BIK is a pro-apoptotic BCL-2 family member and is the founding member of a subfamily of pro-apoptotic proteins known as “BH3-alone” proteins. Ectopic expression of BIK induces apoptosis in variety of mammalian cells. BIK complexes with various anti-apoptotic BCL-2 family proteins such as adenovirus E1B-19K and BCL-2 via the BH3 domain. However, the heterodimerization activity of BIK alone is insufficient for its apoptotic activity. Previous studies have shown that phosphorylation regulates the functional activity of both anti-apoptotic and pro-apoptotic members of the BCL-2 family. Here, we have examined phosphorylation of BIK and its effect on the apoptotic activity of BIK. We show that BIK exists as a phosphoprotein and is phosphorylated at residues 33 (threonine) and 35 (serine). Mutation of the phosphorylation sites, in which the Thr and Ser residues were changed to alanine residues, reduced the apoptotic activity of BIK without significantly affecting its ability to heterodimerize with BCL-2. Our results suggest that phosphorylation of BIK is required for eliciting efficient apoptotic activity. Partial purification of the protein kinase from HeLa cell cytoplasmic extracts suggest that BIK may be phosphorylated by a casein kinase II-related enzyme.

Apoptosis is a physiological process of cell death shared by all multicellular organisms and is critical for removing unwanted cells during development. This process is essential for the maintenance of normal tissue homeostasis. Cell death or survival is dependent on the receipt of continuous signals from the extracellular environment. These signals are then transduced through the cell surface to intracellular molecules that regulate apoptotic cell death. A number of such regulators have been identified. Among them, the BCL-2 family proteins play an important role in both induction and suppression of apoptosis (reviewed in Ref. 1). The anti-apoptotic members include the cellular proteins such as BCL-2, BCL-xL, MCL-1, BFL-1, and BCL-w and viral proteins such as Epstein-Barr virus BHRF1 and adenovirus E1B-19K proteins. These cellular and viral proteins suppress apoptosis induced by diverse stimuli (13). Taken together, it can be concluded that the activities of BCL-2 and BCL-xL can be influenced by phosphorylation. Phosphorylation of a serine residue (Ser-70) within this region, which is located between the BH3 and BH4 domains, is required for its full anti-apoptotic activity (11). Deletion of the variable region of BCL-2 has been shown to relieve a novel proliferation restraining activity of BCL-2 (12). Most other pro-apoptotic proteins such as BAD, BID, BIK, BIM, BNIP3, BNIP1, HRK, and NOXA share only a single domain (BH3) with BCL-2 and are hence designated “BH3-alone” proteins; the human BIK protein is the founding member of this family. In Caenorhabditis elegans, a BH3-alone protein Egl-1 has been shown to be essential for developmentally programmed death of somatic cells (2). An interesting feature of the BCL-2 family of proteins is the ability of the pro-apoptotic members to heterodimerize with the anti-apoptotic members (reviewed in Ref. 1). This suggests that one of the mechanisms by which anti-apoptotic members of the BCL-2 family suppress apoptosis may be through heterodimerization with pro-apoptotic members. However, at least in the case of BIK, heterodimerization with anti-apoptotic proteins such as BCL-2 and BCL-xL is insufficient for induction of apoptosis (3), suggesting that other factors may also influence the apoptotic activity of BIK.

Certain apoptotic stimuli appear to modulate the expression and activity of the BCL-2 family proteins at the level of transcription as well as by post-translational modifications. For example, expression of the BH3-alone pro-apoptotic gene Noxa is activated during p53-mediated apoptosis (4). Hypoxic conditions have shown to activate the expression of another pro-apoptotic protein BNIP3 (5). The BH3-alone protein BIM (6) has been shown to be essential for cytokine withdrawal-induced apoptosis in hemopoietic cells (7). BIM is normally sequestered to the microtubules in an inactive form and is released during apoptosis (8). During apoptosis mediated by the death receptors such as CD95, the BH3-alone protein BID is proteolytically cleaved by caspase-8 to a pro-apoptotic form (tBID) from an inactive form (9, 10). Post-translational modifications such as phosphorylation also play important roles in regulating the activity of both anti-apoptotic and pro-apoptotic BCL-2 family proteins. BCL-2 contains several potential serine/threonine phosphorylation sites within the “variable region,” which is located between the BH3 and BH4 domains. Phosphorylation of a serine residue (Ser-70) within this region of BCL-2 has been shown to be required for its full anti-apoptotic activity (11). Deletion of the variable region of BCL-2 has been shown to relieve a novel proliferation restraining activity of BCL-2 (12). Both BCL-2 and BCL-xL have been shown to be phosphorylated in cells following treatment with microtubule disrupting agents (13). Taken together, it can be concluded that the activities of BCL-2 and BCL-xL can be influenced by phosphorylation. Similarly, phosphorylation of the pro-apoptotic protein BAD on Ser-112, Ser-136, and Ser-155, following inter-allelic programmed death of somatic cells (2). An interesting feature of the BCL-2 family of proteins is the ability of the pro-apoptotic members to heterodimerize with the anti-apoptotic members (reviewed in Ref. 1). This suggests that one of the mechanisms by which anti-apoptotic members of the BCL-2 family suppress apoptosis may be through heterodimerization with pro-apoptotic members. However, at least in the case of BIK, heterodimerization with anti-apoptotic proteins such as BCL-2 and BCL-xL is insufficient for induction of apoptosis (3), suggesting that other factors may also influence the apoptotic activity of BIK.

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rylate the BCL-2 family members. The pro-apoptotic protein BAD is a target for phosphorylation by the survival promoting protein kinase Akt (19) and mitochondrial protein kinase A (15). Similarly, phosphorylation of non-BCL-2 members also modulates apoptosis. Phosphorylation of pro-caspase 9 by Akt appears to inhibit activation of this caspase by proteolysis (20). Exposure to DNA damaging agents that leads to p53-mediated apoptosis also induces phosphorylation of p53 at Ser-392 by casein kinase II (CKII) (21). In this article, we report that the pro-apoptotic protein BIK is a phosphoprotein and that phosphorylation is required for the full apoptotic activity of BIK.

**EXPERIMENTAL PROCEDURES**

**BIK and BIK Mutants**—The HA-tagged BIK expression plasmid (pcDNA3HA-BIK) has been described (22). Plasmids pET21b-BIK and mutant BIK were constructed by cloning polymerase chain reaction-amplified BIK cDNA encoding aa 1–134 in expression vector pET21b (Novagen) in frame with the His6 tag at the C terminus. All BIK mutants were constructed by polymerase chain reaction according to a method described by Taylor et al. (23).

**Purification of Recombinant BIK Protein**—His-tagged wt and mutant BIK proteins were expressed in Escherichia coli strain BL21 and purified by Ni-NTA-agarose resin. One liter of bacterial culture was centrifuged and resuspended in 10 ml of sonication buffer (50 mM Tris-Cl, pH 7.5, 50 mM NaCl, 0.5 mM DTT, 1% Nonidet P-40). The cell suspension was sonicated for four minutes with one each. The lysates were centrifuged at 12,000 × g for 30 min at 4 °C. The supernatant was diluted with sonication buffer without Nonidet P-40 to adjust the Nonidet P-40 concentration to 0.5%. The extracts were loaded onto the Ni2+-NTA columns equilibrated with sonication buffer containing 0.5% Nonidet P-40. The proteins were eluted with a linear gradient of 0–3 M imidazole.

**In Vivo Labeling and Immunoprecipitation of BIK**—The wt and mutant (T33A/S35A) BIK proteins were expressed in BSC40 cells using vaccinia/T7 RNA polymerase vector system (24). Briefly, cells were transfected with 10 μg of pcDNA2-based plasmids expressing either HA-tagged wt or mutant (T33A/S35A) BIK and infected with 10 pox/cell vaccinia virus vTF7-3. The cells were labeled either in phosphate-free DMEM with 100 μCi/ml [32P]orthophosphoric acid (PerkinElmer Life Sciences) or in methionine-free DMEM with [35S]methionine-cysteine mixture (PerkinElmer Life Sciences) for 4 h. Eighteen to 20 h after infection, cells were lysed with 50 mM Tris, pH 7.4, 150 mM NaCl, 0.1% SDS, 1% Nonidet P-40 with the protease inhibitors leupeptin and aprotinin. Cell lysates were subjected to immunoprecipitation using HA antibody and analyzed by SDS-PAGE (Fig. 1A). The proteins were visualized by autoradiography.

**In Vitro Kinase Assay**—Purified wt BIK (1–134 amino acids) or mutant BIK (T33A/S35A) proteins were phosphorylated in vitro in 40 mM Hepes, pH 8.0, 2 mM DTT, 10 μg/ml EGTA, 50 μM ATP, 5 μCi of [γ-32P]ATP by 5 μl of crude HeLa cell cytoplasmic extract or partially purified HeLa cell extract. The mixture was incubated at 30 °C for 30 min following removal of unincorporated 32P by precipitation with trichloroacetic acid, the BIK protein was separated by SDS-PAGE and examined by autoradiography.

**Phosphoamino Acid Analysis**—Recombinant BIK (an 1–134) was phosphorylated in vitro using HeLa cell extract (poorly-Sepharose fractions). The phosphorylated protein was separated by SDS-PAGE, and the gel slice containing protein BIK was excised and washed sequentially with 25% isopropanol, 10% methanol, and 50 mM NH4HCO3 (pH 8.8). The protein was digested with 100 μg of chymotrypsin in 50 mM NH4HCO3, pH 8.0, 24 h at 37 °C. The trypsin peptides were hydrolyzed in 6 N HCl at 110 °C for 3 h under vacuum. Nonradioactive phosphoserine, phosphothreonine, and phosphotyrosine were added to the sample, which were together applied onto a TLC plate and separated by electrophoresis at 1000 V for 3 h with pH 1.9 buffer (25). The locations of phosphoamino acids were determined by ninhydrin staining and autoradiography.

**Transient Cell Death Assay**—HeLa cells in 12-well (10° cells/well) plates were transfected with 0.5–3.0 μg of HA epitope-tagged wt BIK or mutant BIK (T33A/S35A) expressed from the pcDNA3 vector, along with 0.1–0.6 μg of the reporter plasmid pCMV-lacZ by the calcium phosphate method. The cells were transfected with 8–10 μl after transfection and stained with X-gal. Briefly, the cells were washed with PBS and fixed with 0.5% glutaraldehyde for 5 min. After washing the cells twice with PBS for 5 min, they were stained with 2 ml of staining solution (2 mg MgCl2 × 5 μm potassium ferricyanate + 5 μm potassium ferrocyanate + 1 mg/ml X-gal in PBS) at 37 °C overnight. The stained cells were microscopically examined and counted. Blue color cells (100–200) were scored as round (apoptotic) and flat (viable) cells.

**Transformation Assay**—EI-AFT24 ras oncogene cooperative transformation of mammalian cells was examined by Western blot analysis of BIK. Extracts of BSC40 cells transfected with pcDNA3HA-BIK and infected with the vaccinia virus vTF7-3, labeled with 35S or 32P, immunoprecipitated with the HA antibody, and analyzed on 15% SDS-PAGE and autoradiography. C, phosphorylation of endogenous BIK. SW480 (ATCC) cells were labeled with 35S or 32P, immunoprecipitated with a BIK-specific peptide antibody (Santa Cruz, SC-1710), and analyzed on 15% SDS-PAGE.

**RESULTS**

**BIK Is a Phosphoprotein**—When the human BIK protein expressed in mammalian cells was examined by Western blot analysis, it generally migrated as a doublet of 24–25 kDa (Fig. 1A). To determine whether one of the bands may represent the phosphorylated form of BIK, we metabolically labeled BIK either with [35S]methionine-cysteine mixture or with [32P]orthophosphoric acid. For this purpose, HA-tagged BIK was expressed in BSC40 cells using the vaccinia virus expression system (24). The proteins were immunoprecipitated with the HA antibody and analyzed by SDS-PAGE (Fig. 1B). The BIK protein that was metabolically labeled with 35S migrated as a doublet, as in the case of Western blot analysis. Comparison of BIK labeled with 32P revealed that the slower moving band may be the phosphorylated form of BIK (Fig. 1B). To determine if endogenous BIK is also phosphorylated, we metabolically labeled SW480 cells either with 35S or 32P, immunoprecipitated BIK with a BIK antibody (N-19) and analyzed by SDS-PAGE (Fig. 1C). This analysis also revealed that endoge-
BIK Phosphorylation and Apoptosis

Various BIK mutants were analyzed by Western blot. A number of different analyses are summarized in Table I. Mutants that expressed both the slow and fast migrating forms of BIK were considered positive for phosphorylation, and the mutant that expressed only the fast migrating form was considered negative for phosphorylation.

| Mutant     | Phosphorylation |
|------------|-----------------|
| T29A       | +               |
| T33A/S35A  | S2A/S8A/T15A/Y18A |
| S47A/S54A  | +               |
| S71A       | +               |
| S81A       | +               |
| S87A       | +               |
| S106A/T112A/T113A | + |
| S124A/S129A/S132A | + |
| Y94A/T97A  | +               |

In addition to the transient apoptosis assay, the effects of wt

**Fig. 2. In vitro phosphorylation of BIK.** A, phosphorylation by total cytoplasmic extract. One hundred ng of BIK (aa 1–134) was incubated in the presence (+) or absence (−) of 5 μl of crude HeLa cell extract and 5 μCi of [γ-32P]ATP in a 20-μl reaction volume. The phosphorylated protein was precipitated with trichloroacetic acid, run on 12% SDS-PAGE, and analyzed by autoradiography. B, phosphorylation by partially purified extract. Five-μl aliquots of various SP-Sepharose fractions of the cytoplasmic extract were assayed for the kinase activity using BIK (1–134) as in A. The reactions were carried out in the presence of 100 ng of BIK (indicated by +) or absence of BIK (indicated by −). C, phosphoamino acid analysis. One μg of BIK was phosphorylated by 10 μl of pooled HeLa cell fractions 5–11 as in A and hydrolyzed, and phosphoamino acids were separated by TLC. Circled areas represent the location of phosphoserine (Ser), phosphothreonine (Thr), and phosphotyrosine (Tyr) markers indicated by ninhydrin staining.

nous BIK is phosphorylated. These results indicate that BIK is a phosphoprotein.

**In Vitro Phosphorylation of BIK—**To characterize the phosphorylation of BIK further, we determined whether BIK could be phosphorylated in vitro. For this purpose, a His-tagged version of BIK that lacks the C-terminal hydrophilic tail (aa 1–134) was expressed in bacteria, purified using Ni2+-NTA-agarose affinity matrix, and used as the substrate for phosphorylation. The BIK protein that was incubated with a cytoplasmic extract from HeLa cells was efficiently phosphorylated while BIK incubated without the extract was not phosphorylated (Fig. 2A). The cytoplasmic extract was also further fractionated through a cation exchange (SP-Sepharose) column and the various fractions were assayed to determine their ability to phosphorylate the recombinant BIK (1–134) protein. These results indicated that fractions 5–11 contained the kinase activity that phosphorylated BIK (Fig. 2B). To identify the phosphorylated amino acids of BIK, we carried out phosphoamino acid analysis of in vitro phosphorylated BIK. For this purpose SP-Sepharose fractions 5–11 were pooled and used to phosphorylate BIK. The phosphoamino acid analysis indicated that Ser and Thr residues were phosphorylated and no detectable phosphorylation of Tyr was observed (Fig. 2C). Similar results were also observed using in vivo phosphorylated BIK (not shown).

**Mapping of the Phosphorylation Site—**To identify the phosphorylation site(s), we performed site-directed mutagenesis by converting all serine, threonine, and tyrosine residues contained within the N-terminal 134-amino acid region of BIK to alanine residues (Table I). The various mutants were tagged with the HA epitope and transiently expressed in 293T cells. The protein extracts were analyzed by Western blot and probed with the HA antibody. All the mutant proteins except mutant S2A/S8A/T15A/Y18A were expressed as stable proteins. The various mutant proteins, except mutant T33A/S35A were expressed as doublets, suggesting that they are not defective in phosphorylation (summarized in Table I). On the other hand, mutant T33A/S35A had only the fast migrating band, suggesting that this mutant may be defective in phosphorylation. To confirm that mutant T33A/S35A is defective in phosphorylation, we performed metabolic labeling of 293T cells transiently transfected with wt BIK or mutant with 32P and 35S. Analysis of the proteins labeled with 35S indicated that both wt and mutant proteins were expressed at comparable levels, while there was no significant incorporation of 32P in the mutant protein (Fig. 3A, rightmost lane). These results suggest that mutant T33A/S35A is strongly defective in phosphorylation under conditions where the wt BIK protein is phosphorylated efficiently. The T33A/S35A mutation was also found to abolish phosphorylation of purified BIK (aa 1–134) protein in vitro (Fig. 3B). These results suggest that residues Thr-33 or/and Ser-35 of BIK are required for phosphorylation. To determine which of the two residues constitute the phosphorylation site of BIK, we constructed two different single amino acid substitution mutants of BIK in which either Thr-33 or Ser-35 was mutated to an Ala residue (mutants T33A and S35A). The mutant proteins were metabolically labeled with 32P and 35S and analyzed (Fig. 3C). Both mutants (T33A and S35A) were found to be defective in phosphorylation, suggesting that both residues are required for phosphorylation of BIK. However, these results do not distinguish between the possibility that both residues may be phosphorylated perhaps in a cooperative manner or only one is phosphorylated and the other residue plays a structural role. Since the phosphoamino acid analysis indicates that both Thr and Ser residues are phosphorylated, it is possible that both Thr-33 and Ser-35 may be target for phosphorylation.

**Effect of Phosphorylation on BIK Activities—**To investigate the role of phosphorylation on BIK activities, we first determined the apoptotic activities of wt BIK and mutant T33A/S35A in a transient apoptosis assay. HeLa cells were transfected with plasmids expressing wt BIK or mutant T33A/S35A along with a plasmid that expresses the E. coli lacZ reporter gene. In cells transfected with the empty vector (pcDNA3HA) less than 10% of the cells (that expressed the lacZ gene) exhibited apoptotic features at all DNA concentrations examined (0.5–3.0 μg), whereas, in cells transfected with wt BIK or mutant BIK, apoptosis increased as a function of DNA concentration (Fig. 4A). In cells transfected with wt BIK, about 30–50% of the transfected cells exhibited apoptosis, depending on the DNA concentration. In cells transfected with the mutant BIK, there was significantly lower cell death at all DNA concentrations, suggesting that the mutation reduces the apoptotic activity of BIK.

In addition to the transient apoptosis assay, the effects of wt
BIK and the mutant BIK were also examined in a cell survival assay (27). This survival assay is based on the principle that coexpression of a pro-apoptotic gene along with the transforming oncogenes (E1A and T24 ras) in primary BRK cells reduces the formation of transformed foci. In BRK cells transfected with the transforming oncogenes and wt BIK, formation of transformed colony formation was severely reduced as compared with cells transfected with the oncogenes and the empty vector (Fig. 4B). In cells transfected with the oncogenes and the mutant, there was consistent increase in the number of foci formation compared with cells expressing wt BIK, suggesting that the mutant induced less efficient cell death. Both the transient apoptosis assay (Fig. 4A) and the cell survival assay (Fig. 4B) suggest that phosphorylation of BIK is required for the manifestation of efficient cell death activity.

We also examined the effect of phosphorylation of BIK on its ability to heterodimerize with the cellular anti-apoptosis protein BCL-2. For this purpose, 293T cells were transfected with HA-tagged wt BIK or mutant (T33A/S35A) BIK or empty vector (pcDNA3-HA) and BCL-2. Protein extracts were immunoprecipitated either with the HA antibody or with the BCL-2 antibody. The immunoprecipitates were subjected to Western blot analysis using the BCL-2 antibody (Fig. 5). This analysis revealed that the phosphorylation-deficient mutant T33A/S35A complexed with BCL-2 at ratios similar to that of wt BIK, suggesting that phosphorylation of BIK at residues Thr-33 and Ser-35 apparently does not significantly influence its affinity for BCL-2. In addition, we have also observed that the T33A/S35A mutant had neither an altered half-life nor an abnormal subcellular localization pattern compared with wt BIK (data not shown).

Characterization of the Kinase Activity—To determine the identity of the protein kinase responsible for phosphorylation of BIK, we analyzed the primary amino acid sequences of BIK for the putative consensus phosphorylation sites for known Ser/Thr protein kinases. Computer-assisted analysis revealed that BIK contains four putative CKII sites and two PKC sites (Fig. 6A). Interestingly, two of the predicted CKII sites (Thr-33 and Ser-35) correspond to the phosphorylation sites determined by mutational analysis of BIK, raising the possibility that CKII may be a candidate kinase for phosphorylation of BIK. It should be noted that mutations of other potential CKII and PKC phosphorylation sites did not affect phosphorylation of BIK (Table I).

To gain further insight into the nature of the protein kinase,
pooled HeLa cell cytoplasmic fractions contain the 40- and 44-kDa subunits of the CKII subunits. Western blot analysis indicated that the molecular sizes of two of these proteins (40 and 44 kDa) are similar to the size of CKII (Fig. 6C). It is interesting to note that molecular sizes of 44 kDa (Fig. 6E) was phosphorylated by three proteins of about 36, 40, and 44 kDa as the substrate. This analysis revealed that BIK-(1–134) was efficiently phosphorylated by CKII while the T33A/S35A mutant was defective in phosphorylation. These results raise the possibility that CKII may be the candidate kinase for phosphorylation of BIK. We then tested the effect of a CKII inhibitor, DRB, on phosphorylation of BIK-(1–134) by purified CKII and HeLa cell extract. DRB inhibited BIK phosphorylation by CKII in a dose-dependent manner (Fig. 6E). However, phosphorylation of BIK by HeLa cell extracts (SP-Sepharose fractions 5–11) was not significantly affected by DRB, although the extracts contain CKII (see Fig. 6C). Taken together, the above result implicates that a kinase related to CKII, but insensitive to DRB, may be the candidate kinase for phosphorylation of BIK.

**DISCUSSION**

We have demonstrated that the human pro-apoptotic protein BIK is a phosphoprotein and that it is phosphorylated on consensus CKII phosphorylation sites (Thr-33 and/or Ser-35). We have observed that phosphorylation is required for efficient BIK-mediated cell death. This is opposite of the role of phosphorylation on another BH3-alone pro-apoptotic protein BAD. In the case of BAD, survival factors induce phosphorylation via survival-promoting kinases Akt and protein kinase A (19) and negatively influence its apoptotic activity. The precise mechanism by which phosphorylation plays a role in the apoptotic activity of BIK is not known. It has been proposed that BH3 proteins mediate apoptosis primarily by interaction with BCL-2 family anti-apoptosis proteins (see Ref. 28). In the case of BAD, phosphorylation of a Ser residue within the BH3 domain affects its ability to interact with BCL-2 (17, 29), which may contribute at least partially toward inactivation of BAD activity. Here, we have observed that the phosphorylation defect of BIK interacts with BCL-2 at levels similar to that of wt BIK, despite its lower apoptotic activity. We have also observed similar results in interaction studies between the phosphorylation-defective mutant (T33A/S35A) and BCL-xL (data not shown). These observations lend support to our earlier observation (3) that heterodimerization of BIK with cellular anti-apoptosis proteins is alone insufficient for its pro-apoptotic activity. It is possible that phosphorylation of BIK may play role in interaction with other potential cellular targets. In case of pro-apoptotic proteins BAK and BID, the BH3 domain becomes exposed in response to apoptotic stimuli (9, 10, 30). In the case of BID, N-terminal protein processing appears to expose the BH3 domain. It is possible that phosphorylation of BIK may confer certain conformational changes that expose the BH3 domain.

Our results suggest that BIK may be a target for phosphorylation by a kinase related to CKII. Although CKII-related kinases have been shown to phosphorylate apoptosis modulators such as p53 and c-Myc (33–36), BIK would be the first BCL-2 family protein target for CKII. Previously, BIK-induced apoptosis has been shown to be suppressed by expression of the survival-promoting kinase, Akt (37). It appears that the effect of Akt on BIK activity may be indirect as Akt suppresses the activity of a number of other pro-apoptotic stimuli. Recently, it has been reported that Akt may inhibit BAD and BIK-induced apoptosis indirectly by inducing expression of BCL-xL (31).

BIK is constitutively expressed in several different human tissues and appears to be expressed at elevated levels in skeletal and cardiac muscles (32). It is possible that the activity of BIK may be activated by phosphorylation in response to different apoptotic stimuli in various human tissues. The effect of various apoptotic stimuli on phosphorylation of BIK remains to be investigated.

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