The PI3K-Akt pathway suppresses Bax translocation to mitochondria

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Summary

Bax, a pro-apoptotic 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 SGK, suppressed staurosporine-induced translocation of Bax, while dominant negative Akt moderately 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 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.
Introduction

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 anti-apoptotic members that possess up to four conserved Bcl-2 homology domains designated BH1, BH2, BH3 and BH4 (5, 6). Many of the anti-apoptotic members, including Bcl-2 and Bcl-X\textsubscript{L}, contain all four domains, while the pro-apoptotic members, including Bax and Bak, lack the BH4 domain, and other the pro-apoptotic 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, anti-apoptotic members such as Bcl-2 and Bcl-X\textsubscript{L}, and pro-apoptotic members such as Bak localize predominantly at mitochondria, and regulate the mitochondrial membrane integrity and cytochrome c release. On the other hand, other pro-apoptotic members such as Bax, Bid and Bad reside in cytoplasm in healthy cells (5). In response to apoptotic stimuli, these cytosolic pro-apoptotic 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-X\textsubscript{L} (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)\textsuperscript{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 \textgamma-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) P$_2$ and PtdIns (3,4,5) P$_3$. Both PtdIns (3,4) P$_2$ and PtdIns (3,4,5) P$_3$ 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 pro-apoptotic proteins such as Bad, caspase-9, forkhead and Nur77, and by activating anti-apoptotic proteins such as NF-$\kappa$B and CREB (9, 10, 34-40). Recent studies have suggested that Akt inhibits apoptosis at a premitochondrial level, since it inhibits cytochrome c release and alteration of mitochondrial membrane potential (41, 42). However, the mechanisms by which Akt inhibits the premitochondrial events of apoptosis are not fully understood (41).

In this study, we asked which molecules activated by growth factors are responsible for regulation of Bax subcellular localization. We found that PI3K activity is essential for retaining Bax in the cytoplasm, and also demonstrated that Akt, but not SGK, is capable of suppressing Bax translocation to mitochondria. These results suggest that the PI3K-Akt pathway plays a critical role in inhibiting Bax translocation to mitochondria.
Experimental Procedures

Materials

We obtained LY294002 from Calbiochem, U0126 from Promega, Staurosporine from ICN Biomedicals, MitoTracker CMXRos, Hoechst33258 and Hoechst33342 from Molecular Probes and Z-VAD-CH₂DCB from Phoenix Pharmaceuticals INC. Anti-Bax (N-20, SantaCruz), anti-VDAC (31HL, Calbiochem), anti-MAPK (K-23, SantaCruz), anti-phospho MAPK (Promega), anti-Akt (Cell Signaling), anti-phospho Akt (Cell Signaling), anti-tubulin (DM1A, SIGMA), anti-Bcl-2 (Bcl-2/100, PharMingen), anti-Bcl-X₅ (L-19, SantaCruz), anti-FKHRL1 (H-144, SantaCruz), anti-phospho FKHRL1 (Cell Signaling), anti-HA (3F10, Roche), anti-JNK (C-17, SantaCruz) and anti-phospho JNK (Cell Signaling) were used for immunoblotting, and anti-Bax (G206-1276, PharMingen) and Alexa 488 anti-rat IgG (Molecular Probes) were used for immunocytochemistry.

Plasmid construction

Mouse Bax cDNA was amplified by PCR, and cloned into EcoRI and KpnI sites of pEGFP-C2 vector (CLONTECH). The constructs encoding p35, constitutively active Akt (myristylated Akt lacking the pleckstrin homology domain, residues 4-125), dominant negative (3A) Akt with a K179A/T308A/S473A mutation, p110CAAX (the membrane-targeted catalytic subunit of PI3K), constitutively active MEK with a S218D/S222D mutation, constitutively active SGK with a S422D mutation, dominant negative (2A) SGK with a T256A/S422A mutation and FKHRL1 have been described previously(33, 43, 44). Active and 2A SGK clones were inserted into the BglII site of pCS4-HA. DsRed-Mit was constructed by inserting synthetic double-stranded oligonucleotides spanning a mitochondrial targeting sequence of human cytochrome c oxidase into BamHI and NheI sites of the pDsRed-N1 vector (CLONTECH).

Immunoblotting

HeLa cells were incubated with 50 µM Z-VAD-CH₂DCB in the absence of serum for 6 hours, treated with or without 10 µM LY294002 or 10 µM U0126 for 30 min, and then stimulated with 10% serum. After 10 or 90 min, cells were washed twice with phosphate...
buffered saline (PBS), and lysed in an extraction buffer (20 mM Tris pH 7.5, 150 mM NaCl, 10 mM β-glycerophosphate, 5 mM EGTA, 1 mM Na₄P₂O₇, 5 mM NaF, 1 mM Na₃VO₄, 0.5% Triton X-100 and 1 mM DTT) supplemented with protease inhibitors (1 mM PMSF, 5 µg/mL leupeptin, 5 µg/mL pepstatin A and 5 µg/mL aprotinin), and then subjected to immunoblotting. COS-1 cells were transfected with GFP-Bax and p35, together with various constructs using FuGENE6 Transfection Reagent (Roche) for 10 hours. Cells were next treated with 1 µM of staurosporine for 3 or 6 hours, and then subjected to immunoblotting.

**Immunocytochemistry**

HeLa cells were grown on poly-D-lysine coated coverslips in 6 well plates. Cells were incubated with or without 50 µM Z-VAD-CH₂DCB in the absence of serum for 6 hours, treated with or without 10 µM LY294002 or 10 µM U0126 for 30 min, and then stimulated with 10% serum. After 48 hours, cells were pretreated with 100 nM MitoTracker CMXRos for 30 min, washed with PBS and fixed with 3.7% formaldehyde in PBS for 10 min at room temperature. The coverslips were soaked with a blocking solution (PBS containing 5% BSA and 0.4% Triton X-100) for 30 min, incubated with anti-Bax antibody for 1 hour, and then with Alexa 488 anti-rat IgG antibody for 30 min in the blocking solution. The cells exhibiting strong punctate staining of Bax, which overlapped with the distribution of MitoTracker CMXRos, were counted as the cells with mitochondrially localized Bax. These cells usually had very low levels of diffuse Bax staining in the cytoplasm.

**GFP-Bax translocation assay**

COS-1 cells were transfected with GFP-Bax and p35, together with various constructs, and incubated for 10 hours. Cells were then treated with 10 µM LY294002, 10 µM U0126 or staurosporine for the indicated times. The localization of GFP-Bax was detected by fluorescence microscope.

**Subcellular fractionation**

COS-1 cells were transfected with various constructs for 1 day, and treated with 1 µM staurosporine for the indicated times. Then the cells were washed with PBS, scraped into an isotonic buffer (200 mM mannitol, 70 mM sucrose, 1 mM EDTA, 10 mM HEPES pH 6.9 and
1 mM DTT) supplemented with the protease inhibitors described above, and homogenized using a Potter-Elvehjem homogenizer. Nuclei and unbroken cells were separated by centrifugation at 600 × g for 10 min. The supernatant was further centrifuged at 10,000 × g for 10 min to collect a heavy membrane pellet, which contained mitochondria. The pellet was washed with the isotonic buffer, resuspended into RIPA buffer (PBS containing 1% Nonidet P40, 0.5% sodium deoxycholate, 0.1% SDS and 1 mM DTT) supplemented with protease inhibitors, and the debris was then removed by a brief centrifugation.
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, since 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. 1A and 1B). Addition of 50 µM of Z-VAD-CH₂DCB, a wide spectrum caspase inhibitor, had little effect on Bax translocation induced 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 (the activator of MAPK) 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 of 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 of U0126, which effectively blocked MAPK phosphorylation under the same conditions, had no effect on Bax localization (Fig. 2A and 2B). 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, while 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, since 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. 3A and 3B). In contrast, GFP-Bax gradually redistributed to mitochondria in response to LY294002 (10 µM) treatment (Fig. 3A and 3B). 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 within several hours, but expression of p110CAAX, a membrane-targeted catalytic subunit of PI3K, resulted in a marked inhibition of Bax translocation (Fig. 3A and 3C). In contrast, expression of active MEK with a S218D/S222D mutation, had no effect on Bax translocation (Fig. 3A and 3D). 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 active Akt, which had a myristylation sequence at the amino terminus and lacked the pleckstrin homology domain, inhibited GFP-Bax translocation to mitochondria induced by staurosporine treatment (Fig. 4A and 4B). 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-X<sub>L</sub>, 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-X<sub>L</sub> proteins. 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 hours 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 approximately 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 hours 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 very similar to Akt (33). Because SGK also appears to play a role in promoting cell survival (33, 44), we examined the possible involvement of SGK in regulation of Bax localization. Expression of active SGK, with a S422D mutation, did not inhibit GFP-Bax translocation to mitochondria induced by staurosporine treatment (Fig. 7A). In addition, a dominant negative (2A) SGK, with a T256A/S422A mutation, did not enhance Bax translocation (Fig. 7B). We confirmed the expression of these SGK mutants by immunoblotting and phosphorylation of co-transfected FKHRL1, a substrate of SGK (Fig. 7C). Therefore, SGK appears not to mediate the effect of PI3K to suppress Bax
translocation.

In sympathetic neurons, NGF inhibits premitochondrial steps of apoptosis signaling (26). Recently, Tsui-Pierchala et al 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 (45). 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. Since our data indicates 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 alkalinization and Bax dimerization (27, 49). However, we found that GFP-Bax translocation was not induced by cytoplasmic pH 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 (Arg x) Arg x x Ser/Thr 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. Since Bax is a key molecule regulating apoptosis, our results may have revealed an important nexus between apoptotic and survival signals.
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Footnotes

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1 The abbreviations used are: VDAC, voltage-dependent anion channel; NGF, nerve growth factor; IL-3, interleukin-3; IL-7, interleukin-7; PI3K, phosphatidylinositol-3-OH kinase; PDK1, 3-phosphoinositide-dependent kinase-1; SGK, serum and glucocorticoid induced protein kinase; NF-κB, nuclear factor-κB; JNK, c-jun N-terminal kinase; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; MAPK, mitogen-activated protein kinase; GFP, green fluorescence protein; DTT, dithiothreitol; DMSO, dimethyl sulfoxide; PMSF, phenylmethylsulfonyl fluoride.


Figure legends

Figure 1. Serum deprivation induces endogenous Bax translocation to mitochondria. (A) HeLa cells were incubated without serum in the presence of 50 µM Z-VAD-CH2DCB for 6 hours, and then stimulated with or without 10% serum for 48 hours. The cells were treated with 100 nM MitoTracker CMXRos for 30 min before immunocytochemistry to stain mitochondria. (B) The number of cells exhibiting diffuse or punctate Bax was assessed by fluorescence microscope, and the percentage of cells displaying punctate Bax determined by counting approximately 20 to 150 cells for each condition. Data represent the mean ± SD (n=5) of three independent experiments. P values for significantly different pairwise comparisons are indicated.

Figure 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 hours, 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 hours, and then with or without serum for 48 hours. 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 ± SD (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 hours, HeLa cells were incubated with or without serum for 48 hours 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.

Figure 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 hours. Cells were treated with vehicle (DMSO), 10 µM LY294002, 10 µM U0126 or 0.1 µM staurosporine for 3 hours. A typical image of each condition is shown. (B) COS-1 cells were transfected with GFP-Bax and p35, and incubated for 10 hours. Cells were treated with vehicle (DMSO), 10 µM LY294002 or 10 µM U0126 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 ± SD (n=5) of two independent experiments. (C) (D) COS-1 cells were transfected with GFP-Bax and p35, together with the indicated constructs and incubated for 10 hours. 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 ± SD (n=5).

Figure 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 hours. Cells were treated with 1 µM staurosporine for 3 hours. 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 hours. Cells were treated with 1 µM staurosporine (STS), and assessed by counting the number of cells with mitochondrial Bax. Data represent the mean ± SD (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 hours. Data represent the mean ± SD (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 EGFP, and incubated for 48 hours in the presence or absence of serum. Apoptosis was then judged by piknotic nucleus after staining with Hoechst33342.

Figure 5. Akt does not effect on the protein levels of Bax, Bcl-2 and Bcl-XL. COS-1 cells were transfected with GFP-Bax and p35, together with various constructs, and incubated for 10 hours. The transfection efficiency was about 75%, measured by monitoring the expression
of GFP-Bax. Cells were treated with 1 μM of staurosporine for 3 hours, and then subjected to immunoblotting with anti-Bax, anti-Bcl-2, anti-Bcl-XL and anti-tubulin antibodies.

Figure 6. Active Akt inhibits Bax translocation to mitochondrial fraction.
COS-1 cells were transfected with vector (control), active Akt or 3A Akt, together with EGFP. The transfection efficiency was about 75%, measured by monitoring the expression of EGFP. Cells were treated with 1 μM staurosporine for 0, 6 or 12 hours, and subjected to subcellular fractionation. The amount of endogenous Bax was assessed by immunoblotting with anti-Bax antibody. The amount of VDAC, a mitochondrial marker, was also detected by immunoblotting. Each lane represents either total cell lysate (total) or mitochondrial fraction corresponding to 1.0 x 10^6 cells.

Figure 7. Expression of SGK does not affect Bax localization. (A) COS-1 cells were transfected with GFP-Bax and p35, together with or without active SGK, and incubated for 10 hours. Cells were treated with 1 μM staurosporine (STS), and assessed by counting the number of cells with mitochondrial Bax. Data represent the mean ± SD (n=5) of four independent experiments. (B) COS-1 cells were transfected with GFP-Bax and p35, together with or without dominant negative (2A) SGK, for 16 hours, and assessed by counting the number of cells with mitochondrial Bax. Data represent the mean ± SD (n=5) of two independent experiments. (C) COS-1 cells were transfected with GFP-Bax, p35 and FKHRL1 together with each SGK construct for 10 hours, and treated with 1 μM staurosporine for 3 hours. Cell extracts were subjected to immunoblotting with anti-phospho FKHRL1, anti-FKHRL1 or anti-HA.

Figure 8. Akt does not affect the levels of JNK phosphorylation. COS-1 cells were transfected with GFP-Bax and p35 together with various constructs, and incubated for 10 hours. Cells were treated with 1 μM of staurosporine for 3 or 6 hours, and subjected to immunoblotting with anti-phospho JNK antibody, which reacts with p46 and p54 JNKs, and with anti-JNK antibody, which reacts with p46 JNK.
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**Figure 3**

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