Role of serine-10 phosphorylation in p27 stabilization revealed by analysis of p27 knock-in mice harboring a serine-10 mutation

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Running title: Analysis of p27(S10A) knock-in mice
SUMMARY

The inhibition of cyclin-dependent kinase activity by p27 contributes to regulation of cell cycle progression. Serine-10 is the major phosphorylation site of p27, and its phosphorylation has been shown to affect the stability and nuclear export of p27 at the G₀-G₁ transition in transfected cultured cells. To investigate the physiological relevance of p27 phosphorylation on Ser¹⁰, we generated p27 "knock-in" mice that harbor a Ser¹⁰-to-Ala (S10A) mutation in this protein. Mice homozygous for the mutation (p27S¹⁰A/S¹⁰A mice) were normal in body size, but the abundance of p27 was decreased in many organs, including brain, thymus, spleen, and testis. The stability of p27 in G₀ phase was markedly reduced in lymphocytes of p27S¹⁰A/S¹⁰A mice compared with that in wild-type cells, whereas p27 stability in S phase was similar in cells of the two genotypes. The degradation of p27 in cells of the mutant mice at G₀ phase was prevented by a proteasome inhibitor. These data indicate that the physiological role of p27 phosphorylation on Ser¹⁰ is to stabilize the protein in G₀ phase. Unexpectedly, the nuclear export of p27 at the G₀-G₁ transition occurred normally in p27S¹⁰A/S¹⁰A mouse embryonic fibroblasts, indicating that phosphorylation of Ser¹⁰ is dispensable for this process.
INTRODUCTION

Progression of the cell cycle in eukaryotic cells is regulated by a series of protein complexes composed of cyclins and cyclin-dependent kinases (CDKs),\(^1\) the activity of which is in turn controlled by a group of CDK inhibitors (CKIs) (1,2). Among these CKIs, p27 plays a pivotal role in the control of cell proliferation (3,4). In normal cells, the amount of p27 is high during G\(_0\) phase of the cell cycle, but it decreases rapidly on the mitogen-induced reentry of cells into G\(_1\) phase (5,6). Forced expression of p27 results in cell cycle arrest in G\(_1\) phase (3,4), and, conversely, inhibition of p27 expression by antisense oligonucleotides increases the proportion of cells in S phase (7). Moreover, we and others have shown that mice with a homozygous deletion of the p27 gene (p27) are larger than normal mice and exhibit both multiple organ hyperplasia as well as a predisposition to the development of spontaneous and radiation- or chemical-induced tumors (8-11). These observations support the notion that p27 is a key determinant of both body size and organ size as a result of its role in the control of cell proliferation and that the loss of p27 function may lead to carcinogenesis. Indeed, many studies have shown that the expression of p27 is deregulated in various human cancers (12).

The abundance of p27 is thought to be controlled by multiple mechanisms that operate at the level of the synthesis (transcription and translation) (13,14), proteolysis (15,16), and localization of this protein (17). The proteolysis of p27 is mediated
predominantly by the ubiquitin-proteasome pathway (15). The protein is phosphorylated on Thr^{187} by the cyclin E–CDK2 complex (18,19), and the phosphorylation of this residue is required for binding of p27 to Skp2, an F-box protein that is thought to function as the receptor component of an SCF ubiquitin ligase complex; such binding then results in the ubiquitylation and degradation of p27 (20-25). We have shown that the degradation of p27 at the G_0-G_1 transition is independent of Skp2, however, and occurs in the cytoplasm, whereas the Skp2- and Thr^{187} phosphorylation–dependent degradation of p27 occurs at S and G_2 phases in the nucleus (26). These observations suggest that the nuclear export of p27 may be critical for its down-regulation early during reentry of quiescent cells into the cell cycle.

We previously identified Ser^{10} as the major phosphorylation site of p27, showing that it accounts for ~70% of the total phosphorylation of this protein; the extent of phosphorylation at this site was 75 times that at Thr^{187} (27). The extent of Ser^{10} phosphorylation is markedly increased in cells in G_0 phase of the cell cycle compared with that apparent for cells in S or M phase. Mutational analysis suggested that phosphorylation of Ser^{10}, like that of Thr^{187}, contributes to regulation of p27 stability. The p27 protein is translocated from the nucleus to the cytoplasm at the G_0-G_1 transition of the cell cycle. We and others showed that substitution of Ser^{10} with Ala (S10A) markedly reduced the extent of p27 export from the nucleus at the G_0-G_1 transition. Furthermore, phosphorylation on Ser^{10} is required for the binding of p27 to CRM1, a carrier protein for nuclear export (28,29). These observations suggest that
phosphorylation on Ser\(^{10}\) is essential for nuclear export of p27. Residues 32 to 45 of p27 constitute a putative nuclear export sequence, the mutation of which reduces both the interaction of p27 with CRM1 as well as the nuclear export and degradation of p27 (30). Human kinase-interacting stathmin (hKIS) has been identified as a nuclear serine-threonine kinase that binds to the COOH-terminal region of p27 and phosphorylates p27 on Ser\(^{10}\) both in vitro and in vivo, thereby promoting its export from the nucleus to the cytoplasm (31).

Most studies that have evaluated the effect of Ser\(^{10}\) phosphorylation on p27 function have been based on overexpression of exogenous mutant proteins in cultured cell lines. Under such conditions, however, it is difficult to exclude the possibility that physiologically irrelevant levels of p27 inhibit cell cycle progression, resulting in apparent inhibition of the nuclear export of p27. It is therefore important that the effects of mutant proteins expressed under the control of the endogenous promoter of p27 be examined. To this end, we have adopted a "knock-in" strategy to express the S10A mutant of p27 in mice. We now show that the stability of the mutant protein is markedly reduced in certain cell types of \(p27^{S10A/S10A}\) mice at G\(_{0}\) phase. Unexpectedly, the export of p27 from the nucleus at the G\(_{0}\)-G\(_{1}\) transition appeared to be unaffected by the S10A mutation. Our data suggest that phosphorylation of Ser\(^{10}\) is important for p27 stability in G\(_{0}\) phase but is not required for nuclear export of this CKI at the G\(_{0}\)-G\(_{1}\) transition.
EXPERIMENTAL PROCEDURES

Generation of p27^{S10A/S10A} knock-in mice

We isolated p27 from a 129/Sv mouse genomic library as described previously (10). The codon for Ser^{10} in exon 1 was mutated to an alanine codon (GGG AGC → GGC GCC) to create the p27^{S10A} allele. The targeting vector was constructed by insertion of a loxP-flanked PGK-neo-poly(A) cassette into the HindIII site of intron 1 of p27 and ligation of a PGK-TK-poly(A) cassette at the 5' end of the insert (Fig. 1A). The maintenance, transfection, and selection of mouse embryonic stem (ES) cells were performed as described (10). The loxP-flanked PGK-neo-poly(A) cassette was deleted in transfected ES cells by infection with an adenovirus encoding Cre recombinase. For detection of mutated ES clones, genomic DNA was digested with BamHI and subjected to Southern hybridization with a 0.9-kb NdeI-EcoRI probe (Fig. 1A). The mutant ES cells were microinjected into C57BL/6 blastocysts, and the resulting male chimeras were mated with female C57BL/6 mice. Heterozygous offspring were intercrossed to produce homozygous mutant animals. All mice were maintained in a specific pathogen–free animal facility.

Immunoblot analysis

Tissues from p27^{+/+} or p27^{S10A/S10A} mice were homogenized in homogenization buffer [50 mM Tris-HCl (pH 7.5), 0.25 M sucrose, 1 mM EDTA] supplemented with 0.4 mM
Na$_3$VO$_4$, 0.4 mM EDTA, 10 mM NaF, 10 mM sodium pyrophosphate, as well as antipain, pepstatin, chymostatin, leupeptin, and phenylmethylsulfonyl fluoride, each at a concentration of 10 $\mu$g/ml. The resulting homogenates as well as cultured primary lymphocytes or mouse embryonic fibroblasts (MEFs) were lysed in radioimmunoprecipitation assay buffer supplemented as for the homogenization buffer. Lysates were incubated on ice for 15 min and then centrifuged at 20,000 $\times$ g for 15 min at 4°C; after determination of its protein concentration with the Bradford assay (Bio-Rad), the resulting supernatant (50 $\mu$g of protein) of each lysate was subjected to SDS–polyacrylamide gel electrophoresis (PAGE). The separated proteins were transferred to a Hybond P membrane (Amersham Biosciences) and subjected to immunoblot analysis with antibodies (1 $\mu$g/ml) to p27 (Transduction Laboratories) or to glycogen synthase kinase–3β (GSK-3β) (Transduction Laboratories). A rabbit polyclonal antibody specific for p27 phosphorylated on Ser$^{10}$ (Zymed) could not be used because it reacted with p27(S10A) (Supplementary Information, Fig. S1). Immune complexes were detected with appropriate horseradish peroxidase–conjugated secondary antibodies and SuperSignal West Pico chemiluminescence reagents (Pierce). Band intensity was measured with an LAS-1000 chemiluminescence imager (FujiFilm).

**Cell culture**

Single-cell suspensions of lymphocytes were prepared from the lymph nodes of
p27+/+ or p27^S10A/S10A^ mice and cultured (1.0 \times 10^7 cells in 5 ml) in RPMI 1640 medium supplemented with 10% fetal bovine serum; the cells were exposed to 10 nM phorbol 12,13-dibutyrate (PDBu) (Sigma), 300 nM ionomycin (Sigma), 10 \mu M MG132 (Peptide Institute), cycloheximide (10 \mu g/ml), or vehicle (dimethyl sulfoxide), as indicated. Primary MEFs were isolated on embryonic day 13.5 and cultured as described (10). For analysis of synchronized cells, MEFs were arrested at G_0 phase by serum deprivation (incubation in medium supplemented with 0.1% fetal bovine serum) for 96 h; they were then cultured for the indicated times in medium containing 20% serum.

**Cell cycle analysis by flow cytometry**

Lymphocytes cultured as described above were exposed to 10 \mu M bromodeoxyuridine (BrdU) (Sigma) for 30 min, harvested, fixed overnight in 70% ethanol at -20 °C, and denatured for 30 min at room temperature in 2 M HCl containing 0.5% Triton X-100. After neutralization with borax buffer (pH 8.5), the cells were subjected to dual-color staining with fluorescein isothiocyanate (FITC)–conjugated antibodies to BrdU (Becton Dickinson) and propidium iodide (5 \mu g/ml). They were then analyzed with a FACSCalibur flow cytometer and Cell Quest software (Becton Dickinson).

**Two-dimensional PAGE**
Lymphocytes, MEFs, and tissues were lysed in a solution containing 8 M urea, 2% Triton X-100, 65 mM dithiothreitol, and 0.2% Ampholine (pH 3.5 to 9.5) (Amersham Pharmacia Biotech). Lysates containing 50 to 150 μg of protein were applied to an Immobiline DryStrip (13 cm, pH 4 to 7) (Amersham Pharmacia Biotech) and subjected to isoelectric focusing for 12 h at 0 V, 1 h at 500 V, 1 h at 1000 V, and 2 h at 8000 V. The DryStrip was then equilibrated with a solution containing 50 mM Tris-HCl (pH 6.8), 6 M urea, 30% glycerol, 2% SDS, and 65 mM dithiothreitol before the separated proteins were resolved in the second dimension by standard PAGE on an 11% gel and subjected to immunoblot analysis.

**32P- and 35S-labeling of p27 in vivo**

Freshly isolated lymphocytes were incubated for 2 h in phosphate-free RPMI 1640 medium supplemented with dialyzed fetal bovine serum and then metabolically labeled with [32P]orthophosphate (Amersham Pharmacia Biotech) at a concentration of 1 mCi/ml for 8 h at 37°C in the same medium with or without 10 nM PDBu and 300 nM ionomycin. Alternatively, the cells were incubated for 8 h with or without PDBu and ionomycin and labeled with [35S]methionine and [35S]cysteine (Amersham Pharmacia Biotech) at a concentration of 80 mCi/ml for the final 1 h. After extensive washing with radioisotope-free medium, the cells were lysed and subjected to immunoprecipitation with antibodies to p27. The resulting precipitates were fractionated by SDS-PAGE and subjected to autoradiography with a BAS-2000 image analyzer (FujiFilm).
**Immunofluorescence analysis of p27 expression**

MEFs were grown on glass cover slips and subjected to immunofluorescence staining as described previously (32). Endogenous p27 was stained with a monoclonal antibody to p27 (Transduction Laboratories) and immune complexes were detected with Alexa488-conjugated goat antibodies to mouse immunoglobulin G (Molecular Probes). Nuclei were stained with Hoechst 33258 dye.

Lymphocytes were plated on glass cover slips precoated with poly-L-lysine (200 μg/ml) and were then subjected to immunofluorescence staining as described previously (32). Endogenous p27 was stained with polyclonal antibodies to p27 (C-19 and N-20, Santa Cruz Biotechnology; Ab-2, Neomarkers) and immune complexes were detected with Alexa488-conjugated goat antibodies to rabbit immunoglobulin G (Molecular Probes). Cell nuclei were stained with propidium iodide. Confocal fluorescence images were obtained with a Radiance2000 microscope (Bio-Rad).
RESULTS

Introduction of an S10A mutation into mouse p27

We designed a targeting construct to replace the wild-type p27 allele with an allele in which the codon for Ser$^{10}$ was changed to an alanine codon (Fig. 1A). The linearized targeting vector was introduced into mouse ES cells by electroporation, and transfectants selected on the basis of their ability to grow in the presence of both G418 and gancyclovir were screened for homologous recombination events by the polymerase chain reaction and Southern blot analysis (data not shown). The loxP-flanked neomycin resistance cassette was then deleted by infection of selected ES cells with an adenovirus vector encoding Cre recombinase, after which the mutant p27 allele was amplified by the polymerase chain reaction and confirmed by DNA sequencing (data not shown). The targeted ES cells were then injected into C57BL/6 blastocysts, and chimeric male mice that transmitted the mutant allele in the germ line were obtained. Heterozygotes (p27$^{+/S10A}$) were bred to produce p27$^{S10A/S10A}$ mice, which were identified by Southern blot analysis of tail DNA (Fig. 1B).

Phenotype of p27$^{S10A/S10A}$ knock-in mice

Heterozygote matings yielded wild-type (p27$^{+/}$), heterozygous (p27$^{+/S10A}$), and homozygous mutant (p27$^{S10A/S10A}$) offspring in a ratio similar to that expected for Mendelian inheritance, indicative of no substantial embryonic lethality of the mutant
allele. We have previously shown that p27−/− mice are larger than normal mice and exhibit multiple organ hyperplasia (10). In contrast, the body size (Fig. 1C) and organ size (data not shown) of p27S10A/S10A mice appeared normal. The increase in body weight with age also did not differ markedly among p27+/+, p27+/S10A, and p27S10A/S10A mice (Fig. 1D). This difference in phenotype between p27−/− and p27S10A/S10A mice suggests that the S10A mutant of p27 is not functionally null. Consistent with this notion, we previously showed that mutation of Ser10 in p27 does not affect the CDK-inhibitory function of this protein (27).

We next examined the abundance of p27 in various organs by immunoblot analysis. The amount of p27(S10A) was reduced in the brain, thymus, spleen, and testis of p27S10A/S10A mice compared with that of the wild-type protein in p27+/+ animals, whereas the expression of p27 in liver, heart, lung, and skeletal muscle was not affected by the mutation (Fig. 2A, B). For all organs examined, the abundance of p27 mRNA was similar in p27S10A/S10A and p27+/+ mice (data not shown). Two-dimensional PAGE revealed that the proportion of p27 molecules that were phosphorylated in various organs of wild-type mice varied from 30 to 70% (Fig. 2C) and was not correlated with the extent of p27 down-regulation apparent in the corresponding organs of p27S10A/S10A mice. No signal corresponding to phosphorylated p27 was detected in thymus or lung of p27S10A/S10A mice (Fig. 2C), indicating that the signal observed in wild-type animals was attributable to phosphorylation of p27 on Ser10. These data suggest that phosphorylation of Ser10 affects the abundance of p27 in
some, but not all, organs.

**Cell cycle–dependent phosphorylation of p27 on Ser^{10} in primary lymphocytes**

We have previously shown that p27 is phosphorylated on Ser^{10} at G_0-G_1 phase of the cell cycle in cultured HeLa and NIH 3T3 cells, whereas the extent of this phosphorylation is markedly reduced in cells in S and M phases (27,29). We examined the phosphorylation status of Ser^{10} of p27 in primary lymphocytes isolated from wild-type mice. Almost all mature lymphocytes are in G_0 phase in the absence of mitogenic stimulation (Fig. 3A). Stimulation of the cells with the combination of PDBu and ionomycin for 12 h (G_1 phase) or 24 h (S phase) resulted in a substantial reduction in the abundance of p27 (Fig. 3B). We evaluated the relative amounts of phosphorylated and nonphosphorylated forms of p27 in lymphocytes and MEFs by two-dimensional PAGE (Fig. 3C, D). Similar to our previous observations with HeLa and NIH 3T3 cells (27,29), about half of p27 molecules were phosphorylated in wild-type lymphocytes at G_0 phase, whereas no such phosphorylation was detected in p27^{S10A/S10A} lymphocytes (Fig. 3C). These data suggest that most phosphorylation of p27 at G_0 phase is attributable to Ser^{10}. Only ~8% of p27 molecules were phosphorylated in wild-type lymphocytes at S phase, suggesting that the phosphorylated molecules present in G_0 phase had been dephosphorylated or selectively degraded. We obtained similar results with MEFs (Fig. 3D).

The detection of marked phosphorylation of p27 on Ser^{10} at G_0 phase does not
necessarily indicate that p27 undergoes phosphorylation during G0 phase. It might become phosphorylated before the entry of cells into G0 phase. To test this hypothesis, we labeled lymphocytes freshly isolated from wild-type or p27\textsuperscript{S10A/S10A} mice with \(^{32}\text{P}\)orthophosphate for 8 h in the absence or presence of mitogenic stimulation (Fig. 3E). In the absence of stimulation (G0 phase), neither wild-type nor p27\textsuperscript{S10A/S10A} cells incorporated \(^{32}\text{P}\)orthophosphate into p27. In contrast, p27 present in both types of cells was labeled with \(^{32}\text{P}\)orthophosphate in response to mitogenic stimulation, although the extent of labeling was greater for wild-type cells than for p27\textsuperscript{S10A/S10A} cells. To exclude the possibility that the resting lymphocytes were metabolically inactive, the cells from wild-type mice were labeled with \(^{35}\text{S}\)methionine and \(^{35}\text{S}\)cysteine. Lymphocytes in G0 or G1 phases incorporated the radiolabeled amino acids to similar extents. These data suggest that p27 undergoes phosphorylation on Ser\textsuperscript{10} not during G0 phase but during the cell cycle before entry of cells into G0 phase.

**Destabilization of p27 by the S10A mutation specifically at G0 phase**

The predominance of the Ser\textsuperscript{10}-phosphorylated form of p27 in G0 phase suggested that phosphorylation of this residue might be responsible for the stabilization of p27 apparent in quiescent cells. To test this hypothesis, we measured, as described previously (33), the half-life of p27 in lymphocytes isolated from wild-type or p27\textsuperscript{S10A/S10A} mice. In G0 phase, whereas wild-type p27 was stable, p27(S10A) was
markedly unstable (Fig. 4A). In contrast, wild-type p27 and p27(S10A) were degraded at similar rates in S-phase cells (Fig. 4B). Treatment of p27^{S10A/S10A} lymphocytes with the proteasome inhibitor MG132 largely prevented the degradation of p27(S10A) at G_0 phase (Fig. 4C), suggesting that this proteolysis is mediated by the ubiquitin-proteasome system. These data indicate that phosphorylation of Ser^{10} contributes to the stabilization of p27 apparent during G_0 phase.

Degradation of p27 at G_0-G_1 independent of Ser^{10} phosphorylation

p27 is rapidly degraded by the ubiquitin-proteasome pathway at the G_0-G_1 transition. This process is largely independent both of SCFSkp2 and of phosphorylation of p27 on Thr^{187} (26,33). We examined whether the degradation of p27 at the G_0-G_1 transition is dependent on phosphorylation of Ser^{10}. Both wild-type and p27^{S10A/S10A} lymphocytes exhibited similar kinetics of p27 down-regulation at the G_0-G_1 transition, even though the original abundance of p27(S10A) was lower than that of wild-type p27 at G_0 phase (Fig. 5A). The timing of entry of cells into S phase after mitogenic stimulation also did not differ substantially between wild-type and p27^{S10A/S10A} lymphocytes (Fig. 5B). These data suggest that the phosphorylation of p27 on Ser^{10} is not required for the degradation of p27 at the G_0-G_1 transition.

Nuclear export of p27 in p27^{S10A/S10A} cells

We and others previously showed that the S10A mutation reduced the efficiency of
nuclear export of p27 (28-30). Given that these previous experiments were performed with overexpressed recombinant proteins in cultured cells, however, interpretation of their results was not necessarily straightforward. The mouse knock-in model developed in the present study provided a more physiological system for determination of the effect of the S10A mutation on the nuclear export of p27. MEFs prepared from wild-type or p27^{S10A/S10A} mice were synchronized in G_0 phase by serum deprivation for 96 h and were then stimulated to enter the cell cycle by reexposure to serum. Immunofluorescence analysis revealed that wild-type p27 and the p27(S10A) mutant were localized in the nucleus of G_0 cells (Fig. 6A). Unexpectedly, serum stimulation resulted in the translocation of p27(S10A) from the nucleus to the cytoplasm, as it did with wild-type p27. Quantitative analysis indicated that the efficiency of nuclear export was virtually indistinguishable between wild-type p27 and p27(S10A) (Fig. 6B).

We generated Skp2^{−/−};p27^{S10A/S10A} mice by crossing Skp2^{−/−} mice and p27^{S10A/S10A} mice and then compared the stabilities of wild-type or mutant p27 in MEFs derived from Skp2^{+/+};p27^{+/+}, Skp2^{+/+};p27^{S10A/S10A}, Skp2^{−/−};p27^{−/−}, or Skp2^{−/−};p27^{S10A/S10A} mice (Fig. 6C). The stability of p27 at G_0 phase was as greatly reduced in Skp2^{−/−};p27^{S10A/S10A} MEFs as in Skp2^{+/+};p27^{S10A/S10A} MEFs compared with that in Skp2^{+/+};p27^{+/+} or Skp2^{−/−};p27^{+/+} MEFs, providing genetic evidence that Skp2 does not contribute to the turnover of p27 in G_0 phase. We therefore conclude that phosphorylation of p27 on Ser^{10} is an important determinant of the stability of p27 at G_0.
G₀ phase but is dispensable for the translocation of p27 from the nucleus to the cytoplasm at the G₀-G₁ transition.

We also examined the nuclear export of p27 in lymphocytes by confocal laser-scanning microscopy. Three different antibody preparations were used for the immunostaining of resting lymphocytes (Supplementary Information, Fig. S2). With all of these antibody preparations, the p27 signal was detected in both the nucleus and the cytoplasm of wild-type lymphocytes. The distribution of p27 in the nucleus did not appear to be homogeneous. Stimulation of wild-type and p27⁶¹⁰A⁰/S¹⁰A lymphocytes with the combination of PDBu and ionomycin resulted in a gradual decrease in the amounts of p27 present in both the nucleus and the cytoplasm (Supplementary Information, Fig. S3), but the translocation of p27 from the nucleus to the cytoplasm was not as evident in the lymphocytes of either genotype as was that apparent in MEFs. We thus conclude that, in lymphocytes, nuclear export of p27 might not be the major pathway of p27 degradation, although phosphorylation of Ser¹⁰ plays an important role in the stabilization of p27 in G₀ phase in both lymphocytes and MEFs.
Regulation of the cell cycle at the G₁-S boundary is thought to be important for the control of cell proliferation. Kinase activity associated with two G₁ cyclins, cyclins D and E, is essential for this transition, largely because of the requirement for phosphorylation of Rb and the consequent termination of its inhibition of cell cycle progression (1,2). Among the mechanisms responsible for regulation of G₁ cyclin–associated kinase activity, control of the abundance of p27 by external mitogenic signals appears important (34,35). The amount of p27 is relatively high in quiescent (G₀) cells and decreases on entry of cells into the cell cycle (5,6). The abundance of p27 is regulated predominantly by posttranslational modification, which affects protein stability, rather than by transcriptional control (13,15). The phosphorylation state of many proteins affects their stability, and phosphorylation of p27 on Thr¹⁸⁷ is essential for its binding to Skp2, the F-box protein component of an SCF (Skp1-Cul1–F-box protein) ubiquitin ligase complex; such binding results in the ubiquitylation and degradation of p27 (20-25). Moreover, we previously showed that phosphorylation on Ser¹⁰ markedly increased p27 stability (27). In p27⁻¹⁰/⁻¹⁰ mice, the expression of p27 was reduced in the brain, thymus, spleen, and testis compared with that in wild-type mice, suggesting that phosphorylation of Ser¹⁰ is also important for p27 stability in these organs. However, the expression of p27 was normal in the liver, heart, lung, and skeletal muscle of p27⁻¹⁰⁻¹⁰ mice. Although the reason for this
organ specificity in the down-regulation of p27 in p27<sup>S10A/S10A</sup> mice remains unclear, the affected organs (brain, thymus, spleen, testis) overlap with those affected by hyperplasia in p27<sup>+/−</sup> mice (10). Such organs express the p27-related CKI p57 only at low levels, if at all (36), and appear to depend solely on p27 for maintaining cellular quiescence, whereas cell quiescence in other organs is controlled by both p27 and p57. The mechanism responsible for the regulation of p27 stability in G<sub>0</sub> phase also may differ between the two groups of organs.

p27 is located in the nucleus and is stable at G<sub>0</sub> phase, but the mechanism of this stabilization has not been clear. The p27<sup>S10A/S10A</sup> knock-in mouse has now provided insight into this mechanism. We have thus shown that the defect in Ser<sup>10</sup> phosphorylation resulted in proteolysis of p27 via the ubiquitin-proteasome pathway in G<sub>0</sub> cells. The ubiquitylation of p27(S10A) at G<sub>0</sub> phase is likely mediated by a ubiquitin ligase other than SCF<sup>Skp2</sup>, given that the expression of Skp2 is not detected at this stage (26) and that the rate of degradation of p27(S10A) at G<sub>0</sub> in MEFs derived from p27<sup>S10A/S10A</sup> mice was not affected by loss of Skp2. Phosphorylation of Ser<sup>10</sup> may interfere with the recognition of p27 by this putative ubiquitin ligase.

By overexpressing wild-type or mutant p27 proteins in cultured cell lines, we and others previously showed that p27 is translocated from the nucleus to the cytoplasm at the G<sub>0</sub>-G<sub>1</sub> transition in a manner dependent on Ser<sup>10</sup> phosphorylation (28-30). Our present observations with the p27<sup>S10A/S10A</sup> knock-in mouse, however, do not support the notion that Ser<sup>10</sup> phosphorylation is essential for the nuclear export of
p27. The previous analyses of transiently transfected cell lines might thus have revealed nonphysiological effects of p27 overexpression. Overexpressed p27(S10A) may suppress cyclin D–associated kinase activity and inhibit the progression of cells from G₀ to G₁ phase, resulting in an apparent block of the nuclear export of the p27 mutant. On the other hand, genetic engineering in intact animals, such as that applied to produce the knock-in model of the present study, might give rise to skewed results, given that related proteins or selective expansion of resistant cells during development may compensate for the loss of function of the target protein (37). A system for inducible expression in conditional knock-in mice might ameliorate such potential problems.

The biochemical activity of p27 suggests that the protein functions as a tumor suppressor. Indeed, mice lacking p27 are prone to spontaneous and induced tumorigenesis (8-11). On the other hand, mutations in p27 appear to be rare in human cancers (38-46). Reduced expression of p27 has nevertheless been correlated with poor prognosis in cohorts of individuals with breast, colorectal, or stomach carcinoma (47-53). Tumors with low levels of p27 expression have also been shown to exhibit relatively high rates of p27 degradation (and vice versa) (49). It is unlikely that this increased degradation of p27 is due to nonspecific enhancement of general protein degradation, because degradation of neither p21 nor cyclin A was affected in the same tumors. The mechanisms responsible for the specific control of p27 stability thus appear important in cancer development. Identification of components of the
degradation machinery that determines the turnover rate of p27 may thus provide insight into the altered expression of this protein in tumor cells as well as into whether such altered expression is a cause or a consequence of cell transformation. Indeed, Skp2 is overexpressed in many human cancer cell lines (54), suggesting that p27 degradation mediated by Skp2 may be related to carcinogenesis. It is thus possible that the Ser\(^{10}\) phosphorylation–dependent mechanism of p27 stabilization is also deregulated in some cancer cells.
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FOOTNOTE

1Abbreviations: CDK, cyclin-dependent kinase; CKI, CDK inhibitor; ES, embryonic stem; MEF, mouse embryonic fibroblast; PAGE, polyacrylamide gel electrophoresis; GSK-3β, glycogen synthase kinase–3β; PDBu, phorbol 12,13-dibutyrate; BrdU, bromodeoxyuridine; FITC, fluorescein isothiocyanate.
**FIGURE LEGENDS**

**Figure 1.** Generation of knock-in mice harboring an S10A mutation in p27. (A) Structure of the targeting vector, the wild-type p27 allele, the mutated allele after homologous recombination [S10A(Neo)], and the mutated allele after deletion of the neomycin resistance cassette (neo) by Cre recombinase (S10A). Exons are depicted by filled boxes. Restriction sites: B, BamHI; E1, EcoRI; H, HindIII; K, KpnI. tk, thymidine kinase gene. "S" and "A" in the first exon denote the codons encoding Ser\(^{10}\) (wild type) and Ala\(^{10}\) (mutant), respectively. The position of a probe used for Southern blot analysis is shown. (B) Southern blot analysis of genomic DNA extracted from the tail of mice of the indicated genotypes. The DNA was digested with BamHI and subjected to hybridization with the probe depicted in (A). (C) Appearance of p27\(^{S10A/S10A}\) mice. The mutant and wild-type animals were 12-week-old littermates. (D) Growth curves of wild-type (+/+), heterozygous (+/S10A), and homozygous mutant (S10A/S10A) littermates.

**Figure 2.** Reduced expression of p27 in certain organs of p27\(^{S10A/S10A}\) knock-in mice. (A) Immunoblot analysis of the indicated organs of p27\(^{+/+}\) and p27\(^{S10A/S10A}\) mice with antibodies to p27 or to GSK-3\(\beta\) (control). Band intensity was measured with a chemiluminescence imager, normalized by that of GSK-3\(\beta\), and expressed as a percentage of the corresponding normalized value for p27\(^{+/+}\) mice in each pair of lanes. (B) Relative abundance of p27 in organs of p27\(^{S10A/S10A}\) mice compared with the
corresponding values for p27+/+ mice. Data are means of values from three mice. (C) Two-dimensional PAGE and immunoblot analysis with antibodies to p27 of the indicated organs of p27+/+ and p27S10A/S10A mice. The positions of spots corresponding to nonphosphorylated (p27) and phosphorylated (pp27) forms of p27 are indicated, as are the amounts of each of these two forms of the protein expressed as a percentage of total p27. pI, isoelectric point.

**Figure 3.** Cell cycle–dependent phosphorylation of p27 on Ser10 in primary lymphocytes and MEFs. (A) Lymphocytes freshly isolated from wild-type mice were stimulated with 10 nM PDBu and 300 nM ionomycin for the indicated times, labeled with 10 μM BrdU for 30 min before harvesting, stained with FITC-conjugated antibodies to BrdU and propidium iodide, and analyzed by flow cytometry. The percentages of cells in G0-G1, S, and G2-M phases of the cell cycle are shown below each panel. (B) Lymphocytes isolated from wild-type mice were stimulated for the indicated times as in (A), after which cell extracts were subjected to immunoblot analysis with antibodies to p27 or to GSK-3β. (C) Lymphocytes isolated from p27+/+ or p27S10A/S10A mice were stimulated for the indicated times as in (A), after which cell extracts were subjected to two-dimensional PAGE and immunoblot analysis with antibodies to p27. The amount of lysate protein analyzed (50 to 150 μg) was adjusted to ensure that the total amounts of p27 were similar at the different times. The positions corresponding to nonphosphorylated (p27) and phosphorylated (pp27)
forms of p27 are indicated, as are the amounts of each of these two forms of the protein expressed as a percentage of total p27. (D) MEFs derived from p27+/+ or p27^{S10A/S10A} mice were synchronized in G\textsubscript{0} phase by serum deprivation for 96 h and then stimulated to reenter the cell cycle by exposure to 20% serum for the indicated times. Cell extracts were subjected to two-dimensional PAGE and immunoblot analysis as in (C). (E) Lymphocytes isolated from p27+/+ or p27^{S10A/S10A} mice were incubated for 8 h with [\textsuperscript{32}P]orthophosphate in the absence (G\textsubscript{0} phase) or presence (G\textsubscript{1} phase) of 10 nM PDBu and 300 nM ionomycin (left panel). Alternatively, lymphocytes isolated from p27+/+ mice were incubated for 7 h in the absence (G\textsubscript{0} phase) or presence (G\textsubscript{1} phase) of PDBu and ionomycin and then for 1 h in the additional presence of [\textsuperscript{35}S]methionine and [\textsuperscript{35}S]cysteine (right panel). Cell lysates were then subjected to immunoprecipitation (IP) with antibodies to p27 (\alpha-p27), and the resulting precipitates were subjected either to autoradiography (upper panels) or to immunoblot analysis (IB) with antibodies to p27 (lower panels).

**Figure 4.** Effect of mutation of Ser\textsuperscript{10} on the stability of p27. (A) Lymphocytes isolated from p27+/+ or p27^{S10A/S10A} mice were incubated with cycloheximide (10 \textmu g/ml) for the indicated times, after which cell extracts were subjected to immunoblot analysis with antibodies to p27 or to GSK-3\textbeta (left panel). Band intensity was measured, normalized by that of GSK-3\textbeta, and expressed as a percentage of the corresponding normalized value for time zero (right panel). (B) Lymphocytes isolated from p27+/+ or p27^{S10A/S10A}
mice were stimulated with 10 nM PDBu and 300 nM ionomycin for 22 h, after which
cycloheximide was added to the cells for the indicated times. Cell extracts were
subjected to immunoblot analysis and p27 band intensity was determined as in (A).
(C) Lymphocytes isolated from p27S10A/S10A mice were incubated with cycloheximide in
the absence or presence of 10 μM MG132 for the indicated times, after which cell
extracts were subjected to immunoblot analysis as in (A).

Figure 5. Normal degradation of p27(S10A) in lymphocytes at the G₀-G₁ transition.
(A) Lymphocytes isolated from p27+/+ or p27S10A/S10A mice were stimulated with the
combination of 10 nM PDBu and 300 nM ionomycin for the indicated times, after
which cell extracts were subjected to immunoblot analysis with antibodies to p27 and
to GSK-3β (left panel). Band intensity was measured, normalized by that of GSK-3β,
and expressed as a percentage of the corresponding normalized value for time zero
(right panel). (B) Lymphocytes isolated from p27+/+ or p27S10A/S10A mice were
stimulated with PDBu and ionomycin for the indicated times, labeled with 10 μM BrdU
for 30 min before harvesting, stained with antibodies to BrdU and propidium iodide,
and analyzed by flow cytometry. The percentage of cells in S phase (BrdU-positive
cells) was determined.

Figure 6. Nuclear export of p27 independent of Ser¹⁰ phosphorylation. (A) MEFs
derived from p27+/+ or p27S10A/S10A mice were synchronized in G₀ phase by serum
deprivation for 96 h and then stimulated to reenter the cell cycle by exposure to 20% serum for the indicated times. Cells were subjected to immunostaining for endogenous p27 (green), and cell nuclei were revealed by staining with Hoechst 33258 (blue). (B) Quantitative analysis of the subcellular localization of p27 in experiments similar to that described in (A). At least 300 cells were scored for each sample. Data represent the percentage of cells in which p27 was localized predominantly in the nucleus and are means ± SE of values from three independent experiments. (C) MEFs derived from Skp2+/+;p27+/+, Skp2+/+;p27S10A/S10A, Skp2–/–;p27+/+, or Skp2–/–;p27S10A/S10A mice were synchronized in G0 phase by serum deprivation for 96 h, after which cycloheximide (10 μg/ml) was added to the cells for the indicated times. Cell extracts were subjected to immunoblot analysis with antibodies to p27 or to GSK-3β (left panel). Band intensity was measured, normalized by that of GSK-3β, and expressed as a percentage of the corresponding normalized value for time zero (right panel).
SUPPLEMENTARY FIGURE LEGENDS

Supplementary Figure S1. Specificity of an antibody to Ser\textsuperscript{10}-phosphorylated p27. (A) HEK293T cells were transfected with an empty expression plasmid (mock) or the same plasmid encoding Flag epitope–tagged forms of wild-type (WT) p27 or its S10A or S10D mutants. Cell extracts were subjected to immunoblot analysis with antibodies to p27 (\textit{\textalpha}-p27) or to p27 phosphorylated on Ser\textsuperscript{10} (\textit{\textalpha}-pSer-10). The positions of Flag-p27 and phosphorylated Flag-p27 are indicated. (B) Thymic extracts derived from p27\textsuperscript{+/+} or p27\textsuperscript{S10A/S10A} mice were subjected to immunoblot analysis as in (A).

Supplementary Figure S2. Confocal microscopy of p27 expression in primary lymphocytes at G\textsubscript{0} phase. (A–C) Lymphocytes isolated from p27\textsuperscript{+/+} or p27\textsuperscript{−/−} mice were subjected to immunostaining of endogenous p27 (green) with C-19 (Santa Cruz Biotechnology) (A), N-20 (Santa Cruz Biotechnology) (B), or Ab-2 (Neomarkers) (C) polyclonal antibodies specific for this protein. Cell nuclei (red) were revealed by staining with propidium iodide (PI).

Supplementary Figure S3. Confocal microscopy of p27 expression in primary lymphocytes at the G\textsubscript{0}-G\textsubscript{1} transition. Lymphocytes isolated from p27\textsuperscript{+/+} or p27\textsuperscript{S10A/S10A} mice were stimulated with the combination of 10 nM PDBu and 300 nM ionomycin for the indicated times, after which they were subjected to immunostaining of
endogenous p27 (green) with polyclonal antibodies to this protein (C-19; Santa Cruz Biotechnology). Cell nuclei (red) were revealed by staining with propidium iodide (PI).
Kotake et al. Figure 1
Kotake et al. Figure 3
Kotake et al. Figure 4
Kotake et al. Figure 5
Cells with nuclear localization of p27 (%)

Time (h)

+/+
S10A/S10A

Cells with nuclear localization of p27 (%)

Time (h)

+/+
S10A/S10A

Skp2+/+, p27+/+
Skp2+/+, p27S10A/S10A
Skp2−/−, p27S10A/S10A
Skp2−/−, p27+/+ 

GSK-3β

p27 remaining (%)

Time (h)

Skp2+/+, p27+/+
Skp2+/+, p27S10A/S10A
Skp2−/−, p27S10A/S10A
Skp2−/−, p27+/+ 

Kotake et al. Figure 6
A

Mock  WT  S10A  S10D

\( \alpha-p27 \)

\( \alpha-p\text{Ser}-10 \)

\( \text{Flag-pp27} \)

\( \text{Flag-p27} \)

B

\( \alpha-p27 \)

\( \alpha-p\text{Ser}-10 \)

\( S10A\text{S10A} \)
A

-/-  +/+  

p27

PI

Merge

B

-/-  +/+  

p27

PI

Merge

C

-/-  +/+  

p27

PI

Merge

Kotake et al. Supplementary Figure S2
Kotake et al. Supplementary Figure S3
Role of serine-10 phosphorylation in p27 stabilization revealed by analysis of p27 knock-in mice harboring a serine-10 mutation
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