AKT/PKB Phosphorylation of p21<sup>Cip/WAF1</sup> Enhances Protein Stability of p21<sup>Cip/WAF1</sup> and Promotes Cell Survival*

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p21<sup>Cip/WAF1</sup> (p21), a p53-inducible protein, is a critical regulator of cell cycle and cell survival. p21 binds to and inhibits both the DNA synthesis regulator proliferating cell nuclear antigen and cyclin A/E-CDK2 complexes. Recently, p21 has also been shown to be a positive regulator of cell cycle progression as p21 is necessary for the assembly and activation of cyclin D1-CDK4/6 complexes. Furthermore, elevated p21 protein levels have been observed in various aggressive tumors as well as linked to chemoresistance. Here we demonstrate that p21 is directly phosphorylated by AKT/PKB, a survival kinase that is hyperactivated in many late stage tumors. Two sites (Thr<sup>145</sup> and Ser<sup>146</sup>) in the carboxyl terminus of p21 are phosphorylated by AKT/PKB in <i>vivo</i> and <i>in vitro</i>. Phosphorylation of Thr<sup>145</sup> inhibits PCNA binding, whereas phosphorylation of Ser<sup>146</sup> significantly increases p21 protein stability. Glioblastoma cell lines with activated AKT/PKB show enhanced p21 stability, and they are more resistant to taxol-mediated toxicity. Finally, AKT/PKB controls the assembly of cyclin D1-CDK4 complexes through modulation of p21 and cyclin D1 levels. These data imply that enhanced levels of p21 in tumors are due, in part, to phosphorylation by activated AKT/PKB. Furthermore, they suggest that one mechanism of AKT/PKB regulation of tumor cell survival and/or proliferation is to stabilize p21 protein.

Tumors utilize a diversity of genetic mechanisms to allow for survival under the onslaught of the immune system or radiation and chemotherapy. The most notable modifications are found in the deletion of various tumor suppressor genes that are involved with a diversity of cellular functions ranging from control of cell cycle progression to regulation of apoptotic cell death. For example, the loss of p53 is a common genetic lesion in tumor cells, and this deletion results in changes in the cell's response to DNA damage such that the transformed cells become less prone to apoptosis when their genomes are toxically modified (1). Because p53 is a transcription factor, its loss results in changes in the expression of downstream target genes. One such target gene is p21<sup>Cip/WAF1</sup> (p21), which encodes a protein that regulates cell cycle and DNA synthesis (see below) (2). The cell cycle of tumor cells can also be modulated by the deletion of the retinoblastoma protein, a substrate molecule of cyclin-dependent kinase (CDK),<sup>1</sup> which negatively modulates the mitosis-inducing E2F transcription factors (3). Recently, the homozygous loss of the PTEN/MMAC tumor suppressor, which appears to positively modulate tumor cell survival, has been identified in a diversity of late stage cancers including glioblastoma and prostate tumors (4, 5). PTEN/MMAC is a lipid phosphatase that regulates the levels of two phospholipid products of the pro-survival PI3 kinase pathway, phosphatidylinositol phosphate-3,4 and -3,4,5, by dephosphorylation at position 3 (6). These phospholipids are required for activation of AKT/PKB, a kinase that down-regulates apoptosis by phosphorylating various substrates including BAD, caspase 9, and most importantly, forkhead family transcription factors. The phosphorylation of the forkhead family proteins by AKT/PKB results in their cytoplasmic localization (7–9). Because these translocation factors mediate the expression of pro-apoptotic genes, their phosphorylation and then cytoplasmic sequestration result in the inhibition of cell death (10). Thus, the loss of PTEN/MMAC results in enhanced AKT/PKB kinase activity along with decreased forkhead transcription activity and enhanced cell survival. Finally, although it is likely that the AKT/PKB pathway is involved with the regulation of cell cycle, very little has been observed along these lines, although an increase in the p21<sup>Cip/WAF1</sup> cyclin inhibitor protein is found as a result of forkhead-induced transcription (11).

p21 was initially isolated as a p53-inducible protein. Because p53 was known to function in cell cycle regulation, p21 was proposed to be a regulator of cell cycle progression (2). Subsequent studies revealed that p21 played a dual role in cell cycle progression by regulating both DNA synthesis and CDK activity. p21 bound to various cyclin-CDK complexes via an amino-terminal binding site, and this interaction acted to inhibit the activity of these complexes, thus resulting in a block in cell cycle progression (12). The versatility of p21 was revealed by studies demonstrating that the protein also bound to PCNA, a co-activator of DNA polymerase δ and ε activities (13), by a carboxyl-terminal interaction (14). This interaction resulted in an inhibition of the ability of PCNA to assist DNA polymerases, which led to inhibition of DNA synthesis (15). Although these data are consistent with the suggestion that p21 acts as an inhibitor of cell cycle progression, several recent studies have revealed the p21 could also function as a positive regulator of cell cycle. Studies from Sherr et al. (17) and others (16, 18) have demonstrated that despite its inhibition of cyclin E-CDK2 complexes, p21 mediates the assembly and activation of cyclin D1-CDK4/6 complexes, which function as sensors of growth factors at G<sub>i</sub>-S phase complexes. In addition to the functions in the cell cycle, p21 has also been suggested to play a role in enhancing cell survival. For example, terminally differentiated...
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cells, such as muscle cells (19), hematopoietic stem cells (20) and macrophages (21), were found to contain elevated levels of p21, and the inhibition of the up-regulation of this protein resulted in apoptotic cell death. p21 may act as a coordinator of DNA synthesis and cell cycle arrest, as cells lacking p21 undergo DNA damage-mediated DNA replication, resulting in endoreduplication and subsequent apoptosis (22, 23). Furthermore, p21 can protect various types of cells from death following anticancer treatments. p21 overexpressed by inducible expression systems or adenovirus gene transfer increases cell survival against prostat glandin A2 or p53 overproduction (24, 25). The reduction or absence of p21 expression, however, sensitizes cells to adriamycin, camptothecin, etoposide, vincristine or adenovirus gene transfer increases cell progression and cell survival (18, 29–32).

Three recent papers have suggested that p21 is modulated by the Akt/PKB survival pathway (33–35). Mammalian p21 isolated from baculovirus-infected insect cells was found to be phosphorylated on two residues, Thr145 and Ser146 (33). Interestingly, these phosphorylation sites were found in the PCNA binding site, and it was shown that phosphorylation at these sites disrupted PCNA binding to p21 in vitro. Although these residues could be weakly phosphorylated in vitro by protein kinases A and C, these investigators proposed another, unknown kinase as the actual mediator of phosphorylation. Examination of these residues suggested that they were consensus phosphorylation sites for Akt/PKB kinases, and it was subsequently shown that one of the residues, Thr145, could be phosphorylated by Akt/PKB in vitro as well as in cells overexpressing the HER-2 oncogene or moderately activated Akt/PKB (34, 35). An examination of the effects of this phosphorylation event suggested that it mediated a change in the subcellular localization of p21, such that the phosphorylated form was found in the cytoplasm (34). However, another group demonstrated that this phosphorylation event was not involved with subcellular localization but was instead involved with regulation of PCNA binding and cyclin-CDK assembly (35). Here we demonstrate that both Thr145 and Ser146 sites are targets for Akt/PKB phosphorylation in vivo. We also demonstrate that phosphorylation at Ser146 results in a significant increase in p21 protein stability and protection of cells from taxol-induced apoptosis. We confirm and extend the data suggesting that the phosphorylation at Thr145 results in an inhibition of p21-PCNA binding in vivo. Finally, we demonstrate that p21 induced by Akt/PKB activation does not inhibit cyclin E-CDK2 but binds with and activates cyclin D1-CDK4 complex. Together, these data suggest that a novel mechanism for Akt/PKB-mediated regulation of cell survival and/or cell proliferation is by phosphorylation and up-regulation of p21.

**EXPERIMENTAL PROCEDURES**

**Materials**—Anti-Flag (M2) and anti-Myc (9E10) antibodies were from Sigma and Roche Molecular Biochemicals, respectively. The anti-AKT/PKB and anti-phospho AKT/PKB (T508) were purchased from Cell Signaling. Antibodies recognizing p21(C-19 and 187), CDK2, cyclin D1, and CDK4 were from Santa Cruz Biotechnology, Inc. The polyclonal antibody against p21(AB5) used for immunoprecipitation was from Oncogene. The anti-β-tubulin and anti-PCNA antibodies were from BD PharMingen and Transduction Laboratories, respectively. Kinase substrates Histone H1 and GST-Rb were from Roche Molecular Biochemicals and Santa Cruz Biotechnology, Inc., respectively. DNA plasmids encoding Myc-tagged Myc-Akt and Akt-KD were purchased from Upstate Biotechnology, Inc. and taxol was from Sigma.

**Plasmid Constructs**—Using standard PCR protocols, Flag-tagged p21 was cloned into the pcDNA3.1 vector (Invitrogen). The putative Akt phosphorylation site (Thr145 or Ser146) was mutated to Ala by site-directed mutagenesis using the QuickChange kit from Stratagene. The presence of the introduced mutations was confirmed by sequence analysis. To generate constructs for expression of GST-tagged p21 in *Escherichia coli*, wild type or mutants of p21 cDNA fragments were subcloned into vector pGEX4T-3 (Amersham Biosciences Inc.). The GST fusion proteins were expressed in *E. coli* strain BL21 and purified by glutathione-Sepharose chromatography based on manufacturer’s instructions (Amersham Biosciences Inc.).

**Cell Culture**—The glioblastoma cell lines U-87 MG, U-118 MG, A172, DBTRG-05MG, and T98G and the human epithelial cell line 293E were obtained from ATCC and cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum (Invitrogen) and antibiotics (50 units/ml penicillin and 50 µg/ml streptomycin). The MCF7 human breast carcinoma cell line was from ATCC and grown in RPMI (Invitrogen) supplemented with 10% heat-inactivated fetal calf serum (Invitrogen) and antibiotics. Stable MCF7 cells overexpressing Myc-tagged Myc-AKT/PKB and Akt/PKB-KD were generated by transfecting the cells with plasmids and selecting in the RPMI medium containing 500 µg/ml G418.

**Transfection**—DNA plasmids were transfected using FuGENE™6 reagent (Roche Molecular Biochemicals). Phosphorothioate oligodeoxynucleotides (250 nm) were transfected with Oligofectamine™ reagent (Invitrogen) following manufacturer’s instructions (Invitrogen). The p21+/−/WAF1 antisense oligo and control oligo were purchased from Molecular Research Laboratories, LLC. The antisense oligo, 5'-CCAACTCATTCCCGGCTCGC-3', is complementary to the coding region of p21. The control oligo, 5'-AAACCCCTTCCGGCCCTCGA-3', is a scrambled sequence.

**In Vitro AKT/PKB Kinase Assay**—2 µg of GST-tagged wild type or mutant p21 protein was incubated with 0.5 µg of active Akt (Upstate Biotechnology, Inc.) in the presence of 10 µCi [γ-32P]ATP and 50 µM cold ATP in the kinase buffer (20 mM Hepes, pH 7.2, 25 mM sodium β-glycerophosphate, 5 mM EGTA, 1 mM dithiothreitol, and 10 mM MgCl₂) for 30 min at 30 °C. The reaction was terminated by 10 mM EDTA and SDS sample buffer. The reaction products were separated on 4–20% SDS-PAGE and transferred to nitrocellulose filters. 32P-incorporated proteins were detected by autoradiography.

**[32P]Orthophosphate Labeling**—293E cells were cotransfected with Myc-tagged Myc-Akt and Flag-tagged p21 (wild type or mutants). 16 h after transfection, the cells were serum-starved for 12 h and then grown in phosphate-free medium for 1.5 h. 1.5 µCi/µl [32P]Orthophosphate was then added to the medium. At 4 h after the labeling, p21 was immunoprecipitated with anti-Flag antibody and resolved by SDS-PAGE. The 32P-labeled p21 was then detected by autoradiography and quantified by phosphorimaging.

**Western Blotting, Immunoprecipitation, and CDK Kinase Assays**—Cells were washed with cold phosphate-buffered saline and lysed in ice-cold lysis buffer (50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 10 mM sodium β-glycerophosphate, 50 mM NaF, 5 mM sodium pyrophosphate, 1 µM Microcystin-LR, 0.27 mM sucrose, and protease inhibitors). After lysates were centrifuged at 14,000 rpm for 15 min at 4 °C, 50 µg proteins were electrophoresed by 4–20% SDS-PAGE and transferred to nitrocellulose filters. The membrane was blocked in 5% nonfat milk and probed with different antibodies. Expression of β-tubulin was used as an equal loading control. For assessment of CDK2 and CDK4 kinase activities, endogenous CDK2 or CDK4 was immunoprecipitated from 1 µg of cell lysate proteins. The immunoprecipitated CDK2 and CDK4 were incubated with 2 µg of histone H1 and 2 µg of GST-Rb, respectively, in the presence of 100 Ci of [γ-32P]ATP and 20 µM cold ATP in the kinase buffer (20 mM Hepes, pH 7.2, 25 mM sodium α-glycerophosphate, 5 mM EGTA, 1 mM dithiothreitol, and 10 mM MgCl₂).

**Trypan Blue Assay**—48 h after taxol challenge, floating cells were transferred to a tube. The adherent cells were treated with trypsin and then mixed with the floating cells. The cells were collected by centrifugation at 1500 rpm for 5 min. After being stained with 0.4% trypan blue (Sigma) for 5 min at room temperature, the numbers of trypan blue-positive dead cells and trypan blue-negative alive cells were counted on a hemocytometer under a microscope.
RESULTS

AKT/PKB Physically Interacts with and Phosphorylates p21 at Thr^{145} and Ser^{146}.—Examination of the p21 sequence revealed two overlapping sites in the carboxyl terminus for phosphorylation by the AKT/PKB kinase. Fig. 1A shows that one site at Thr^{145} is preceded by the known consensus sequence RXRXX, while a second adjacent site at Ser^{146} is preceded by a highly conserved site, KXRXX. In addition, the second site at Ser^{146} is followed by a hydrophobic residue (Met^{147}), a type of residue commonly found in AKT/PKB phosphorylation sites. To examine whether AKT/PKB physically interacted with p21, coprecipitation studies (an important, although not necessary, component of AKT/PKB-mediated phosphorylation) were performed. Fig. 1B shows that p21 could be effectively coprecipitated with AKT/PKB, and mutation of the consensus phosphorylation sites to alanine did not appear to affect this molecular interaction. Examination of endogenous proteins also revealed that AKT/PKB and p21 interacted in vivo (Fig. 1C).

During the preparation of this manuscript, three groups reported that p21 is a phosphoprotein (33–35). In one study, it was shown that p21 isolated from actively growing baculovirus-infected insect cells was phosphorylated at Thr^{145} and Ser^{146} although the in vivo kinase responsible for this event was not identified (33). Two other groups demonstrated that the AKT/ PKB kinase phosphorylated the Thr^{145} residue (34, 35). In one case, it was shown that activation of the HER-2 receptor tyrosine kinase signaling pathway was involved with AKT/ PKB-mediated p21 phosphorylation, while in the other case, p21 was a substrate for AKT/ PKB phosphorylation in transfected cells. Interestingly, the second group demonstrated residual AKT/ PKB-dependent phosphorylation on p21 that was mutated to alanine at the Thr^{145} position, suggesting at least one other AKT/ PKB phosphorylation site in p21 (35). To examine whether the residual phosphorylation was at position Ser^{146}, transfection experiments were performed using p21 mutated at either or both of the potential AKT/ PKB phosphorylation sites. Fig. 1D demonstrates that p21 is indeed phosphorylated in cells transfected with either the highly oncogenic myristoylated form of AKT/ PKB (Mys-AKT) or with an epitope-tagged form of the cellular protein (V5-AKT). Fig. 1E shows that in vitro phosphorylation of bacterially produced p21 by purified AKT occurs on both the Thr^{145} and the Ser^{146} positions, with the Thr^{145} position showing a moderately higher efficiency of phosphorylation. These in vitro data were recapitulated in the transfection studies, where it was again demonstrated that both Thr^{145} and Ser^{146} served as substrates for AKT/ PKB phosphorylation (Fig. 1F). These results suggest that the residual phosphorylation that was previously observed in the Thr^{145} mutant is likely to be due to AKT/ PKB-mediated phosphorylation at the Ser^{146} residue.

AKT/ PKB Stabilizes p21 by Phosphorylation at Ser^{146}.—While examining the potential effects of AKT/ PKB on p21, we observed that elevated levels of p21 were found in cells cotransfected with a plasmid expressing the activated, but not kinase dead (KD) forms of AKT/ PKB (Fig. 2A). We further tested whether endogenous AKT/ PKB activity can increase the p21 protein level in vivo. As shown in Fig. 2B (left panel), the level of endogenous p21 protein in MCF7 cells was significantly higher in the presence of 10% serum when AKT/ PKB was fully active. The addition of LY 294002, a specific PI3 kinase inhibitor, reduced p21 protein level comparable with that under serum free conditions. In contrast, overexpressing active AKT/ PKB in the serum-starved MCF7 cells was able to restore the level of p21 protein (Fig. 2B, right panel).

The protein levels of p21 are known to be regulated by post-translational degradation as well as by transcriptional activation, although the exact mechanism of protein degradation is controversial (36). Interestingly, two previous studies demonstrated that the steady state levels of p21 appeared to be modulated by the PI3 kinase pathway (37, 38). To examine whether the elevated levels of p21 in AKT/ PKB transfected cells was due to protein stabilization, protein degradation studies were performed. Fig. 2C shows that p21 is rapidly degraded after the addition of cycloheximide in either nontransfected or AKT-KD transfected cells. In contrast to these results, p21 appears to be stabilized in cells expressing the oncogenic form of AKT/ PKB (Mys-AKT). These results suggested that phosphorylation of p21 by AKT/ PKB might affect the stability of the protein. To examine this question, we transfected either wild type or forms of p21 that were mutated at Thr^{145}, Ser^{146}, or both sites into cells together with AKT/ PKB and examined the stability of each protein. Fig. 2D shows that whereas the T145A mutant showed stability kinetics that were similar to wild type p21 (Fig. 2C), mutation at Ser^{146} resulted in a protein half-life that was indistinguishable from that observed in AKT/ PKB nontransfected cells or in cells transfected with KD AKT/ PKB. These results demonstrated that phosphorylation of p21 at Ser^{146} by AKT/ PKB was involved with protein stability. Finally, analysis of endogenous p21 protein stability in MCF7 cells demonstrated that expression of oncogenic AKT/ PKB resulted in an increase in protein stability (Fig. 2E). Together, these data suggested that one of the functions of AKT/ PKB is to phosphorylate p21 at position Ser^{146} and increase protein stability and the resultant steady state p21 levels.

p21 Stability Correlates with AKT Activation in Glioblastomas—Advanced stage tumors such as glioblastoma multiformes have been found to express elevated levels of p21 (29). These same tumor types have been shown to contain hyperactivated AKT/ PKB, the result of the loss of the PTEN tumor suppressor phosphatase, a protein that down-regulates AKT/ PKB activity (7, 8). To examine whether the levels of activated AKT/ PKB correlated with p21 stability, five different glioblastoma cell lines were examined. Fig. 3A shows that two of these lines, U-87 MG and U-118 MG, showed high levels of AKT/ PKB activation, whereas the three other lines showed little if any activation of the kinase. Examination of the stability of endogenous p21 revealed an exact correlation between p21 stability and AKT/ PKB activation. As shown in Fig. 3, B and C, p21 was much more stable in cell lines with higher levels of activated AKT/ PKB than in those lines with lower AKT/ PKB activation levels. Although this suggests that stability may play a major role in p21 levels, the results are made more complex by the fact that p21 levels are also transcriptionally modulated by p53 (26). Thus, the overall steady state levels of p21 in tumor cells (Fig. 3A) are achieved by a combination of both transcriptional regulation by p53 as well as by the induction of post-translational stability induced by AKT/ PKB phosphorylation. Because these glioblastoma cell lines are all actively dividing, these data also suggest that tumor cells are capable of cell cycle progression even in the presence of high levels of the cyclin kinase inhibitor p21.

AKT/ PKB Regulates Taxol-induced p21 Levels—Previous studies have demonstrated that taxol induces p21, and this induction is in part responsible for protecting cells from taxol-mediated cytotoxicity (39, 40). To examine whether AKT/ PKB was involved with the modulation of p21 levels in taxol-treated cells, the levels of the protein were examined in cells transfected with either the oncogenic form of AKT/ PKB or the kinase dead version of the enzyme, after which they were exposed to taxol for 48 h. Fig. 4A shows that, in nontransfected cells, the p21 levels are significantly increased after taxol treatment in the presence of serum. The withdrawal of serum eliminates the
FIG. 1. AKT/PKB interacts with p21 and phosphorylates it at Thr\(^{145}\) and Ser\(^{146}\). A, schematic diagram of the functional domains of p21. The potential AKT/PKB phosphorylation sites of p21 are compared with that of other known AKT/PKB substrates. The two overlapping sites are shown in red and green. B, AKT/PKB interacts with p21. 293E cells were transfected with wild type (WT) or KD mutant of Myc-tagged AKT/PKB and wild type or mutants (T145A, S146A, or T145A,S146A) of p21. The cell lysates were immunoprecipitated with anti-Flag antibody and then detected with anti-Myc antibody. IP, immunoprecipitate; WB, Western blot. C, endogenous interaction between AKT/PKB and p21 in MCF7 cells. Endogenous p21 was immunoprecipitated with a polyclonal antibody against p21 (Ab5) or with a control IgG. The bound AKT/PKB was detected with an anti-AKT/PKB antibody. D, AKT/PKB phosphorylates p21 in vitro. Myc-tagged Mys-AKT/PKB (constitutively active), V5-tagged wild type AKT/PKB, or Myc-tagged KD AKT/PKB was transfected into 293E cells. Flag-p21 was also overexpressed in 293E cells. 48 h after transfection, AKT/PKB immunoprecipitated with anti-Myc antibody or anti-V5 antibody was incubated with p21 immunoprecipitated with anti-Flag antibody in the kinase buffer containing 10 \(\mu\)Ci of \([\gamma-32P]\)ATP for 30 min at 30 °C. The kinase reaction was terminated with 10 mM EDTA and SDS-PAGE buffer, and the samples were assayed by autoradiography as shown in the top panel. E, AKT/PKB phosphorylates p21 at Thr\(^{145}\) and Ser\(^{146}\) in vitro. 2 \(\mu\)g of GST-fused wild type (WT) or mutant p21 purified from E. coli was incubated with 0.5 \(\mu\)g of active AKT/PKB in kinase buffer containing 10 \(\mu\)Ci of \([\gamma-32P]\)ATP for 30 min at 30 °C. The autoradiography of \(32P\)-labeled protein is shown in the upper panel, the Coomassie staining of GST-p21 proteins is shown in the lower panel; the histogram shows the radioactive counts (cpm) of the labeled GST-p21. F, AKT/PKB phosphorylates p21 at Thr\(^{145}\) and Ser\(^{146}\) in vivo. Flag-tagged p21 (wild type or mutants) was cotransfected with active AKT/PKB (Mys-AKT/PKB) or inactive AKT/PKB (AKT/PKB-KD) into 293 cells. 36 h after the transfection, cells were labeled with 1.5 mCi/ml of [\(^{32}\)P]orthophosphate for 4 h. p21 was immunoprecipitated with anti-Flag antibody and then analyzed by autoradiography or Western blotting. The histogram shows the ratio of the amount of \(32P\)-labeled p21 versus the total amount of p21 protein.
FIG. 2. AKT/PKB stabilizes p21 by phosphorylating it at Ser\textsuperscript{146}. A, AKT/PKB kinase activity increases the protein level of p21. 293E cells were transfected with Flag-tagged p21 and Myc-tagged active AKT/PKB (Mys-AKT/PKB), kinase dead AKT/PKB (AKT/PKB-KD), or control vector. As a control, cells transfected with control vector were also treated with 2 μM of MG-132, a proteasome inhibitor, for 16 h. 36 h after transfection, the protein levels of p21 were assayed by Western blotting with anti-Flag antibody. B, AKT/PKB kinase activity increases the protein level of endogenous p21 in MCF7 cells. MCF7 cells were grown in the absence of serum, or in the presence of 10% serum or 10% serum with 20 μM LY294002 for 24 h, and the protein levels of endogenous p21 were assayed by Western blotting as shown in the left panel. In the right panel, the protein levels of p21 were determined in stable MCF7 cells lines expressing control vector, Mys-AKT/PKB, or AKT/PKB-KD grown under serum-free conditions. C, AKT/PKB kinase activity stabilizes p21. 293E cells were transfected with Flag-tagged p21 and Myc-tagged active AKT/PKB (Mys-AKT/PKB), kinase dead AKT/PKB (AKT/PKB-KD) or control vector. 16 h after transfection, cells were treated with 20 μg/ml cycloheximide for 0, 0.5, 1, 2, 4, and 6 h. The degradation of p21 protein was followed by Western blotting with anti-Flag antibody. The graph shows the quantitation of the Western blot data. D, Ser\textsuperscript{146} is important for AKT/PKB stabilization of p21. 293E cells were transfected with Flag-tagged p21 mutants (T145A, S146A, or T145A,S146A) and Mys-AKT/PKB, AKT/PKB-KD, or control vector. The graph shows the stability of p21 proteins determined as in A. E, AKT/PKB stabilizes endogenous p21 in MCF7 cells. Stable MCF7 cell lines expressing control vector or Mys-AKT/PKB were treated with μg/ml cycloheximide for 0, 0.25, 0.5, 1, and 2 h. The protein level of p21 was assayed by Western blotting with anti-p21 antibody. The graph shows the quantitation of the Western blot data.
induction of p21 protein by taxol. The addition of LY 294002 (a specific PI3 kinase inhibitor) to the medium containing serum has the similar effect as serum starvation. Serum induces the PI3 kinase pathway with resultant activation of AKT/PKB, and these results suggest that AKT/PKB might be required for taxol induction of p21 protein levels. Indeed, when the onco-
genic form of AKT/PKB, a serum-independent kinase (41), was overexpressed in serum-starved cells, it restored the taxol-
induction of p21 protein levels. In contrast, the KD form of the enzyme, which can act as a dominant negative protein by
inhibiting the activation of endogenous AKT/PKB, blunts the taxol induced elevation of p21 in both the presence and absence
of serum.

Examination of the U-87 MG glioblastoma (Fig. 4B), which has a hyperactivated AKT/PKB, shows a clear induction of endogenous p21 by taxol treatment. The induction was blocked by treatment with LY 294002, a PI3 kinase inhibitor. Further-
more, in the other two glioblastoma cell lines (Fig. 4C), both of which have little or no AKT/PKB activation, taxol was not able
to increase the p21 protein level. Together, these data support the hypothesis that taxol-induced p21 elevation is, at least in part, due to the stabilizing effect of the AKT/PKB kinase.

Fig. 4. AKT/PKB is required for induction of p21 protein by
taxol treatment. A, Stable MCF7 cell lines expressing Mys-AKT/PKB,
AKT/PKB-KD, or control vector were treated with 100 nM taxol for 48 h
in the presence of 10% serum, 10% serum with 20 μM LY 294002, or
under serum-free condition. 50 μg of lysates were used to analyze the
protein expression of p21 and β-tubulin. B, glioblastoma cell line U-87
MG (AKT+/+) was treated with 100 nM taxol for 0, 24, 48, or 72 h in
the presence or absence of 20 μM LY 294002. The protein expression of
p21 and β-tubulin in 50 μg lysates were analyzed by Western blotting.
C, glioblastoma cell lines DBTRG-05MG, or A172 (AKT-) were treated
with 100 nM taxol for 48 h. The protein level of p21 and β-tubulin in 50
μg lysates were determined by Western blotting.
glioblastoma cells with low AKT/PKB activity (Fig. 5D), over-expression of p21 or oncogenic AKT/PKB results in a significant increase in cell survival upon taxol challenge. More importantly, much of the enhanced cell survival observed with activated AKT/PKB could be reversed by an antisense oligonucleotide directed against p21, suggesting that p21 is important for AKT/PKB dependent cell survival. Together, these data suggest that AKT/PKB executes its survival function through several substrates, AKT/PKB-mediated stabilization and resultant elevation of p21 levels are important for cell survival against taxol-induced apoptotic cell death.

**AKT/PKB Phosphorylation Blocks the Interaction between PCNA and p21**—p21 is known to interact with and inhibit the ability of PCNA to mediate DNA replication activity (13). In addition, the interaction site lies within the carboxyl terminus of the p21 protein (14), and structural analysis of the PCNA-p21 complex has revealed that the region encompassing the AKT/PKB phosphorylation sites, Thr<sup>145</sup> and Ser<sup>146</sup>, is essential for PCNA binding (14). Importantly, the p21 isolated from baculovirus-infected insect cells, which is phosphorylated at both of these residues, is unable to bind to PCNA in vitro (33).

Finally, transfection studies demonstrated that cotransfection of wild type p21 with activated AKT/PKB resulted in a block in PCNA interaction, whereas transfection of a nonphosphorylatable T<sup>145A</sup> mutant was still able to bind to PCNA in the presence of activated AKT/PKB (35). Fig. 6A confirms previous results by demonstrating that conversion of the Thr<sup>145</sup> residue to Glu, which mimics the negative charge of the phosphorylation event, inhibits the interaction between p21 and PCNA, whereas a similar mutation of Ser<sup>146</sup> has no such effect. We extend previous data by demonstrating that AKT/PKB indeed regulates the endogenous interaction between p21 and PCNA. Thus, Fig. 6B illustrates that activated AKT/PKB abolishes the interaction between these two endogenous proteins, whereas the KD version of the enzyme has no effect. These data suggest that the elevated levels of p21 in AKT/PKB up-regulated cells should not inhibit the ability of PCNA to mediate DNA replication.

**AKT/PKB Promotes the Assembly of Active Cyclin D1-CDK4-p21 Complex**—p21 is involved with the inhibition of the cyclin E-CDK2 complex as well as the assembly and activation of the cyclin D1-CDK4 complex (12). To examine how AKT/PKB mod-

**Fig. 5.** Up-regulation of p21 is important for AKT/PKB-dependent cell survival after taxol challenge. A, glioblastoma cell lines U-87 MG (AKT+++), and DBTRG-05MG (AKT++) were treated with various concentrations of taxol for 48 h. The percentage of apoptotic cells was determined by trypan blue staining as described under “Experimental Procedures.” B, antisense oligo of p21 sensitizes U-87 MG to taxol killing. U-87 MG was transfected with a p21 antisense oligo (250 nM) or a control oligo (250 nM) with scrambled sequence. 16 h after transfection, the cells were treated with 100 nM taxol. Cell viability was assayed 48 h after taxol challenge by trypan blue staining. C, antisense oligo of p21 blocks p21 induction by taxol. The protein expression of p21 in the cells treated in B was determined by Western blotting with anti-p21 antibody. D, determination of taxol-induced apoptosis in glioblastoma cell line DBTRG-05MG (AKT++) transfected with Flag-tagged p21, Myc-tagged Mys-AKT, Mys-AKT in the presence of p21 antisense oligo or Mys-AKT with control oligo.
FIG. 6. AKT/PKB blocks PCNA-p21 interaction by phosphorylating p21 at Thr\(^{145}\). A, mutation of Thr\(^{145}\) to Glu (TE) abolishes PCNA-p21 interaction. Flag-tagged wild type or mutant forms (T145A, T145E, S146A, S146D, Ala\(^{145}-146\), and Glu\(^{145}-\text{Asp}^{146}\)) of p21 were transfected into 293E cells. Cells were lysed after 48 h, and p21 was immunoprecipitated with Flag-antibody. p21-bound PCNA was detected with a mouse anti-PCNA antibody. B, AKT/PKB blocks the endogenous interaction between PCNA and p21. Stable MCF7 cell lines expressing Mys-AKT/PKB, AKT/PKB-KD, or control plasmid were lysed, and the endogenous p21 was immunoprecipitated with a rabbit anti-p21 antibody. The bound PCNA (marked with an arrow above the IgG heavy chain band) was detected with a mouse anti-PCNA antibody.

ulation of p21 levels might affect these various complexes, we analyzed their assembly in the presence of various forms of p21 and AKT/PKB. We first sought to determine whether phosphorylation of p21 at Thr\(^{145}\) or Ser\(^{146}\) might affect the assembly and inhibition of the CDK2 complex. Fig. 7 illustrates that either mutation of these two residues to Ala or to the phospho-like Glu or Asp did not affect either the binding to (Fig. 7A) or the inhibition of (Fig. 7B) the CDK2 complex. This result suggested that p21 phosphorylated by AKT/PKB was still capable of interacting with and inhibiting the CDK2 kinase and then possibly blocking the cell cycle progression. In tumor cells or stable MCF7 cells containing highly active AKT/PKB, however, cells are actively dividing even in the presence of high levels of p21, suggesting that the stabilized p21 in these cells does not act as a cell cycle inhibitor. Indeed, as shown in Fig. 7C, although overexpression of AKT/PKB in MCF7 cells elevates the level of p21 protein, it does not increase the amount of p21 protein bound with cyclin E-CDK2 complex nor does it inhibit the CDK2 kinase activity.

Because p21 can function not only as a potent inhibitor of cyclin E-CDK2 but also as a positive regulator of cyclin D-CDK4 kinase (12), we examined complex formation and activity of cyclin D1-CDK4 in the MCF7 cells with activated AKT/PKB. Fig. 7C illustrates that, besides up-regulating p21, the activated AKT/PKB increases the level of cyclin D1, whereas the KD form of the enzyme acts as a dominant negative inhibitor of steady state cyclin D1 levels. It has been known that AKT/PKB reduces the degradation of cyclin D1, probably through modulation of the GSK3\(^{\beta}\) kinase (12). The immunoprecipitation data in this figure demonstrated that, along with the elevated levels of p21, the assembly and activity of cyclin D1-CDK4 complex are enhanced in the presence of the activated AKT/PKB. In the presence of the KD AKT/PKB, however, the amounts of p21 and cyclin D1 associated with CDK4 as well as the kinase activity of this complex are significantly decreased. Our data are consistent with the notion that a noncatalytic function of cyclin D1 is to sequester free p21 away from cyclin E-CDK2 complex, and p21 promotes the assembly of active cyclin D1-CDK4 complex. Together, these data argue that the activated AKT/PKB kinase stabilizes both p21 and cyclin D1; the induced p21 protein does not inhibit cyclin E-CDK2 but binds with and activates cyclin D1-CDK4 complex.

**DISCUSSION**

Although p21 was initially thought of as an inhibitor of cell cycle progression, several recent studies have suggested that this protein might in fact be a positive modulator of cell survival and cell cycle progression. For example, several reports have now clearly demonstrated the involvement of this protein in the assembly and activation of the cyclin D1-CDK4 complex, a critical mediator of G\(_1\)-S progression induced by external mitogenic stimuli (16–18). In addition, a number of studies
have revealed the surprising result that many late stage, rapidly proliferating tumors show a profound elevation in the levels of p21, a result that is counterintuitive in light of the proposed inhibition of cell cycle progression mediated by this protein (18, 29–32). The pro-survival activity of p21 in cells treated with chemotherapeutic agents such as taxol also argues for a role for this protein in the protection of tumors in vivo (39, 40). This pro-survival effect is also observed in terminally differentiated cells such as myoblasts (19), hematopoietic stem cells (20), and macrophages (21), again consistent with a role for p21 as an anti-apoptotic protein. Here, we provide data suggesting that AKT/PKB, a known inhibitor of apoptosis in normal as well as transformed cells (42), appears to provide a mechanistic explanation for many of the observed effects of p21, especially in advanced tumors.

p21 elevation in tumors appears to correlate with enhanced survival and chemoresistance. For example, many late stage glioblastomas have elevated p21 levels (29), and these tumors are known to be extraordinarily resistant to chemotherapy and radiation (8). Importantly, the majority of glioblastoma multiformes contain the PTEN mutation, which is associated with a profound elevation in AKT/PKB kinase activity; it is these advanced tumors that show elevated p21 levels (8, 29, 43). Interestingly, elevated p21 levels in advanced gliomas do not appear to correlate with p53 levels, consistent with a p53-independent mechanism (29). Our analysis of glioblastoma cell lines reported here, although small, is also consistent with this hypothesis. Thus, elevated p21 levels were observed in p53+/+ cell lines with low AKT/PKB levels as well as in p53−/− cell lines with activated kinase levels, suggesting that the loss of p53 can be compensated for by the activation of the AKT/PKB kinase, presumably through the loss of PTEN. The observation of elevated p21 has been extended to a diversity of other tumors, including acute myelogenous leukemia (30) and head and neck (32) and ovarian carcinomas (31). Interestingly, high levels of p21 in acute myelogenous leukemia and head and neck carcinomas were associated with chemoresistance (30). Together, these data argue for a positive role for p21 in tumor cell survival, particularly in the presence of chemotherapeutic agents. Thus, stabilization of p21 by the AKT/PKB survival kinase would endow the tumor with additional protective measures.

A number of previous studies suggested that p21 levels were regulated by p53-independent mechanisms. Notably, many of these studies suggested that serum and other growth factors might be involved in the up-regulation of p21 levels in various cell types (44–46). One study demonstrated that epidermal growth factor, an activator of the PI3 kinase pathway, increased p21 levels by increasing the half-lives of both transcript as well as protein (47). Two recent important studies demonstrated that the PI3 kinase pathway, which is regulated by external growth factors and which up-regulates AKT/PKB kinase activity, was involved with the modulation of p21 levels (37, 38). In both cases, inhibitors of the PI3 kinase pathway, which would be expected to down-regulate AKT/PKB kinase activity, were found to cause a decrease in p21 levels. These earlier studies can now be explained by our observation that the stability of p21 is regulated by AKT/PKB-mediated phosphorylation at residue Ser146. We can only speculate on the mechanism by which this enhanced protein stability is attained. p21 was initially shown to be degraded by the proteasome pathway, and this degradation was assumed to be dependent upon ubiquitination of the protein (48). However, subsequent studies suggested that ubiquitination of p21 was not required for degradation by the proteasome (36). The carboxyl terminus of p21 seems to be essential for p21 protein degradation as deletion of the 24-amo acid peptide within this region stabilizes p21 protein (36). Because we have shown in this study that AKT/PKB phosphorylates this carboxyl-terminal peptide of p21, it might be possible that phosphorylation at residue Ser146 results in inhibited binding by a protein involved with p21 degradation. For example, p53 requires an interaction with the Mdm2 protein for degradation to occur, and phosphorylation of p53 by a diversity of kinases results in a loss of Mdm2 binding and resultant stabilization of p53 (49). The results reported here are consistent with a similar mechanism, although the nature of the potential binding partner is currently unknown.

How might the resultant elevation of p21 by activated AKT/PKB mediate an inhibition of taxol-induced apoptosis? It has been known that taxol blocks cells in G2/M phase and causes cell death through activation of p34cdc2 kinase (39, 50). A chemical inhibitor of p34cdc2 and a dominant-negative mutant of p34cdc2 blocks taxol-induced apoptosis (51, 52). Because p21 is a potent inhibitor of p34cdc2, it is reasonable to propose that p21 protects taxol-treated cells by blocking the activation of p34cdc2. Indeed, an increased level of p21 protein was observed after taxol treatment, and more importantly, high levels of p21 protein were found to be associated with inactive p34cdc2/cyclin B protein complex upon taxol challenge (39). Our current study shows the essential role of AKT/PKB in taxol-dependent p21 induction and demonstrates that p21 played a crucial role in mediating the survival function of AKT/PKB.

How could cells with elevated levels of p21 actively proliferate? The phosphorylation at Thr145 and Ser146 by AKT/PKB together may allow for this effect. p21 is known to be an inhibitor of PCNA-induced DNA synthesis, so that elevated levels of p21 might result in an inhibition of S phase progression (14, 15, 48). However, both others (33, 35) and we have now shown that the result of the phosphorylation of Thr145 is to release PCNA from p21 binding and resultant inhibition. This release allows for elevated p21 levels to exist in cells without inhibiting the cell’s ability to progress through S phase. Perhaps more important is the effect of this increased p21 on other aspects of the cell cycle. A number of previous studies have clearly demonstrated the ability of p21 to inhibit the activity of cyclin E-CDK2 complexes, and we have confirmed here that p21 mutated to mimic the phosphorylated form is capable of binding to and inhibiting CDK2 activity (12, 48). Several recent papers have challenged the notion that p21 is always an inhibitor of cyclin-dependent kinases (16, 17). These results support the hypothesis that elevated levels of p21 are involved with the assembly of active cyclin D1-CDK4 complexes. Importantly, we demonstrate here that AKT/PKB increases the level of cyclin D1 along with p21, and the induced p21 protein does not inhibit cyclin E-CDK2 but rather promotes the assembly and activation of cyclin D1-CDK4 complex. Because this cyclin D1-dependent CDK4 controls the progression through the G1-S interface activity (12), this would result in enhanced cell cycling, which would be beneficial for tumor growth. Finally, whereas previous data suggested that the AKT/PKB-mediated phosphorylation of p21 at Thr145 induced the cytoplasmic localization of the protein (34), another group (35) as well as ourselves (data not shown) have been unable to replicate these findings. In summary, our results provide for a plausible explanation for the up-regulation of p21 levels in many tumors, and they further provide a mechanistic explanation for this effect as well.

In closing, the results provided here, as well as in other recent publications, demonstrate that p21 is a functionally important substrate of the AKT/PKB kinase and reveal that phosphorylating and stabilizing p21 protein is a novel mechanism of AKT/PKB-mediated cell proliferation and survival.
data provide exciting new insights into the mechanisms that tumors utilize to survive under adverse conditions and further underscore the kinase activity of AKT/PKB as an excellent target for the development of chemotherapeutic drugs that induce cytotoxic sensitivity in advanced malignant tumors.

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