI3K inhibitor, or with U0126, a MEK inhibitor, in the presence of serum. Treatment with 10 μM LY294002 resulted in the promotion of Bax translocation to mitochondria (Fig. 2B) under the conditions in which phosphorylation of Akt, but not of MAPK, was effectively blocked (Fig. 2A). In contrast, treatment with 10 μM U0126, which effectively blocked MAPK phosphorylation under the same conditions, had no effect on Bax localization (Fig. 2A and B). These results suggest that PI3K, but not MEK, is required for retaining Bax in the cytoplasm of serum-stimulated HeLa cells. Under the same experimental conditions, both serum deprivation and LY294002 treatment resulted in induction of apoptosis in the absence of caspase inhibitor as judged by pyknotic nuclei, whereas U0126 treatment did not (Fig. 2C, left panel). Moreover, most of the cells with mitochondrial endogenous Bax exhibited pyknotic nucleus, whereas most of the cells with cytoplasmic Bax displayed healthy nucleus (Fig. 2C, right panel). These results confirmed that Bax translocation correlated well with apoptosis in the system analyzed.

To further examine whether PI3K activity is important for retaining Bax in the cytoplasm, we utilized a GFP-Bax fusion protein to monitor Bax localization in real time. In all experiments using GFP-Bax, p35, a pan-caspase inhibitor, was co-transfected to protect cells from undergoing apoptosis because ectopic expression of Bax results in activation of the caspase cascade, which might amplify the upstream apoptotic signals of Bax translocation (46). In control and U0126 (10 μM)-treated COS-1 cells, GFP-Bax typically displayed a diffuse, cytoplasmic localization (Fig. 3, A and B). In contrast, GFP-Bax gradually redistributed to mitochondria in response to LY294002 (10 μM) treatment (Fig. 3, A and B). These results suggest that endogenous PI3K activity, but not MEK activity, is necessary for retaining GFP-Bax in the cytoplasm in this system as well. We next investigated whether activation of PI3K is sufficient for inhibiting Bax translocation induced by staurosporine treatment. Staurosporine induced GFP-Bax translocation to mitochondria (Fig. 3, A and B).

**FIG. 3.** **PI3K activity is essential for retaining GFP-Bax in the cytoplasm.** A, COS-1 cells were transfected with GFP-Bax, DsRed-Mit, and p35 together with the indicated constructs and incubated for 10 h. Cells were treated with vehicle Me2SO (DMSO), 10 μM LY294002, 10 μM U0126, or 0.1 μM staurosporine for 3 h. A typical image of each condition is shown. B, COS-1 cells were transfected with GFP-Bax and p35 and incubated for 10 h. Cells were treated with vehicle Me2SO (DMSO), 10 μM LY294002, or 0.1 μM staurosporine for the indicated times and assessed by counting the number of cells with mitochondrial Bax. GFP-Bax localization at mitochondria was determined based on the overlaps of the GFP-Bax and DsRed-Mit fluorescence images. Data represent the mean ± S.D. (n = 5) of two independent experiments. C and D, COS-1 cells were transfected with GFP-Bax and p35 together with the indicated constructs and incubated for 10 h. Cells were treated with 0.1 μM staurosporine (STS) for the indicated times and assessed by counting the number of cells exhibiting mitochondrial Bax. Data represent the mean ± S.D. (n = 5).
chondria within several hours, but expression of p110CAAX, a membrane-targeted catalytic subunit of PI3K, resulted in a marked inhibition of Bax translocation (Fig. 3, A and C). In contrast, expression of active MEK with a S218D/S222D mutation had no effect on Bax translocation (Fig. 3, A and D). These results suggest that activation of PI3K is sufficient for retaining Bax in the cytoplasm of COS-1 cells.

To dissect the downstream pathway of PI3K, we first tested whether Akt suppresses Bax translocation as Akt often mediates the effects of PI3K in promoting survival (28, 30). In fact, our results suggest that Akt plays an essential role in serum promotion of survival in the system analyzed (see Fig. 4D). We found that expression of Akt, which had a myristylation sequence at the N terminus and lacked the pleckstrin homology domain, inhibited GFP-Bax translocation to mitochondria induced by staurosporine treatment (Fig. 4, A and B). In addition, a dominant negative Akt (3A), with a K179A/T308A/S473A mutation, enhanced GFP-Bax translocation moderately but reproducibly (Fig. 4C). A previous study has shown that expression of active Akt decreases the levels of Bax protein induced by nitric oxide in primary hippocampal neurons (47). However, in this study, the levels of endogenous Bax and GFP-Bax did not alter by expression of Akt (Fig. 5), suggesting that Akt suppression of Bax translocation is not due to a reduction of Bax protein. In primary hippocampal neurons and IL-3-dependent BaF-3 cells, the PI3K-Akt pathway has been shown to induce Bcl-2 and Bcl-XL, which play critical roles in promoting survival of these cells (47, 48). It has recently been shown that expression of Bcl-2 is capable of inhibiting etoposide- and Fas-induced Bax translocation to mitochondria (17, 18). Therefore, it is possible that Akt inhibition of Bax translocation is mediated by an increase in the levels of Bcl-2 and Bcl-XL proteins.

**Fig. 4.** Expression of active Akt inhibits GFP-Bax translocation to mitochondria. A, COS-1 cells were transfected with GFP-Bax, DsRed-Mit, and p35, together with the indicated constructs and incubated for 10 h. Cells were treated with 1 μM staurosporine for 3 h. A typical image of each condition is shown. B, COS-1 cells were transfected with GFP-Bax and p35, together with various constructs, and incubated for 10 h. Cells were treated with 1 μM staurosporine (STS) and assessed by counting the number of cells with mitochondrial Bax. Data represent the mean ± S.D. (n = 5) of three independent experiments. C, COS-1 cells were transfected with GFP-Bax and p35 together with each of the indicated constructs for 16 h. Data represent the mean ± S.D. (n = 5) of two independent experiments. P values for significantly different pairwise comparisons are indicated. D, COS-1 cells were transfected with empty vector, active Akt (Akt with a myristylation site at its N terminus), or 3A Akt (a dominant negative Akt with a K179A/T308A/S473A mutation) with GFP, and incubated for 48 h in the presence or absence of serum. Apoptosis was then judged by pyknotic nucleus after staining with Hoechst33342.
However, the expression of active Akt did not alter the levels of Bcl-2 and Bcl-X<sub>L</sub> proteins under the conditions used in this study (Fig. 5), indicating that the suppression of Bax translocation by Akt is not due to increased expression of these anti-apoptotic members of the Bcl-2 family.

To further confirm the effects of Akt on Bax translocation, localization of endogenous Bax was assessed by subcellular fractionation after transfection of Akt constructs. As shown in Fig. 6, 6 h of staurosporine treatment in control (vector-transfected) cells increased the levels of Bax in the mitochondrial fraction. Twelve hours of staurosporine treatment resulted in redistribution of ~30% of endogenous Bax to the mitochondrial fraction (Fig. 6). In contrast, little Bax protein was detected in the mitochondrial fraction when active Akt, but not kinase-negative (3A) Akt, was expressed in up to 12 h of staurosporine treatment (Fig. 6). The level of the VDAC in the mitochondrial fraction did not alter. These results suggest that active Akt inhibits endogenous Bax translocation to mitochondria.

SGK, another downstream effector of PI3K-PDK1, is a kinase structurally related to Akt and has a substrate specificity.
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COS-1 cells were transfected with GFP-Bax and p35 together with in suppressing Bax translocation.  
rylation of co-transfected FKHRL1, a substrate of SGK (Fig. 5),  
Expression of active SGK, with a S422D mutation, did not enhance Bax translocation (Fig. 7).  
In sympathetic neurons, NGF inhibits premitochondrial steps of apoptosis signaling (26). Recently, Tsui-Pierchala et al. (45) have reported that PI3K activity is required for inhibiting an early death event proximal to c-Jun phosphorylation (most likely catalyzed by JNK) but not for inhibiting Bax translocation in NGF suppression of apoptosis. However, in COS-1 cells, activation of the PI3K-Akt pathway did not block staurosporine-induced JNK activation (Fig. 8) but did antagonize the staurosporine-induced Bax translocation (Fig. 4), suggesting that the main targets of the PI3K pathway in these two systems are different. Because our data indicate that activation of the PI3K-Akt pathway is sufficient for inhibiting Bax translocation, it is plausible that there is a redundant pathway in addition to the PI3K pathway downstream of NGF to block Bax translocation.  
The molecular mechanism of Bax subcellular redistribution is a matter of controversy. Previous reports have suggested that it involves conformational change of Bax induced by cytosolic alkaLization and Bax dimerization (27, 49). However, we found that GFP-Bax translocation was not induced by cytoplasmic pH values ranging between 6.6 and 8.6 when adjusted by nigericin, a proton ionophore, and extracellular pH (data not shown). In addition, it has been shown by NMR experiments that no conformational change of Bax can be detected within a potential intracellular pH range (50). These data may suggest that there is no direct link between intracellular pH change and Bax translocation to mitochondria. Therefore, we do not assume that Akt regulates Bax localization via controlling intracellular pH. Thus far, we have not been able to determine which Akt target mediates its regulation of Bax. It is unlikely that Bax is a direct target of Akt because Bax does not contain the RXRXY(S/T) motif, the consensus sequence required for Akt phosphorylation (51). How Akt regulates Bax awaits further investigation.

In conclusion, we show in this study that the PI3K-Akt pathway regulates Bax translocation to mitochondria in serum-stimulated HeLa and GFP-Bax-expressing COS-1 cells. This finding may account for why Akt inhibits cytochrome c release at a premitochondrial level. Because Bax is a key mol-
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