Phosphorylation of Bax Ser\(^{184}\) by Akt Regulates Its Activity and Apoptosis in Neutrophils*

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Although important for apoptosis, the mechanism of Bax regulation is poorly understood. This study demonstrates that phosphorylation of Ser\(^{184}\) regulates Bax activity. The phosphorylation required phosphatidylinositol 3-kinase/Akt activation and appeared to be mediated by Akt itself. In the serine-phosphorylated form, Bax was detected in the cytoplasm, could not be immunoprecipitated with the activation-specific antibody 6A7, and promoted heterodimerization with Mcl-1, Bcl-x\(_L\), and A1. Apoptotic neutrophils possessed reduced levels of serine-phosphorylated Bax correlating with an increase in activated Bax as well as an increase in the amount of Bax found translocated to the mitochondria. We suggest that Bax is regulated by phosphorylation of Ser\(^{184}\) in an Akt-dependent manner and that phosphorylation inhibits its Bax effects on the mitochondria by maintaining the protein in the cytoplasm, heterodimerized with anti-apoptotic Bcl-2 family members.

Apoptosis is a natural and required process that allows normal development, removal of damaged or mutated cells, and remodeling of tissues (1). Pro- and anti-apoptotic signals are carefully balanced in the neutrophil, a cell whose life span both \textit{in vivo} and \textit{in vitro} is only a few hours. The major role of the neutrophil is to kill and eliminate microorganisms. During inflammation, however, this intrinsically beneficial function may go awry, so proteases and oxidants are released into the environment, where they begin to destroy the surrounding tissue. The extent of damage is thought to be controlled in part by neutrophil apoptosis. Initiation of the apoptotic process inhibits the ability of neutrophils to move, degranulate, and produce superoxide (2, 3) and leads to rapid removal of the cells before cytolysis ensues.

Thus, understanding how neutrophil apoptosis is controlled, especially at the site of inflammation, is critical. As with most cell types, neutrophil apoptosis is regulated by Bcl-2 family members, a complex group of proteins composed of members that can either promote apoptosis such as Bax, Bak, Bad, and Bim or that inhibit apoptosis such as Bcl-2, Bcl-x\(_L\), Bcl-w, Mcl-1, and A1 (4). Although the neutrophil does not express Bcl-2 and contains only a limited amount of Bad, it does contain the pro-apoptotic proteins Bak and Bax and the anti-apoptotic proteins, Mcl-1, Bcl-x\(_L\), and Al (5, 6). There exists some evidence indicating that Bax is necessary for inducing neutrophil apoptosis and that this effect can be down-regulated by anti-apoptotic stimuli (6, 7). There are also reports indicating that Bax function is altered in chronic inflammatory diseases and that, under these conditions, neutrophil apoptosis is significantly attenuated (8).

Bax is a 21-kDa protein that is important in controlling cell death, particularly in hematopoietic cells. Cells that overexpress Bax show enhanced apoptosis (9), whereas Bax-null cells show resistance to both spontaneous and induced apoptosis. Bax expression has also been associated with tumor development and hematopoietic malignancies (10, 11). The protein is normally found in the cytoplasm heterodimerized to anti-apoptotic Bcl-2 family members such as Mcl-1 and Bcl-x\(_L\); however, once the cell is exposed to an apoptotic stimulus, Bax translocates to the mitochondria (12–14), where it is thought to form oligomers. These promote apoptosis by forming large pores, resulting in the loss of mitochondrial membrane potential and the release of cytochrome c (15, 16).

Regulation of Bcl-2 family members can occur by a number of mechanisms, including up-regulation of synthesis (17), enhancement of degradation, and phosphorylation. Both anti- and pro-apoptotic Bcl-2 family members may be controlled by phosphorylation. For example, the pro-apoptotic ability of Bad is inhibited by Akt-mediated phosphorylation, which induces it to pair with the 14-3-3 protein instead of heterodimerizing with anti-apoptotic Bcl-2 family members. Another pro-apoptotic family member, Bik, is regulated in a similar manner. Bcl-2 and Bcl-x\(_L\) also become phosphorylated, and this inactivates their anti-apoptotic abilities (18). Although phosphorylation of Bax as a means for regulation has not been well documented, Lewis \textit{et al.} (19) have demonstrated that recombiant Bax lacking the C-terminal tail is phosphorylated \textit{in vitro} by MAPK\(^3\) and protein kinase A. In addition, Ser\(^{184}\) has been

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1 The abbreviations used are: MAPK, mitogen-activated protein kinase; PI3K, phosphatidylinositol 3-kinase; MEK1, mitogen-activated protein kinase/extracellular signal-regulated kinase-1; TNF-\(_\alpha\), tumor necrosis factor-\(_\alpha\); GM-CSF, granulocyte/macrophage colony-stimulating factor; LPS, lipopolysaccharide; BSA, bovine serum albumin; PBS, phosphate-buffered saline; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; MOPS, 4-morpholinepropanesulfonic acid; ERK, extracellular signal-regulated kinase.
implicated as being important for regulating Bax activity. Thus, it seems likely that Bax could be regulated by phosphorylation, but this has not yet been demonstrated within cells.

One of the key enzymes involved in regulating apoptosis is Akt/protein kinase B. Akt, a 60-kDa serine/threonine kinase expressed in most cell types (20), has been shown to inhibit both spontaneous and stress-induced apoptosis in neutrophils (21, 22). It appears to be responsible for regulating growth factor-mediated survival in most cell types (23–25). Akt is activated by a variety of growth factors via PI3K-generated phosphatidylinositol(3,4,5)-trisphosphate induces recruitment of Akt to the plasma membrane, where it is serine/threonine-phosphorylated. Active Akt is thought to inhibit apoptosis in a variety of ways, both upstream and downstream of mitochondrial perturbation. It can lead to inhibition of caspase-9 activity, phosphorylation of pro-apoptotic Bcl-2 family members such as Bad, or regulation of transcription factors such as cAMP-responsive element-binding protein and NF-xB and members of the Forkhead family (20, 24, 27). These multiple anti-apoptotic effects of Akt make it a prime kinase candidate for inducing phosphorylation of Bax and thus inhibiting its pro-apoptotic abilities.

This study focuses on the mechanism by which Bax is regulated, particularly in neutrophils. It is suggested that anti-apoptotic stimuli lead to the activation of Akt and Ser184 phosphorylation of Bax. This phosphorylation promotes its sequestration to the cytoplasm and promotes its ability to heterodimerize with the anti-apoptotic Bcl-2 family members Mcl-1 and Bcl-xL, thus inhibiting its pro-apoptotic abilities.

EXPERIMENTAL PROCEDURES

Material and Antibodies—[32P]Orthophosphate and [γ-32P]ATP were obtained from Amersham Biosciences. The PI3K inhibitors LY-294002 and wortmannin were obtained from Sigma and used at 20 μM and 50 nM, respectively. The MEK1 inhibitor PD 98059 (Calbiochem) was used at 25 μM. Murine TNF-α-human TNF-α (R&D Systems, Minneapolis, MN) were used at 1000 units/ml, and GM-CSF (R&D Systems) was used at 100 ng/ml. Anti-phosphoserine and anti-phosphothreonine monoclonal antibodies were obtained from Alexis (San Diego, CA). Monoclonal antibody 4G10. Akt constructs, and active and inactive Akt proteins were obtained from Upstate Biotechnology, Inc. (Lake Placid, NY). Anti-β2 integrin clustering antibody 2LPM19 was obtained from Dako Corp. (Carpinteria, CA) and used at 2 μg/ml. Anti-Bax antibody, antibody 6A7 (which recognizes only the open form of Bax), and anti-human and anti-mouse Bax, anti-Mcl-1, and anti-Bcl-xL antibodies were obtained from PharMingen. Mouse monoclonal antibody 1273 against human mitochondria was raised against a 4G10, Akt constructs, and active and inactive Akt proteins were obtained from Amersham Biosciences. The PI3K inhibitors LY-294002 and wortmannin were obtained from Sigma and used at 20 μM and 50 nM, respectively. The MEK1 inhibitor PD 98059 (Calbiochem) was used at 25 μM. Murine TNF-α-human TNF-α (R&D Systems, Minneapolis, MN) were used at 1000 units/ml, and GM-CSF (R&D Systems) was used at 100 ng/ml. Anti-phosphoserine and anti-phosphothreonine monoclonal antibodies were obtained from Alexis (San Diego, CA). Monoclonal antibody 4G10. Akt constructs, and active and inactive Akt proteins were obtained from Upstate Biotechnology, Inc. (Lake Placid, NY). Anti-β2 integrin clustering antibody 2LPM19 was obtained from Dako Corp. (Carpinteria, CA) and used at 2 μg/ml. Anti-Bax antibody, antibody 6A7 (which recognizes only the open form of Bax), and anti-human and anti-mouse Bax, anti-Mcl-1, and anti-Bcl-xL antibodies were obtained from PharMingen. Mouse monoclonal antibody 1273 against human mitochondria was raised against a

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RESULTS

Neutrophils from Bax-null Mice Display Resistance to Both Spontaneous and Stress-induced Apoptosis—Neutrophils either isolated from the peripheral blood or elicited by sterile infection undergo rapid, unstimulated apoptosis. This is inhibited by activation of either the PI3K/Akt or MAPK pathway. Recently, Bel-2 family members have been implicated in regulating neutrophil apoptosis, in particular the pro-apoptotic Bel-2 homology 3 domain-containing molecule Bax (39). To examine a potential modulatory role for Bax in neutrophil apoptosis, cells from Bax+/− mice and littermate controls were isolated from a sterile inflammatory site induced by thioglycolate injection, and the spontaneous rate of apoptosis was assessed. Cells were also treated with the PI3K inhibitor LY-294002 to investigate the importance of the PI3K pathway in spontaneous apoptosis. After 12 h of culture, neutrophils from Bax+/− mice displayed 50% less spontaneous apoptosis compared with their littermate controls (Fig. 1). Additionally, the PI3K inhibitor LY-294002 enhanced the number of apoptotic cells in control mice while having only a slight enhancing effect on cells in Bax-null mice. This suggests that the Akt pathway is not as important in preventing death in Bax+/− cells as in control cells.

The spontaneous apoptotic process in neutrophils can be enhanced by pro-apoptotic stress stimuli such as TNF-α and anti-Fas IgM (40, 41). Cells isolated from control mice had 30% enhanced apoptosis when treated with TNF-α (Fig. 1). However, the apoptosis of Bax-null cells was not significantly affected. These data suggest an important role for Bax in regulating both spontaneous and stress-induced apoptosis of neutrophils.

Anti-apoptotic Stimuli Induce Phosphorylation of Bax—Protein phosphorylation is a potent and widely diverse means by which cells regulate signaling. Phosphorylation of Bax has been implicated as a means of regulation, but this has never been demonstrated. To pursue the possibility that phosphorylation could inhibit Bax activity, neutrophils were cultured in the presence or absence of the anti-apoptotic stimulus GM-CSF. Cells were metabolically labeled with [32P]orthophosphate prior to stimulation. Bax was then immunoprecipitated, and [32P] incorporation was detected by autoradiography. Bax from GM-CSF-stimulated cells displayed a significant increase in [32P] incorporation/phosphorylation compared with control cells (Fig. 2A). These data indicate that anti-apoptotic conditions result in Bax phosphorylation and support the possibility that phosphorylation can regulate Bax activity.

To determine whether the phosphorylation occurs at threonine, serine, or tyrosine, neutrophils were again cultured with [32P] in the presence of GM-CSF. Samples were lysed, and Bax

FIG. 1. Neutrophils from Bax-null mice show decreased spontaneous and stress-induced apoptosis. Thioglycolate-elicited peritoneal neutrophils were isolated from Bax+/− mice and littermate controls. Isolated neutrophils were incubated in the presence or absence of the PI3K inhibitor LY-294002 or stimulated with murine TNF-α for 12 h. Apoptosis was assessed by morphology. Bars represent percent apoptotic cells ± S.D. (n = 3).
was immunoprecipitated and analyzed for phosphoamino acids. Using internal standards visualized by ninhydrin staining and Akt (a molecule that is serine-phosphorylated) as a positive control (data not shown), we observed that GM-CSF induced serine phosphorylation of Bax (Fig. 2B). There was no significant evidence for phosphothreonine or phosphotyrosine.

**Anti-phosphoserine Antibodies Specifically Recognize Bax**—To establish a practical experimental system for analyzing Bax phosphorylation, a specific anti-phosphoserine antibody for Bax was sought. After examining a number of anti-phosphoserine antibodies (all of which showed some reactivity with Bax in GM-CSF-stimulated neutrophils), the IgG clone 7F12 (Alexis) was chosen (Fig. 3A). Although the IgM clones 4H4, IL8, and LB4 demonstrated reactivity with Bax, we selected the IgG clone 7F12 for ease of use in subsequent experiments. Control IgM and IgG antibodies as well as anti-phosphothreonine and anti-phosphotyrosine antibodies were ineffective in immunoprecipitating Bax (Fig. 3B). This provided additional evidence that Bax is selectively serine-phosphorylated.

As shown in Fig. 3C, three unrelated anti-apoptotic stimuli (GM-CSF, LPS, and anti-β2 integrin clustering antibody 2LPM19c) each induced Bax serine phosphorylation. By contrast, TNF-α, acting as a pro-apoptotic stimulus, decreased Bax phosphorylation.

**Activation of P13K and Akt Induces Bax Phosphorylation**—Two major anti-apoptotic pathways that inhibit neutrophil apoptosis are the P13K/Akt and MEK1/ERK pathways. However, the three anti-apoptotic stimuli (GM-CSF, LPS, and anti-β2 integrin clustering antibody) are more effective stimulators of P13K/Akt. To confirm P13K/Akt involvement in Bax phosphorylation, we pretreated neutrophils with the P13K inhibitor LY-294,002 or the MEK1 inhibitor PD 98059 for 20 min prior to addition of GM-CSF. Cells were lysed after 4 h, and Bax phosphorylation was analyzed with anti-phosphoserine antibody. Cells treated with GM-CSF showed a significant increase in Bax phosphorylation compared with control cells. This phosphorylation was inhibited in the presence of the P13K inhibitor, but not upon addition of the MEK1 inhibitor. P13K-dependent phosphorylation of Bax was confirmed with another P13K inhibitor, wortmannin (data not shown). This supports a role for the P13K pathway independent of the MEK1 pathway.

The role of P13K and Akt was further examined using the promyelocytic cell line PLB-985, which can be differentiated...
into neutrophil-like cells. This cell line was analyzed for Bax phosphorylation via [32P]orthophosphate labeling after stimulation with GM-CSF in the presence or absence of the PI3K inhibitor LY-294,002. GM-CSF treatment enhanced 32P labeling of Bax immunoprecipitates, and this was reduced in the presence of the PI3K inhibitor (Fig. 4B).

To pursue the involvement of Akt in a pathway leading to serine phosphorylation of Bax, dominant-negative and constitutively active Akt plasmids were ligated into the MSCV-1Thy1-1 vector for infection of PLB-985 cells. After 24 h, cells were collected and lysed, and Bax was immunoprecipitated. Cells infected with the empty Thy1 vector demonstrated some endogenous Bax phosphorylation; however, cells that received constitutively active Akt showed a marked increase in Bax phosphorylation, and this was absent in cells transfected with dominant-negative Akt (Fig. 4C).

Akt Leads to Phosphorylation of Bax at Ser184—Based upon the recent work describing the structure of Bax (44) and a study by Nechushtan et al. (45) demonstrating the importance of Ser184 in regulating the ability of Bax to translocate to the mitochondria and to maintain its pro-apoptotic capabilities, the observed serine phosphorylation of Bax might be expected to occur primarily at Ser184. Therefore, PCR-based site-directed mutagenesis of a murine Bax construct with Ser184 mutated to a negatively charged amino acid (glutamate) or a non-charged amino acid (alanine) was used to examine serine phosphorylation in PLB-985 cells. Cells were stimulated with GM-CSF and lysed, and lysates were precleared with anti-human Bax antibody to remove most of the endogenous Bax present in the PLB-985 cells. Lysates were then immunoprecipitated with anti-mouse Bax antibody, and immunoblots were probed with anti-phosphoserine antibody. Cells treated with empty vector demonstrated minimal Bax phosphorylation, whereas cells that received murine wild-type Bax demonstrated significantly increased phosphorylation. This was absent in lysates from both the alanine and glutamate mutants (Fig. 5A).

In response to GM-CSF, transfected murine wild-type Bax again showed enhanced phosphorylation compared with empty vector, and this was reversed with the PI3K inhibitor LY-294,002 (Fig. 5B). These data support the hypothesis that serine phosphorylation is PI3K/Akt-dependent.

Fig. 5C demonstrates that Akt could directly phosphorylate Bax in vitro. Bax was immunoprecipitated from control cells and exposed to either active or inactive Akt in the presence of [γ-32P]ATP, and phosphorylation was determined by autoradiography.

Anti-apoptotic Stimuli Inhibit Bax Translocation to the Mitochondria—To determine the duration of Bax phosphorylation in resting and stimulated cells, neutrophils were incubated with or without GM-CSF for 4 or 8 h, and Bax immunoprecipitates were probed with anti-phosphoserine antibody. Control cells displayed some endogenous Bax phosphorylation at 4 h, and this decreased by 8 h (Fig. 6A). The reduced Bax phosphorylation at 8 h correlated with 40% enhancement in apoptosis. GM-CSF stimulation maintained serine phosphorylation of Bax up to 8 h and even as long as 24 h (data not shown). The maintained Bax phosphorylation correlated with the ability of GM-CSF to inhibit apoptosis, reducing apoptosis to 10% at 8 h. Fig. 6B demonstrates the relative amounts of Bax phosphorylated in control and GM-CSF-stimulated cells. Neutrophils were

![Image of Fig. 4](http://www.jbc.org/)

**Fig. 4. Serine phosphorylation of Bax is PI3K/Akt-dependent.** A, neutrophils were pretreated with either LY-294,002 or PD 98059 prior to addition of GM-CSF (100 ng/ml). Samples were lysed at 4 h, immunoprecipitated (I.P.) with anti-Bax antibody, and run on a 12% SDS-polyacrylamide gel, and probed with anti-phosphoserine antibody. B, PLB-985 cells were orthophosphate-labeled and then preincubated with the PI3K inhibitor LY-294,002 prior to GM-CSF stimulation. Samples were lysed after 4 h; Bax was immunoprecipitated and run on a 12%
initially immunoprecipitated with anti-phosphoserine antibody, and the lysates were then transferred and re-immunoprecipitated with anti-Bax antibody. Both sets of immunoprecipitates were run on a gel and blotted with anti-Bax antibody (Fig. 6B). Most of the Bax present in cells at 0 and 2 h was serine-phosphorylated. By 4 h, however, untreated control cells displayed a reduced level of serine-phosphorylated Bax with a corresponding increase in the non-phosphorylated protein. However, in cells stimulated with GM-CSF, most of the cellular Bax was still serine-phosphorylated at 4 h.

Bax is normally located in the cytoplasm and is not recognized by antibody 6A7, which recognizes only the activated/pro-apoptotic form of Bax. However, upon exposure to a pro-apoptotic stimulus or, in the case of neutrophils, during spontaneous apoptosis, Bax changes its configuration and is recognized by antibody 6A7. Neutrophils were examined for Bax activation with antibody 6A7 by fluorescence microscopy. As apoptosis increased and Bax phosphorylation decreased, cells became positive for “activated” Bax (Fig. 6C). GM-CSF-treated cells displayed little, if any, activated Bax. GM-CSF-mediated inhibition was reversed when cells were pretreated with the PI3K inhibitor wortmannin, but not with the MEK1 inhibitor PD 98059 (data not shown).

Fig. 6D demonstrates that activated Bax immunoprecipitated with antibody 6A7 from control, UV-treated (pro-apoptotic), or GM-CSF-treated (anti-apoptotic) cells was undetectable with anti-phosphoserine antibody. Immunoprecipitates of total Bax confirmed that there was significant Bax phosphorylation in GM-CSF-treated cells. As expected, antibody 6A7 immunoprecipitated a significant amount of Bax from UV-treated and control cells, but less from GM-CSF-treated cells.

**Bax Phosphorylation Promotes Bax Heterodimerization with Anti-apoptotic Bcl-2 Family Members**—The pro-apoptotic potential of Bax is likely blocked by heterodimerization with anti-apoptotic Bcl-2 family members such as Mcl-1 and Bcl-xL. To examine the relationship between serine phosphorylation and heterodimerization, cells were treated with or without GM-CSF for 4 h, and Bax immunoprecipitates were probed for co-immunoprecipitation with Mcl-1, Bcl-xL, or A1. Enhanced heterodimerization was seen after GM-CSF treatment, especially with Mcl-1 and Bcl-xL (Fig. 7A). Similar results were observed if the anti-apoptotic Bcl-2 family member was immunoprecipitated and the blots were probed for Bax (Fig. 8D) (data not shown).

Fig. 7B examines the importance of Ser\(^{184}\) in promoting Bax heterodimerization with anti-apoptotic Bcl-2 family members. PLB-985 cells were transfected with the murine wild-type and Ser\(^{184}\) mutant constructs. Cells were stimulated with GM-CSF and lysed, and lysates were precleared with anti-human Bax antibody prior to immunoprecipitation with anti-mouse Bax antibody. Lysates from cells containing murine wild-type Bax demonstrated significant heterodimerization of Bax with Mcl-1; this did not occur with the S184A mutant. As predicted, although the S184E mutant showed no detectable serine phosphorylation, it did heterodimerize with Mcl-1 and Bcl-xL (Fig. 7B). Ser\(^{184}\) phosphorylation is PI3K-dependent. Wild-type Bax constructs were transfected into PLB-985 cells. After 24 h, cells were pretreated with the PI3K inhibitor LY-294002 or the MEK1 inhibitor PD 98059 and then stimulated with GM-CSF for 4 h. Murine Bax was immunoprecipitated from lysates precleared of human Bax. Immunoblots were probed with anti-phosphoserine antibody and then stripped and reprobed for Bax. C, Bax immunoprecipitates were exposed to active or inactive Akt, and phosphorylation was determined as \(^{32}\)P incorporation. **P-Bax**, serine-phosphorylated Bax.
brane fractions were separated by differential centrifugation. Bax was immunoprecipitated from these fractions and run on an SDS-polyacrylamide gel. After 4 h of cell culture, Bax began to translocate to the membrane fraction in control cells; however, there was little, if any, Bax translocation in GM-CSF-treated cells (Fig. 8A). Anti-phosphoserine antibody recognized Bax located only in the cytoplasmic fractions. These data suggest that serine phosphorylation of Bax helps maintain its localization in the cytoplasm.

The facts that serine-phosphorylated Bax heterodimerized with anti-apoptotic Bcl-2 family members and was located primarily in the cytoplasmic fractions contrasted with studies suggesting that anti-apoptotic Bcl-2 family members reside in the outer mitochondrial membrane (46, 47). To address this potential discrepancy, we looked for the anti-apoptotic Bcl-2 molecules in the cytoplasm of neutrophils. Fig. 8B demonstrates that both Mcl-1 and Bcl-x\textsubscript{L} displayed little, if any, co-localization with the mitochondria in viable cells as determined by fluorescent microscopy. Rather, they appeared to be located in or on discrete, as yet unidentified cytoplasmic structures.

**Fig. 6. Serine phosphorylation decreases as cells begin to undergo apoptosis.** A, neutrophils were cultured in the presence or absence of GM-CSF for 4 and 8 h. Cells were lysed; Bax was immunoprecipitated (I.P.); and blots were probed with anti-phosphoserine antibody. B, Bax is highly phosphorylated in resting cells. Lysates from unstimulated control cells and cells stimulated with GM-CSF for 2 and 4 h were immunoprecipitated with anti-phosphoserine antibody. Lysates were secondarily immunoprecipitated with anti-Bax antibody, and both immunoprecipitates were subjected to SDS-PAGE and probed with anti-Bax antibody. Only in the unprotected (no GM-CSF) cells was a significant amount of non-phosphorylated Bax seen at 4 h. C, the phosphorylated form of Bax (P-Bax) is not activated. Neutrophils were stimulated with GM-CSF for 24 h, and samples were cytospun, fixed and permeabilized. Samples were stained with antibody 6A7 to determine activation of Bax. D, the activated form of Bax is not recognized by anti-phosphoserine antibody. Cells stimulated with GM-CSF (anti-apoptotic) or UV irradiation (pro-apoptotic) were lysed after 4 h and immunoprecipitated with anti-Bax antibody or antibody 6A7 (which recognizes only the activated open form of Bax). Samples were run on an SDS-polyacrylamide gel and probed with anti-phosphoserine antibody.
Serine Phosphorylation of Bax

**A.** Anti-phosphoserine (anti-7F12) Immunoblot

![Bax Immunoblot](image)

**B.** Anti-Bax Immunoblot

![Bcl-2 Immunoblot](image)

**C.** Anti-Mcl-1 Immunoblot

![Anti-Mcl-1 Immunoblot](image)

**D.** Anti-Bcl-xL Immunoblot

![Anti-Bcl-xL Immunoblot](image)

**E.** Bax(Cy5/Mcl-1 (FITC))

![Bax(Cy5/Mcl-1 (FITC))](image)

**F.** Bcl-xL (FITC)

![Bcl-xL (FITC)](image)

**G.** Bax(Cy3)

![Bax(Cy3)](image)

**FIG. 8.** A, serine phosphorylation of Bax occurs only in the cytoplasm. Cells were stimulated with GM-CSF and incubated for 4 h. Cells were lysed and differentially centrifuged, and Bax was immunoprecipitated (I.P.), run on an SDS-polyacrylamide gel, and probed with anti-phosphoserine antibody. Blots were stripped and reprobed for total Bax. B, anti-apoptotic Bcl-2 family members are located in the cytoplasm. Cells were labeled with MitoTracker Red CMXRos (red), cytospun, fixed, and permeabilized. Cells were then stained with anti-Mcl-1 or anti-Bcl-xL antibody followed by a fluorescein isothiocyanate (FITC)-conjugated secondary, and staining was visualized by confocal microscopy. C, anti-apoptotic Bcl-2 family members are associated with neutrophil granules. Granular fractions were run on an SDS-polyacrylamide gel and probed for Mcl-1. Blots were...
The presence of Mcl-1 and Bcl-xL in the cytoplasm and their apparent localization to discrete structures led to the hypothesis that these proteins reside in or on the neutrophil granules. Neutrophils contain few mitochondria, but do contain at least several types of granules, including primary, secondary, tertiary, and secretory. To determine whether Mcl-1 and Bcl-xL are associated with the granules, neutrophils were lysed via nitrogen cavitation, and granular fractions were isolated as described by Kjeldsen et al. (38). Equal amount of proteins from the granular fractions were run on a 12% SDS-polyacrylamide gel and probed for Mcl-1. Mcl-1 was found associated with the secretory and azurophilic granules (Fig. 8C).

To pursue this observation, neutrophils were incubated with or without GM-CSF for 4 h and lysed by nitrogen cavitation. Lysates were separated by differential centrifugation; Mcl-1 or Bcl-xL was immunoprecipitated; and immunoblots were probed with anti-Bax antibody. Bax co-immunoprecipitated predominately with Mcl-1, and Bcl-xL localized to the cytoplasmic fractions (Fig. 8D). Immunoblots reprobed for Mcl-1 and Bcl-xL indicated that they also resided primarily in the cytoplasmic fractions. Immunofluorescence also demonstrated that Bax and Mcl-1 co-localized in discrete cytoplasmic structures (Fig. 8E). Isotype controls (rabbit and mouse) for each antibody was used to ensure specificity. These data indicate that the localization of anti-apoptotic Bcl-2 family members is unique in neutrophils and provide a potential explanation as to why we found serine-phosphorylated Bax in the cytoplasm heterodimerized with Mcl-1 and Bcl-xL.

**DISCUSSION**

A model is presented in which Bax is maintained inactive in the cytoplasm in a phosphorylated closed state, heterodimerized with anti-apoptotic Bcl-2 family members. In the neutrophil, these partners are Bcl-xL, Mcl-1, and A1. Upon induction of apoptosis, Bax appears to become dephosphorylated, loses its ability to heterodimerize, and converts to the open form able to be recognized by antibody 6A7. These events appear to be related to the onset of apoptosis.

An increasing number of Bcl-2 family members are regulated by phosphorylation. However, although previous reports have implicated Bax Ser184 as being involved in controlling Bax activity, direct evidence of this phosphorylation was not demonstrated. Our continuing investigation of mechanisms of apoptosis and its regulation in neutrophils (22, 34) led us to address the potential role for Bax in this process. The literature suggests that Bax may be a key component of apoptosis in neutrophils and in hematopoietic cells in general (6, 48). Neutrophils have a short life span and undergo spontaneous apoptosis in vitro and presumably in vivo as well. Apoptosis can be enhanced by stress stimuli such as Fas ligation, TNF-α, and oxidants (49, 50) and delayed by a host of stimuli as divergent as GM-CSF, chemokines, LPS, and non-activating β2 integrin ligation (34, 50). In this study, a potential role for Bax in this regulation was first shown by examination of neutrophils from Bax-null mice, which demonstrated inhibited apoptosis in comparison with their Bax-expressing littermate controls. In passing, it should be noted that, although Bax levels appear to be important in neutrophil apoptosis, there was no striking alteration in circulating neutrophil numbers in the Bax-null mice. However, it is still not known how the number of circulating granulocytes are sensed and regulated, including the signals that control influx of neutrophils into and efflux from the circulation. Thus, it seems reasonable to suppose that there could be appropriate compensatory mechanisms that maintain constant circulating numbers even in the presence of decreased apoptosis. An alternative possibility is a compensatory effect of the pro-apoptotic family member Bak because it has been shown that mice deficient in both Bax and Bak do display significant neutrophilia (51).

When human neutrophils were examined for Bax phosphorylation, the molecule was [32P]orthophosphate-labeled following anti-apoptotic stimulation. Phosphorylation of serine was confirmed by phosphoamino acid analysis and by increased reactivity with a variety of anti-phosphoserine antibodies. Antibodies against phosphothreonine or phosphotyrosine were inefficient at recognizing Bax. In some of the orthophosphate labeling and antibody experiments, serine-phosphorylated Bax appeared as a doublet. These results could indicate that we are detecting one of the other isoforms of Bax with the immunoprecipitation and that it is also serine-phosphorylated. Alternatively, Bax may be phosphorylated at multiple serine residues; however, it appears that phosphorylation of Ser184 is important for regulating heterodimerization of Bax with anti-apoptotic Bcl-2 family members and therefore is probably the major regulatory phosphorylation event in neutrophils.

Because it is not possible to transfect neutrophils to confirm serine phosphorylation of Bax, we used the myelomonocytic cell line PLB-985, which can be differentiated into neutrophil-like cells in vitro. Transfected wild-type Bax was phosphorylated after stimulation with GM-CSF. Mutation of Ser184 to an alanine or glutamate prevented this, suggesting the presumption that Ser184 is primarily targeted for phosphorylation. Cells transfected with the empty vector control did display slight Bax phosphorylation, indicating that the preclearing of human Bax was not totally efficient and that a small amount of the immunoprecipitated human Bax ended up in the immunoblot. Although some endogenous Bax was detected, the serine phosphorylation was significantly increased with the murine wild-type construct, and the mutant construct phosphorylation was similar to that with empty vector, indicating that any limited contamination did not influence or obscure the observed results. In addition, transfection of murine wild-type Bax and the constructs mutated at Ser184 did not affect the viability of the cells as determined by a WST-1 assay. This could potentially be possible because, as an immortalized cell line, the PLB-985 cells may possess enhanced levels of compensatory molecules such a Bcl-2 and Bcl-xL, which would inhibit the proapoptotic activity of Bax. Additionally, the experiments were performed in the presence of GM-CSF, which can inhibit stress-induced apoptosis in PLB-985 cells and could potentially protect the cells from the adverse affects of the infected Bax constructs.

Bax regulation has been shown to occur in a PI3K/Akt-dependent manner, similar to Bad regulation (52), although the exact process by which Akt is involved has not been demonstrated. Here, using inhibitors of PI3K in neutrophils and mutant forms of Akt in PLB-985 cells, we show that PI3K and, more importantly, Akt is important for initiating serine phosphorylation of Bax. The effect could be direct or involve downstream kinases activated themselves by Akt. The Bax sequence does not suggest an obvious Akt consensus phosphorylation site around Ser184.
However, direct incubation with constitutively active Akt with Bax immunoprecipitated from resting neutrophils did result in \( ^{32}P \) incorporation into Bax, an effect that was not seen with inactive enzyme. The data support the potential ability of Akt to directly phosphorylate Bax, perhaps because of configurational presentation of an appropriate phosphorylation site.

We propose that cytoplasmic Bax is endogenously phosphorylated most likely at Ser\(^{184} \). A role for PI3K/Akt in maintaining phosphorylation is suggested; indeed, inhibition of the PI3K/Akt pathway by LY-294002 (Fig. 4A) led to decreased phosphorylation of Bax and induction of its activation. In support of the concept that cytoplasmic Bax is maintained in a phosphorylated state, immunoprecipitation of neutrophil lysates with anti-phosphoserine antibodies resulted in removal of most of the Bax in viable resting cells and GM-CSF-stimulated cells. The amount of serine-phosphorylated Bax significantly declined as cells began to undergo apoptosis. This creates something of a paradox in that one would not then expect Akt to directly phosphorylate Bax derived from resting cells in vitro. On the other hand, one might hypothesize that there could be constant dephosphorylation and repARATION of Bax in the cell, particularly a short-lived cell such as a neutrophil. An alternative possibility is that Akt phosphorylates Bax at residues other than or in addition to Ser\(^{184} \) and that this phosphorylation allows for Ser\(^{184} \) phosphorylation by an alternative kinase. Although alternative sites cannot be completely ruled out, a number of lines of evidence lead us to suggest this is an unlikely explanation. Unlike wild-type Bax, phosphorylation was not seen in S184A mutants transfected into PLB-985 cells.

Cell fractionation revealed that Bax was phosphorylated only in the cytoplasm (in keeping with this location for the closed and inactive form). During induction of apoptosis, Bax was found in membrane fractions (in keeping with its mitochondrial translocation). Significantly, the membrane-associated form was not detectable with anti-phosphoserine antibodies. These observations were strengthened by the demonstration that Bax immunoprecipitated with antibody 6A7 was undetectable with anti-phosphoserine antibody, even though Bax was present. The data support phosphorylation as a potential mechanism by which Bax is maintained in the cytoplasm. In addition, cytoplasmic phosphorylated Bax heterodimerized with the anti-apoptotic Bcl-2 family members Bcl-x\(_L\), Mcl-1, and, to a lesser extent, A1. Bidirectional co-immunoprecipitation showed Bax association with the dimerization partner. Which comes first is not yet known. We have shown that neutral protein apoptosis induced by stress stimuli is associated with oxidant-induced activation of SHIP and decreased Akt activity (22). Decreased Akt activity might also occur during spontaneous apoptosis in these cells. In keeping with the above-mentioned role of oxidants, spontaneous neutrophil apoptosis is significantly delayed if the cells are maintained in a hypoxic environment (53). On the other hand, activation of appropriate serine phosphatases during apoptosis could achieve the same effect. The observation that broad-spectrum phosphatase inhibitors delay apoptosis (data not shown), whether spontaneous, stimulated, or in the presence of PI3K inhibitors, supports both of these possibilities.

Inhibition of Bax activity by Ser\(^{184} \) phosphorylation may be due in part to alterations in Bax structure upon phosphorylation. Ser\(^{184} \) is located in the C-terminal tail, which is important for insertion of Bax into the mitochondrial membrane. Addition of a negative charge could prevent Bax oligomerization and insertion into the mitochondrial membrane, thus inhibiting its pro-apoptotic abilities. In addition, phosphorylation in the C-terminal tail may induce a conformational change favorable to heterodimerization with anti-apoptotic Bcl-2 proteins.

Phosphorylation of Bcl-2 family members appears to be a generalized phenomenon that occurs with both pro-apoptotic and anti-apoptotic family members. Phosphorylation of anti-apoptotic Bcl-2 family members such as Bcl-2 and Bcl-x\(_L\) has been shown to inhibit their anti-apoptotic capabilities (54), whereas serine phosphorylation of the pro-apoptotic family member Bad inhibits its pro-apoptotic activities (55). Phosphorylation of Bax appears to act similarly, as it promotes Bax heterodimerization with anti-apoptotic Bcl-2 proteins and thus inhibits its activity (56). Interestingly, there is a fundamental difference between Bad and Bax phosphorylation in that Bad phosphorylation inhibits heterodimerization, whereas Bax phosphorylation seems to promote this heterodimeric state. An interesting question raised by these experiments is whether phosphorylation is a means of regulating all Bcl-2 family members. These experiments also suggest the possibility that Bax, which has been shown to have functional homology to Bax, may also be regulated by serine phosphorylation. As such, serine phosphorylation may be a common theme of regulation for multiple pro-apoptotic Bcl-2 family members.

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