Phosphorylation-independent Stabilization of p27kip1 by the Phosphoinositide 3-Kinase Pathway in Glioblastoma Cells*

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The PTEN tumor suppressor gene is a frequent target of somatic mutation, particularly in glioblastoma multiform and prostate cancer. The expression of PTEN in PTEN-mutant glioblastoma cells leads to a cell cycle arrest in G0/G1 that is mediated at least partially by increased p27kip1 levels. Here we show that p27kip1 is not regulated by transcriptional control but that p27kip1 protein shows increased stability after inhibition of the phosphoinositide (PI) 3-kinase pathway. Because p27kip1 protein stability is known to be regulated by phosphorylation, we have examined modifications in the phosphorylation pattern after PI 3-kinase inhibition. Biochemical evidence suggests that p27kip1 is phosphorylated on several serine residues, including Ser-10 and Ser-178, but that phosphorylation is unaltered by PI 3-kinase activity. This is further confirmed by the inducible expression of p27kip1 phosphorylation site mutants, suggesting that p27kip1 is destabilized in a phosphorylation-independent manner by the PI 3-kinase pathway at the G1/S transition.

One of the most common mutations seen in glioblastoma multiform, the most frequent primary brain tumor, occurs in the tumor suppressor gene phosphatase and tensin homologue deleted on chromosome 10 (PTEN) (1). PTEN regulates the activity of the phosphoinositide (PI) 3-kinase pathway, a signaling cascade implicated in cancer development. PTEN dephosphorylates phosphatidylinositol 3,4,5-trisphosphate and phosphatidylinositol 3,4-bisphosphate, the lipid products of PI 3-kinase activity (2). We and other investigators have shown previously (3–5) that the introduction of wild type (WT) PTEN into PTEN-mutant glioblastoma cell lines decreases PI 3-kinase lipid products and the activity of a PI 3-kinase down-regulation factors, which are inactivated after the phosphorylation by Cyclin-Cdk2 complex, it can also act as a substrate. Cdk2-Cyclin E phosphorylates p27kip1 on Thr-187, which leads to the recruitment of the Skp2-containing Skp1-Cul1-Fbox protein complex (a ubiquitin-protein isopeptide ligase) resulting in p27kip1 ubiquitination and degradation by the proteasome (9–11). Therefore, only a small fraction of steady state p27kip1 would predictably be phosphorylated on Thr-187. The major phosphorylation site on p27kip1 is Ser-10, which leads to CRM1-dependent nuclear export (12) and increased protein stability in the cytosol (12, 13).

Recently, AKT has been shown to directly phosphorylate p27kip1 on Thr-157 in breast cancer cell lines, preventing nuclear import (14–16). It is not clear whether this also applies to other tumor types with high AKT activity or whether this regulation is specific for breast cancer. AKT may also phosphorylate Thr-198, promoting 14-3-3 association and cytoplasmic sequestration (17).

Finally, it has been demonstrated that Forkhead transcription factors, which are inactivated after the phosphorylation by AKT, can regulate p27kip1 transcription (8, 18). Nakamura et al. (19) have also proposed that Forkhead transcription factors increase p27kip1 levels by affecting primarily protein stability.

The importance of p27kip1 as an important mediator of PI 3-kinase-induced cell cycle progression is well established, but clearly the mechanism of regulation can be different in different cell types. We therefore explored the mechanisms underlying p27kip1 induction following inhibition of PI 3-kinase in PTEN-mutant glioblastoma cells.

EXPERIMENTAL PROCEDURES

Cell Culture—U87-MG glioblastoma cells were maintained in Dulbecco’s modified Eagle’s medium (Invitrogen) containing 10% fetal bovine serum and supplemented with antibiotics. When cells were to be infected with adenovirus, the medium was changed to 2% fetal bovine serum 1 h prior to infection.

Inducible Cell Lines—Ecdysone-inducible cell lines were established using the ecdysone-inducible mammalian expression system (Invitrogen). Hygromycin-resistant pInd expression vectors containing Myc-tagged p27kip1 and phosphorylation site mutants were transfected using FuGENE 6 (Roche Applied Science) into U87-MG cell lines expressing the ecdysone response receptor. Twenty single cell-derived independent drug-resistant colonies of each form of p27kip1 were cloned and screened for each gene. Exogenous expression levels of each of these genes was monitored by Western blotting, and the highest induced gene expression cell line was chosen for further study. Different ranges of ponasterone A (PonA) concentration were used to induce p27kip1 to different degrees in the different cell lines. The amounts varied from 0.3 to 10 M but were different in the cell lines in a manner that was independent of the generation of phosphorylation sites and were probably more related to clonal variability.

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1 The abbreviations used are: PI, phosphoinositide; CDK, Cyclin-dependent kinase; WT, wild type; PonA, ponasterone A; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid.
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Control and WT PTEN-expressing adenoviruses were made as described previously (7). U87-MG cells were infected routinely at a multiplicity of infection of 10, and cells were harvested 48 h after infection. The infection efficiency achieved by this procedure was >98% (data not shown). Quantitative Real Time PCR—RNA was purified by the RNeasy protocol (Qiagen), and first strand cDNA was synthesized using reverse transcriptase. p27kip1 RNA levels were analyzed by quantitative PCR (Taqman) using the oligonucleotides 5'-GTTAGGAGCAATGCG-3' and 5'-TCACAGAGGGCATTG-3' to amplify the p27kip1 cDNA and the fluorescently labeled internal oligonucleotide 5'-TCACAGAGGGCATTG-3'. After preincubation with 1 μM of anti-p27kip1 antibody (catalog no. SC-528, Santa Cruz Biotechnology) and 20 μL of protein G beads, the precipitate was washed six times in lysis buffer and separated on a 12% SDS gel. After transfer to an Immobilon-P membrane, p27kip1 was quantified using the STORM PhosphorImager. Equal loading was confirmed by immunoblotting for p27kip1.

Antisense Oligonucleotides, (32P)Orthophosphate Labeling, Phosphoamino Analysis, and Phosphopeptide Mapping—Cells were treated with p27kip1 antisense oligonucleotides as described previously (7). Subconfluent U87-MG cells were preincubated in phosphate-free medium for 1 h and labeled with 1 μCi/ml [32P]phosphoric acid for 6 h. Cells were lysed, and p27kip1 was immunoprecipitated as described for pulse-chase analyses.

One-third of the sample was separated on a 12% SDS gel, transferred to an Immobilon-P membrane, and immunoblotted for p27kip1. The membrane was exposed to the STORM PhosphorImager, and radiolabeled p27kip1 was quantified. The membrane containing p27kip1 was excised and subjected to partial hydrolysis in 6 N HCL for 60 min at 110 °C. Two-dimensional phosphoamino analysis was performed as described previously (22).

Two-thirds of the sample were separated on a 12% SDS gel and exposed to the STORM PhosphorImager. The band corresponding to p27kip1 was excised and digested with trypsin, and the phosphopeptides were separated by electrophoresis and thin layer chromatography (22).

Cell Cycle Analysis—U87-MG cells were trypsinized following 48 h of treatment with 20 μM LY294002 and fixed in 70% ethanol at 4 °C overnight. The ethanol-fixed cells were washed twice in phosphate-buffered saline containing 10 μg/ml propidium iodide (Roche Applied Sciences) and 1 μg/ml RNase A, incubated for 30 min at room temperature, and analyzed by fluorescence-activated cell sorting. To aid in the visualization of the effects on the cell cycle, one-half of the samples were treated with nocodazole (70 ng/ml) for 18 h prior to trypsinization to induce an arrest in G2, as described previously (23).

RESULTS

Inhibition of the PI 3-Kinase Pathway by PTEN or LY294002 Leads to p27kip1 Stabilization—The introduction of PTEN leads to a G1 arrest that is mediated by p27kip1 (7). Using an adenovirus expression system >98% of cells were green fluorescent protein-positive by fluorescence microscopy and fluorescence-activated cell sorting, indicating adenoviral gene transduction into >98% of cells (data not shown). We (7) and other investigators (4, 6, 24) have shown previously that p27kip1 levels accumulate in U87-MG cells after the expression of WT PTEN or treatment with LY294002 (Fig. 1A). This accumulation was not caused by transcriptional regulation because quantitative PCR (Fig. 1A) or Northern blotting (data not shown) showed no significant differences in p27kip1 mRNA levels. We therefore examined whether changes in p27kip1 stability could underlie the increased levels seen following the inhibition of PI 3-kinase activity. As depicted in Fig. 1B, adding cycloheximide to prevent protein synthesis resulted in the decay of p27kip1 levels in untreated cells, with a half-life of ~2 h. In contrast, when PI 3-kinase was inhibited by either infection with PTEN adenovirus or treatment with LY294002, cycloheximide addition did not result in a rapid loss of p27kip1 protein. In the inhibited cells, the p27kip1 half-life was greater than the duration of the experiment, i.e. ~8 h. In contrast, the stability of two additional cell cycle proteins, p21kip1 and Cyclin D3, was not affected by PI 3-kinase inhibition (Fig. 1B). p27kip1 stabilization by PI 3-kinase inhibition was independent of AKT and mTOR, because the overexpression of dominant-negative (kinase-dead) AKT or treatment with rapamycin did not dramatically affect p27kip1 stability (Fig. 1C). Increased p27kip1 stability following the inhibition of PI 3-kinase was further confirmed by 32P-SIMet/Cys pulse-chase experiments (Fig. 1D). The half-life of endogenous p27kip1 determined by these experiments was 1.5–2 h, which increased to 4–6 h in the presence of LY294002 or PTEN adenovirus. This is slightly less than the half-life determined by cycloheximide treatment, suggesting that proteins required for p27kip1 degradation may also have a short half-life.

p27kip1 Phosphorylation Pattern or Overall Charge Is Unaltered by PTEN Expression or Treatment with LY294002—Because p27kip1 stability could be regulated by phosphorylation,
we tested whether the inhibition of PI 3-kinase altered p27kip1 stability by changing its phosphorylation state. Immunoprecipitation from [32P]orthophosphate-labeled U87-MG cells demonstrated that endogenous p27kip1 was a phosphoprotein under these conditions (Fig. 2A). Endogenous phosphorylated Cdk2, the identity of which was confirmed by Western blot analysis (data not shown), was also co-immunoprecipitated under these circumstances. However, the stoichiometry of
Phosphorylation of individual p27kip1 phosphorylation sites was not affected significantly by PI 3-kinase activity. In a complementary approach that was applied to analyze the phosphorylation status of p27kip1, as well as to analyze additional post-translational modifications, we performed two-dimensional gel and Western blot analyses. After treatment of U87-MG cells with LY294002 or PTEN, protein lysates were separated by isoelectric focusing followed by SDS-PAGE and blotted for p27kip1. This analysis revealed two major and three minor differently charged forms of p27kip1, which also did not consistently change following PI 3-kinase inhibition (Fig. 2C).

Inducible Expression of WT p27kip1 and Phosphorylation Site Mutants Are Stabilized after Inhibition of the PI 3-Kinase Pathway—Even though the phosphorylation state of p27kip1 did not change following PI 3-kinase inhibition, we reasoned that...
the regulation of Thr-187 phosphorylation could still be controlling p27kip1 stability but that its phosphorylation would cause immediate degradation and therefore would not be detected. We therefore generated U87-MG cell lines that expressed inducible forms of WT Myc-tagged p27kip1 and phosphorylation site mutant derivatives. As depicted in Fig. 3A, left panel, increasing doses of PonA resulted in a dose-dependent increase in WT p27kip1 levels. The inhibition of PI 3-kinase caused increased levels of these exogenously expressed proteins, consistent with their stabilization following the inhibition of this pathway (Fig. 3A, right panel). The p27kip1 proteins were functional, as judged by their ability to inhibit Cdk2 kinase activity (Fig. 3A, bottom left panel), and caused a cell cycle arrest in G1 (Fig. 3B).

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As shown in Fig. 3A, the inducible expression of p27kip1 affected endogenous levels of p27kip1. We suspected that the exogenous expression of p27kip1 would inhibit Cdk2 (as shown in Fig. 3A), leading to a perturbation of the balance between p27kip1 on one hand and Cyclin-Cdk2 on the other. To confirm
that the effects of LY294002 on exogenous \( p27^{kip1} \) were direct and did not result from decreased Cdk2 activity following increased levels of endogenous \( p27^{kip1} \), we utilized \( p27^{kip1} \) antisense oligonucleotides. These can eliminate endogenous \( p27^{kip1} \) but not affect the levels of exogenous \( p27^{kip1} \) because of the introduction of silent mutations in the oligonucleotide binding site. As can be seen in Fig. 4B, the exogenous \( p27^{kip1} \) was stabilized independently of the presence of endogenous \( p27^{kip1} \) after treatment with LY294002. This showed that the titration of inducible WT \( p27^{kip1} \) at physiological levels behaved similarly to endogenous \( p27^{kip1} \).

To gain further insight into the phosphopeptide maps of the endogenous \( p27^{kip1} \) shown in Fig. 2 and to identify which spots corresponded to which sites, we performed two-dimensional phosphopeptide mapping experiments on the exogenous \( p27^{kip1} \) phosphorylation site mutants. Fig. 5A shows the phosphorylation of exogenous \( p27^{kip1} \) was almost identical to that seen for the endogenous \( p27^{kip1} \), with a prominence of spots 1–4 in both cases. Treatment with LY294002 also did not dramatically change the pattern of phosphorylation of the exogenous \( p27^{kip1} \) (Fig. 5A), confirming the results seen with endogenous \( p27^{kip1} \). The phosphorylation site represented by spot 3 did increase slightly following treatment with LY294002. However, because this difference was not apparent in endogenous \( p27^{kip1} \) (Fig. 2B), we are unsure of its importance. Use of the T187A mutant of \( p27^{kip1} \) (Fig. 5B) showed that phosphorylation at Thr-187 was barely detectable, consistent with the results of the phosphoamino acid analysis of endogenous \( p27^{kip1} \). We do not believe that spot 5 represents this site, because its appearance was somewhat variable and seemed to depend on the running conditions of the thin layer chromatography plate. The use of the S10A mutant (Fig. 5, B and C) showed that most of the phosphorylation of \( p27^{kip1} \) occurred on this site and that mutation at this site caused the disappearance of spots 1 and 2. Further mutation of S178A also caused the disappearance of spot 4, suggesting that this residue was also phosphorylated in U87-MG cells under these conditions. There was still one remaining spot present even in the S10A/S178A/T187A triple mutant, suggesting that a further phosphorylation site accounted for approximately 10–15% of \( p27^{kip1} \) phosphorylation. As revealed by phosphoamino acid analysis, this residue was serine-phosphorylated (data not shown) (Fig. 2B).

**DISCUSSION**

Our data showed that \( p27^{kip1} \) protein accumulated after the re-expression of WT PTEN or treatment with LY294002 in PTEN-mutant U87-MG cells. We have shown previously that \( p27^{kip1} \) is important for the PTEN-mediated G1 arrest (7). Transcriptional control of \( p27^{kip1} \) by the PI 3-kinase pathway has been described previously, indicating that activated AKT phosphorylates Foxo transcription factors, causing cytosolic accumulation and thereby preventing Foxo-dependent transcription of \( p27^{kip1} \) mRNA (8). Although AKT was highly activated in U87-MG cells because of the mutation of PTEN, \( p27^{kip1} \) mRNA was readily detectable, and mRNA levels were not affected by the inhibition of the pathway (Fig. 1A). The lack of transcriptional regulation in glioma cells has been described...
previously by us (7) and other investigators (24). Also, p27kip1 protein levels remained constant despite the expression of dominant-negative (kinase-dead) AKT in U87-MG cells (Fig. 1C) (data not shown). Furthermore, the p27kip1 expressed from an exogenous promoter was stabilized after PI 3-kinase inhibition (Fig. 3A), showing the independence from the endogenous promoter. In summary, this suggested that p27kip1 was not transcriptionally regulated by the PI 3-kinase pathway, at least in U87-MG cells.

Translational control of p27kip1 has been observed in normal and tumor cells (25–27). p27kip1 mRNA has also been shown to be translated in a cap-independent manner, accounting for the increased expression of p27kip1 in cells exhibiting decreased cap-dependent translation (28). However, two pieces of evidence argue against a strong contribution of translational control in regulating p27kip1 levels in U87-MG cells. First, rapamycin, which decreases cap-dependent translation, had little effect on endogenous or exogenous p27kip1 levels (Fig. 1C) (data not shown). Second, exogenously expressed p27kip1 lacking the 5'- and 3'-untranslated regions that are normally responsible for translational control was stabilized by PI 3-kinase inhibition in our inducible cell system (Figs. 3A and 4A). However, we cannot exclude some role for translational regulation of WT and phosphorylation site mutants of p27kip1 in our experiments.

Rather, the stability of p27kip1 protein was affected strongly by PI 3-kinase activity (Figs. 1, B and C, and 4A). Surprising to us, we demonstrated that stabilization does not alter any detectable phosphorylation changes or any other post-translational modification that might affect the overall charge of p27kip1 protein (Fig. 2). Mamillapalli et al. (24) have proposed that the restoration of PTEN in PTEN-mutant cells leads to transcriptional down-regulation of Skp2, thereby preventing degradation of p27kip1, which accumulates as a result. Although it is an attractive hypothesis, such a mechanism would result in the accumulation of p27kip1 phosphorylated on Thr-187, which was not observed in U87-MG cells (Fig. 2B). Moreover, the p27kip1 T187A mutant was stabilized equally after inhibition of the pathway. Likewise, the major phosphorylation site on p27kip1, Ser-10, was unaffected by the inhibition of the PI 3-kinase pathway, and mutants at this site responded normally to PI 3-kinase inhibition.

Other investigators have proposed Thr-157 phosphorylation of p27kip1 by AKT as a means of localizing p27kip1 to the...
cytoplastm by preventing nuclear import (14–16). This mechanism has been reported exclusively in breast cancer cells, and it is not known at this time whether this tissue displays a unique mechanism for p27kip1 regulation by the PI 3-kinase pathway. In our studies, p27kip1 was localized primarily to the cytosol, and this localization did not change in response to PI 3-kinase activity (data not shown). In addition, threonine phosphorylation would have been readily detectable in phosphate labeling and phosphoamino acid analysis experiments.

Recently, a second proteolytic pathway for controlling p27kip1 has been described by generating an elegant p27-T187A mouse knock-in model (29). Whereas the S-phase degradation of p27kip1 is Thr-187- and Skp2-dependent, the degradation of p27kip1 at the G1 restriction point is independent of these. Instead, it is controlled by mitogen stimulation, because the addition of serum leads to the degradation of both wild type p27kip1 and the Thr-187 mutant (29, 30). Given that serum and mitogens stimulate the PI 3-kinase pathway and that we observed a G1 arrest in cells after the re-expression of PTEN in PTEN-mutant glioblastoma cells, it is tempting to speculate that this G1-specific proteolytic pathway of p27kip1 would be dependent on PTEN, independent of AKT.

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