Phosphoinositide 3-OH Kinase (PI3K) and PKB/Akt Delay the Onset of p53-mediated, Transcriptionally Dependent Apoptosis*

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The phoshoinositide 3-OH kinase (PI3K)-PKB/Akt signaling pathway has been shown to mediate both Ras- and cytokine-induced protection from apoptosis. In addition, apoptosis induced by the p53 tumor suppressor protein can be inhibited by Ras- and cytokine-mediated signaling pathways. It was therefore of interest to determine if the PI3K-PKB/Akt signaling pathway was capable of conferring protection from apoptosis induced by p53. We demonstrate in this report that constitutively active PI3K and PKB/Akt are capable of significantly delaying the onset of p53-mediated apoptosis. This was manifested as a delay in the kinetics of DNA degradation and cell death as well as a profound attenuation in the accumulation of cells with a sub-G1 DNA content. Moreover, we found that this effect is mediated in the absence of changes in expression of Bcl-2, Bcl-XL, and the pro-apoptotic protein Bax. Our results provide the first direct and unambiguous link between p53-mediated apoptosis and the PI3K-PKB/Akt signaling pathway.

The serine/threonine protein kinase PKB/Akt was originally identified as the cellular counterpart of the v-Akt transforming protein present in AKT8, a retrovirus that causes T cell lymphomas in mice (1). v-Akt was generated by a fusion event that juxtaposes the retroviral glycosaminoglycan protein and the entire coding region of PKB/Akt (2, 3). The fusion protein, designated glycosaminoglycan-PKB, is constitutively active due to a myristoylation signal present within the amino terminus of the glycosaminoglycan protein that targets PKB/Akt to the plasma membrane.

In quiescent or serum-starved cells, PKB/Akt resides within the cytosol in a catalytically inactive state. Upon stimulation of cells with growth factors and cytokines, PKB/Akt is recruited to the plasma membrane and catalytically activated by phosphorylation at threonine 308 and serine 473 (4–8). Phosphorylation of PKB/Akt at threonine 308 is catalyzed by the ubiquitously expressed and constitutively active protein kinase PDK-1 (5, 7). The kinase responsible for phosphorylation of PKB/Akt at serine 473 has not been definitively established, although possible candidates have been proposed (9). Recruitment of both PKB/Akt and PDK-1 to the plasma membrane is mediated by second messenger phosphorylated phosphoinositides generated by the phosphorylation of inositol lipids by phosphoinositide 3-OH kinase (PI3K). Platelet-derived growth factor- and IGF-mediated activation of PKB/Akt is inhibited by two pharmacological inhibitors of PI3K, LY294002 and wortmannin (7, 10, 11). Also, platelet-derived growth factor receptor mutants incapable of activating PI3K are likewise unable to activate PKB/Akt (12). Moreover, PDK-1 potentiates the platelet-derived growth factor-mediated activation of PKB/Akt, and this effect is abrogated by wortmannin (13). Taken together, these results indicate that PI3K can function as an upstream activator of PKB/Akt and can regulate the ability of PDK-1 to modulate PKB/Akt activity.

PI3K is a heterodimeric lipid kinase consisting of a p85 regulatory subunit and a p110 catalytic subunit and is capable of triggering a plethora of biological responses (14–16). PI3K is activated by the interaction of the p85 regulatory subunit with phosphorylated tyrosine residues on activated growth factor receptors (17). The binding of PI3K to upstream signaling molecules leads to the recruitment of p85-p110 heterodimeric complexes to the plasma membrane and the subsequent activation of the p110 catalytic subunit. The activated p110 catalytic subunit phosphorylates inositol lipids at the 3-position of the inositol ring, thereby generating the phospholipid second messenger molecules required for the transposition of PKB/Akt and PDK-1 to the plasma membrane.

Cytokines, growth factors, and certain oncogenes have been shown to be effective inhibitors of apoptosis, and in many situations, this anti-apoptotic effect is mediated by the PI3K-induced activation of PKB/Akt (18–23). IGF-1 is a well-documented activator of the PI3K-PKB/Akt signaling pathway (4, 23, 24). IGF-1 inhibits UV-induced apoptosis in fibroblasts and prevents apoptosis in neuronal cells in response to growth factor withdrawal (21, 23). In both cases, IGF-1-mediated protection from apoptosis is abrogated either by pharmacological inhibitors of PI3K or by dominant-negative PKB/Akt constructs. Moreover, constitutively active PI3K or PKB/Akt mimics the anti-apoptotic function of IGF-1. Ras activates the PI3K-PKB/Akt signaling pathway by interacting directly with the p110 catalytic subunit of PI3K (16). Ras-induced activation of the PI3K-PKB/Akt signaling pathway confers protection from apoptosis in fibroblasts in response to oncogenic Myc and protects epithelial cells from apoptosis induced by anoikis (22, 25, 26). In this respect, PI3K-PKB/Akt-mediated survival contributes to the ability of Ras to function as an oncogene.

The p53 tumor suppressor protein is a transcription factor capable of inducing either growth arrest or apoptosis (27–29). In response to DNA damage, p53 induces a G1-specific cell cycle arrest by transcriptionally up-regulating the expression of the cyclin/CDK inhibitor, p21/WAF1-Cip1. This allows cells time to repair damaged DNA before progressing into S phase (30, 31). However, in response to oncogenic activation and/or growth factor withdrawal, p53 can induce apoptosis. p53-mediated ap-

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† The abbreviations used are: PI3K, phosphoinositide 3-OH kinase; IGF, insulin-like growth factor; IL, interleukin; BRK, baby rat kidney; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; FACS, fluorescence-activated cell sorter.
optosis in certain cell types requires a transcriptionally functional p53. In this respect, the p53-inducible proteins Bax and IGF-binding protein-3 have been shown to be capable of inducing apoptosis. The inhibition of p53-mediated apoptosis in vivo potentiates the rate at which a tumor progresses to the stage of malignancy, and this is thought to be a major reason why p53 is so often mutated in human cancers.

The cytokine IL-3 is a potent inhibitor of p53-mediated apoptosis in erythroleukemia cells (32). In addition, the IL-3-mediated activation of JAK kinase is sufficient to protect myeloid cells from p53-mediated apoptosis in response to γ-radiation (33). IL-3 is also a well established activator of the PI3K-PKB/Akt survival pathway (18). Moreover, p53-mediated apoptosis in baby rat kidney (BRK) cell lines transformed by E1A and tsp53(Val-135) (where ts is temperature-sensitive) is suppressed by oncogenic Ras (34). These findings indicate that p53-mediated apoptosis is inhabitable under conditions in which the PI3K-PKB/Akt survival pathway is activated.

In light of these findings, it was of interest to determine if the PI3K-PKB/Akt signaling pathway was capable of conferring protection from apoptosis induced by p53. To this end, we employed a well characterized cell culture system in which apoptosis is exclusively p53-dependent (35–38). Using this cell culture system, we demonstrate that both constitutively active PI3K and PKB/Akt compromise the onset of p53-mediated apoptosis. Moreover, we find that this effect is mediated in the absence of changes in expression of Bcl-2, Bel-Xl, and the pro-apoptotic protein Bax. These results provide the first unambiguous and compelling evidence that the PI3K-PKB/Akt survival pathway can protect from apoptosis induced by the p53 tumor suppressor protein.

**EXPERIMENTAL PROCEDURES**

**Cell Lines and Tissue Culture**—The p53A and 4B cell lines have been described (35, 37, 38). Briefly, both cell lines were established from primary epithelial BRK cells transfected with genes encoding adenovirus E1A and murine tsp53(Val-135), which is predominantly in the mutant confirmation at the restrictive temperature of 38.5 °C and predominantly in the wild-type confirmation at the permissive temperature of 32 °C. The p53A cell line proliferates at the restrictive temperature of 38.5 °C, but undergoes p53-mediated, transcriptionally dependent apoptosis at the permissive temperature of 32 °C. The 4B cell line also constitutively expresses an ectopically introduced human BCL-2 gene and proliferates at the restrictive temperature of 38.5 °C. This 4B cell line is rescued from p53-mediated apoptosis at the permissive temperature of 32 °C and instead undergoes p53-mediated growth arrest predominantly in the G2/M phase of the cell cycle.

The LXS N, Akt-1, and 110-1 cell lines were generated as follows. p53A cells were seeded at 2.5 × 105 cells/35-mm dish. Twenty-four hours later, 1 ml of supernatant from the GP+E ectopic packaging cell line expressing empty retrovirus vector (LXS N), oncogenic v-Akt (LXS N- v-Akt) (80), or the constitutively active p110 subunit of PI3K (LXS N- p110oxaMyc) was added to the cells in the presence of 8 μg/ml Polybrene (Sigma), and the cells were allowed to incubate at 38.5 °C for 6 h. The supernatants were then aspirated, and the cells were replenished with fresh growth medium and allowed to incubate at 38.5 °C for 24 h. Cells were treated with G418 (Life Technologies, Inc.) at a concentration of 0.5 mg/ml and maintained in this selection medium until a proliferating pool of cells was obtained. All BRK-derived cell lines were maintained at 38.5 °C in Dulbecco's modified Eagle's medium (high glucose) + 5% fetal bovine serum.

**Western Blotting and Antibodies**—Twenty-five micrograms of whole cell extracts prepared from cell lines incubated at 38.5 and 32 °C were subjected to SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes (Millipore Corp., Bedford, MA) using standard procedures. Immunoblotting was performed with the following antibodies: polyclonal anti-Bax (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), polyclonal anti-Bcl-XI (Calbiochem-Novabiochem), monoclonal anti-Bcl-2 (Transduction Laboratories, Lexington, KY), monoclonal anti-actin (Amersham Pharmacia Biotech), polyclonal anti-p110,2 and polyclonal anti-PKB/Akt (Upstate Biotechnology, Inc.).

**RESULTS**

**Constitutively Active PI3K and PKB/Akt Delay the Onset of p53-mediated Apoptosis**—The p53A cell line was generated from primary BRK cells transformed by adenovirus E1A and murine tsp53(Val-135) (35, 37, 38). p53A proliferates at the restrictive temperature of 38.5 °C, but succumbs to p53-mediated, transcriptionally dependent apoptosis at the permissive temperature of 32 °C. Therefore, apoptosis induced in the p53A cell line upon incubation at 32 °C is entirely p53-dependent. Derivatives of the p53A cell line that express either constitutively active PI3K (110-1) or PKB/Akt (Akt-1) were generated from pooled populations of G418-resistant p53A cells infected with the LXS N- p110oxaMyc and LXS N- v-Akt retroviruses, respectively. The LXS N cell line was generated in parallel from pooled populations of p53A cells infected with the LXS N retrovirus alone and therefore serves as an appropriate negative control. All three cell lines were maintained at 38.5 °C. Western blot analysis indicated that the Akt-1 and 110-1 cell lines expressed the v-Akt and p110oxaMyc proteins, respectively, whereas neither protein was expressed in the LXS N control cell line (Fig. 1). Moreover, PKB/Akt activity was significantly higher in the Akt-1 and 110-1 cell lines compared with the LXS N control cell line, thus demonstrating that the p110oxaMyc and v-Akt proteins are catalytically intact (Fig. 1).

In an effort to determine if the constitutively active PI3K and PKB/Akt can abrogate p53-mediated apoptosis, the Akt-1 and 110-1 cell lines were plated out at 38.5 °C and 24 h later were incubated at 32 °C for 12, 24, 48, and 72 h. The 4B cell line has been described and is a derivative of the p53A cell line, which constitutively expresses an ectopically introduced BCL-2 proto-oncogene (37). The 4B cell line is rescued from p53-mediated apoptosis
apoptosis at 32 °C and instead undergoes p53-mediated growth arrest. The 4B cell line was therefore used as a positive control for the inhibition of p53-mediated apoptosis at 32 °C.

The p53A parental and LXSN control cell lines underwent a significant decrease in viability by 12 h at 32 °C (Fig. 2A). The viability of both lines progressively decreased at a comparable rate at subsequent time points, and by 72 h at 32 °C, both the p53A and LXSN cell lines were essentially nonviable. The onset of p53-mediated cell death in the Akt-1 and 110-1 cell lines was significantly delayed by comparison in that a net decrease in cell viability was not evident in either line until after 24 h at 32 °C (Fig. 2A). Interestingly, the decrease in viability observed in the Akt-1 and 110-1 cell lines between 24 and 48 h at 32 °C was dramatic and occurred with accelerated kinetics compared with the p53A and LXSN control lines. The viability of the 4B cell line remained relatively constant at all time points, as previously reported (37). The delayed onset of cell death observed in the Akt-1 and 110-1 cell lines could be due to either an inhibition of apoptosis or a balance between cell growth and cell death. Therefore, additional experiments were performed to distinguish between these two possibilities.

The degradation of genomic DNA into small molecular weight oligonucleosome-sized fragments is a characteristic of cells that succumb to apoptotic cell death (39). Moreover, this hallmark sign of apoptosis is observed in the p53A cell line subsequent to incubation at 32 °C (35, 37, 38). In contrast, the 4B cell line, which is rescued from p53-mediated apoptosis at 32 °C, was devoid of DNA degradation at all time points. With respect to the Akt-1 and 110-1 cell lines, DNA degradation was undetectable at the 0-, 12-, and 24-h time points when cell viability was maintained (Fig. 2B). These results indicate that the preservation of cell viability observed in the Akt-1 and 110-1 cell lines at early time points subsequent to incubation at 32 °C is due to the inhibition of p53-mediated apoptosis.

The percentage of apoptotic cells within a given population can be quantitated by labeling cells with propidium iodide, subjecting them to FACS analysis, and calculating the percentage of cells with a sub-G1 DNA content. p53-mediated apoptosis in BRK cell lines transformed by E1A and tsp53(Val-135) is paralleled by a rapid and dramatic increase in cells with a sub-G1 DNA content (35). Therefore, to quantitate the delayed kinetics of apoptosis observed in the Akt-1 and 110-1 cell lines, both lines were labeled with propidium iodide at 38.5 and 32 °C and subjected to FACS analysis. The population of cells with a sub-G1 DNA content was then calculated and represented as a percentage of the total cell population.

The 4B cell line was virtually devoid of cells with a sub-G1 DNA content at both 38.5 and 32 °C (Fig. 3). Moreover, the 4B cell line gradually underwent a p53-mediated G2/M cell cycle arrest by 72 h at 32 °C, as previously observed (34). The population of cells with a sub-G1 DNA content present in both the p53A and LXSN cell lines had increased dramatically by 12 h at 32 °C and continued to increase at subsequent time points (Fig. 3). These results correspond to the rapid kinetics of DNA degradation and cell death observed in the p53A and LXSN cell lines upon incubation at 32 °C (Fig. 2A). In contrast, neither the Akt-1 nor the 110-1 cell line showed any significant increase in the percentage of cells with a sub-G1 DNA content for up to 24 h at 32 °C. These results correspond to the delayed kinetics in p53-mediated DNA degradation and cell death observed in both lines. In addition, these results further confirm the fact that both constitutively active PI3K and PKB/Akt compromise the onset of apoptosis induced by p53, and, in turn, demonstrate that this is a quantitatively significant event.

The Delayed Onset of p53-mediated Apoptosis Induced by
markers (50–10,000 base pairs) were from Bionexus, Inc. DNA molecular weight (MW) prepared and analyzed in parallel with the viability assay in A110-1 cell lines and the 4B control cell line for 72 h at 32 °C by trypan blue exclusion and is expressed as the percentage of the original viability at the time of shift to 32 °C. B, low molecular weight DNA was prepared and analyzed in parallel with the viability assay in A using procedures described previously (38). DNA molecular weight (MW) markers (50–10,000 base pairs) were from Bionexus, Inc.

Constitutively Active PKB/Akt Is Not Due to Changes in the Levels of Bax, Bcl-2, or Bcl-XI—In BRK cell lines transformed by E1A and tsp53(Val-135), Bax is transcriptionally up-regulated by p53 at 32 °C, and this is sufficient to induce apoptosis (40). In addition, in certain cell types, the survival function of the PI3K-PKB/Akt signaling pathway is due, in part, to the PKB/Akt-mediated up-regulation of Bcl-2 (20, 41). Bcl-2 inhibits apoptosis induced by a variety of stimuli and can function as an oncogene by inhibiting apoptosis induced by p53 (37, 42). The anti-apoptotic protein Bel-XI is a homologue of Bcl-2 and is also capable of inhibiting p53-mediated apoptosis (43). In light of these findings, it was of interest to determine if the delayed onset of p53-mediated apoptosis induced by constitutively active PKB/Akt was due to attenuation of p53-mediated Bax induction or to increased expression of Bcl-2 or Bcl-XI. To this end, whole cell extracts were prepared from the Akt-1 cell line at 38.5 °C and at 12 and 24 h at 32 °C when all signs of induction or to increased expression of Bcl-2 or Bcl-XI. However, Bax protein levels in the Akt-1 cell line continued to increase up to 24 h at 32 °C, although cell viability was still completely maintained (Fig. 4). Bel-2 protein levels were detectable in the LXSN cell line and remained constant at all time points (Fig. 4). Bel-2 was also detectable at both temperatures in the Akt-1 line, but the level of expression appeared to decrease somewhat upon incubation at 32 °C. The Bel-XI protein was detectable in both the LXSN and Akt-1 cell lines and was expressed at a similar level at corresponding time points (Fig. 4). Thus, the delayed kinetics of apoptosis observed in the Akt-1 cell line cannot be explained by a corresponding delay in the p53-mediated up-regulation of Bax or by an increased expression of Bel-2 or Bel-XI. However, it is possible that PI3K and PKB/Akt may regulate the function of these proteins through some other mechanism that has yet to be identified.

DISCUSSION

We demonstrate in this report that both PI3K and PKB/Akt are capable of compromising the onset of apoptosis induced exclusively by the tumor suppressor protein p53. This was manifested as a significant delay in the kinetics of DNA degradation and cell death as well as a profound attenuation in the accumulation of cells with a sub-G1 DNA content. The protection from p53-mediated apoptosis conferred by PI3K and PKB/Akt was not permanent, as both the Akt-1 and 110-1 cell lines ultimately succumbed to cell death at the permissive temperature. One possible explanation for this observation centers around the mechanism by which p53-mediated, transcriptionally dependent apoptosis is induced.

p53-mediated apoptosis in BRK cell lines transformed by E1A and tsp53(Val-135) is transcriptionally dependent and is triggered by a class of enzymes known as caspases (44, 46). Caspases are a family of aspartate-specific proteases that induce apoptosis by cleaving and inactivating cellular substrates, which play an essential role in maintaining cell viability (47). Apoptosis in mammalian cells results from the activation of caspases in a cascade-like fashion, with initiator caspases lying at the apex of the cascade and effector caspases lying farther downstream (48, 49). Caspase-9 is an initiator caspase that becomes activated by the release of cytochrome c from mitochondria in response to many apoptotic stimuli (reviewed in Ref. 50). Recent evidence indicates that PKB/Akt can phosphorylate and inactivate caspase-9 and thereby abrogate caspase-9-mediated apoptosis (51). Thus, in certain cell types, PKB/Akt may abrogate apoptosis through the direct inhibition of caspases. Constitutively active PKB/Akt was capable of abrogating the early stages of p53-mediated apoptosis, but was incapable of conferring protection at later time points (Fig. 2). Therefore, it is tempting to speculate that the early stages of p53-mediated, transcriptionally dependent apoptosis are triggered by initiator caspases such as caspase-9 that, once inhibited by PKB/Akt. A role for caspase-9 in p53-mediated apoptosis per se is implicated by the observation that dominant-negative caspase-9 constructs can inhibit apoptosis induced by E1A and Bax (52, 53). Moreover, a recent study indicates that the inactivation of caspase-9 can substitute for p53 loss in permitting the oncogenic transformation of primary mouse embryonic fibroblasts by c-Myc (54). Experiments to determine if...
Caspase-9 is activated at the permissive temperature in BRK cell lines transformed by E1A and tsp53(Val-135) are in progress.

The pro-apoptotic protein Bad antagonizes the anti-apoptotic function of Bcl-2 and Bcl-Xl by forming inactivating Bad-Bcl-2 and Bad-Bcl-Xl heterodimers (55). Bad has recently been shown to be a target of PKB/Akt-mediated phosphorylation, and the phosphorylation of Bad by PKB/Akt prevents Bad from heterodimerizing with Bcl-2 and Bcl-Xl (55–57). When uncomplexed with Bad, Bcl-2 and Bcl-Xl are capable of abrogating Bax-mediated apoptosis through the formation of Bcl-2/Bcl-Xl-Bax heterodimers (42, 55). Bax is a transcriptional target of p53 in BRK cell lines transformed by E1A and tsp53(Val-135), and its expression is sufficient to induce apoptosis (40). Therefore, by phosphorylating Bad and potentiating the interaction of Bcl-2 and Bcl-Xl with Bax, PKB/Akt could conceivably protect from apoptosis induced by p53. However, Bad expression was completely undetectable in the Akt-1 cell line at both 38.5 and 32 °C (data not shown). Therefore, the delayed onset of p53-mediated apoptosis observed in the Akt-1 cell line at both 38.5 and 32 °C (data not shown). The percentage of cells with a sub-G1 DNA content is represented numerically in each box. The positions of sub-G1, G1, S, and G2/M DNA contents are indicated.

We have demonstrated that PI3K and PKB/Akt can promote cell survival by compromising the kinetics of apoptosis induced by p53. It would therefore be of interest to determine if PI3K-PKB/Akt-mediated survival can restrict the efficacy of anticancer interventions that function by triggering p53-induced apoptosis. Indeed, p53 has been shown to mediate apoptosis in response to γ-radiation and certain chemotherapeutic reagents (60–64). This concern may be especially relevant in the treatment of malignancies such as ovarian cancer, in which the PI3K-PKB/Akt survival pathway has been shown to be aberrantly activated (65–67).

The abrogation of p53-mediated apoptosis through inactivating mutations represents an important driving force in tumor development (68–70). However, the inactivation of p53 is typically not involved in tumor initiation, as it is frequently observed to be a late-onset event in human cancers (71). Thus,
there may exist other oncogenic mechanisms that function to modulate the impact of p53-mediated apoptosis at the early stages of cancer development. The transformation of colorectal epithelium to carcinomas is associated with a progressive inhibition of apoptosis, and p53 inactivation occurs near the transition from benign to malignant growth (72). In contrast, Ras mutations occur most often during the early adenomatous stages of colorectal cancer development. The transformation of colorectal epithelium to carcinomas is associated with a progressive inhibition of apoptosis, and p53 inactivation occurs near the transition from benign to malignant growth (72). In contrast, Ras mutations occur most often during the early adenomatous stage of the disease (73). Thus, it is conceivable that Ras-mediated activation of the PI3K-PKB/Akt survival pathway may limit the extent of apoptosis induced by p53 during the early premalignant stages of colon cancer. This, in turn, would facilitate the progression of tumor growth until p53-mediated apoptosis is completely abrogated by inactivating mutations. Ras is capable of suppressing p53-mediated apoptosis by p53, as this might help to explain how Ras functions as an oncogene in vivo.

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