Degradation of p27\textsuperscript{Kip1} at the G\textsubscript{0}-G\textsubscript{1} Transition Mediated by a Skp2-independent Ubiquitination Pathway*

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Progression of the cell cycle in eukaryotic cells depends on the activity of a series of protein complexes composed of cyclins and cyclin-dependent kinases (CDKs). The activity of cyclin-CDK complexes is regulated by various mechanisms, including association of the kinase subunit with the regulatory cyclin subunit, phosphorylation-dephosphorylation of the kinase subunit, and association of the complex with a group of CDK inhibitors (CKIs) (1, 2). The interaction of CKIs with cyclin-CDK complexes is triggered by a variety of antimitogenic signals and results in inhibition of the catalytic activity of the complexes and consequent restraint of cell cycle progression (3, 4).

A key question with regard to regulation of the cell cycle concerns the mechanism by which cells undergo the transition from the resting state (G\textsubscript{0}) to proliferation. The CKI p27\textsuperscript{Kip1} plays a pivotal role in the control of cell proliferation. Transition from G\textsubscript{0} phase to S phase of the cell cycle is promoted by complexes of G\textsubscript{1} cyclins with CDKs, and p27\textsuperscript{Kip1} inhibits the activity of these complexes directly by binding to them (5). In normal cells, the amount of p27\textsuperscript{Kip1} is high during G\textsubscript{0} phase, but it rapidly decreases on reentry into G\textsubscript{1}-S phases triggered by specific mitogenic factors (6, 7). Forced expression of p27\textsuperscript{Kip1} results in cell cycle arrest in G\textsubscript{1} phase (8, 9); conversely, inhibition of p27\textsuperscript{Kip1} expression by antisense oligonucleotides increases the number of cells in S phase (10). Moreover, we and others have demonstrated that mice homozygous for deletion of the p27\textsuperscript{Kip1} gene are larger than normal mice, and that they exhibit multiple organ hyperplasia as well as a predisposition to both spontaneous and radiation- or chemical-induced tumors (11–14).

The ubiquitin-proteasome pathway plays an important role in the degradation of short-lived regulatory proteins, including those that participate in the cell cycle, cellular signaling in response to stress and to extracellular signals, morphogenesis, the secretory pathway, DNA repair, and organelle biogenesis (15). The concentration of p27\textsuperscript{Kip1} is thought to be regulated predominantly by this proteolytic pathway (16–18). The ubiquitin-mediated proteolysis of many proteins is regulated by phosphorylation of the target, which increases its susceptibility to degradation (19–22). The proteolysis of p27\textsuperscript{Kip1} may also be regulated by such a mechanism, given that degradation of the protein is promoted by its phosphorylation on Thr\textsuperscript{187} by the cyclin E-CDK2 complex (23–25). Recent data have also suggested that Skp2, an F-box protein that is thought to function as the receptor component of an SCF ubiquitin ligase complex, binds to p27\textsuperscript{Kip1} in conjunction with Cks1 only when Thr\textsuperscript{187} of p27\textsuperscript{Kip1} is phosphorylated; such binding then results in the ubiquitination and degradation of p27\textsuperscript{Kip1} (26–30). These biochemical observations are supported by genetic evidence that p27\textsuperscript{Kip1} accumulates to high levels in the cells of mice that lack either Skp2 or Cks1 (29–31).

We now show that, although the degradation of p27\textsuperscript{Kip1} during S and G\textsubscript{2} phases of the cell cycle is markedly impaired in Skp2\textsuperscript{−/−} cells, the degradation of this protein at the G\textsubscript{0}-G\textsubscript{1} transition occurs normally in these cells. Our data suggest that the existence of a second pathway of p27\textsuperscript{Kip1} degradation that is also mediated by the ubiquitin-proteasome system. This second pathway may be critical for regulation of cell cycle progression from the resting state to proliferation, and may be important in mechanisms of carcinogenesis.

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‡ The abbreviations used are: CDK, cyclin-dependent kinase; CKI, cyclin-dependent kinase inhibitor; PDBu, phorbol 12,13-dibutyrate; GSK-3, glycogen synthase kinase-3\textbeta; BrdUrd, bromodeoxyuridine; RT, reverse transcription; GST, glutathione S-transferase; PBS, phosphate-buffered saline; DTT, dithiothreitol; MEF, mouse embryonic fibroblast; SCF, skp1-cull-f-box protein.
**EXPERIMENTAL PROCEDURES**

**Immunoblot Analysis**—Single-cell suspensions of lymphocytes were prepared from the lymph nodes of 4-month-old Skp2−/− or Skp2+/− mice and were cultured (1.0 × 10^6 cells in 5 ml) for the indicated times in RPMI 1640 medium supplemented with 10% fetal bovine serum, 10 mM phenol red, 12,13-dibutylate (PBu) (Sigma), and 300 mM iomycin (Sigma). In some experiments, 10 μg lactacystin (Kyowa Medics), 10 μM MG132 (Peptide Institute), or vehicle (dimethyl sulfoxide) was added to the culture medium. The cells were subsequently harvested and lysed in 50 μl of radioimmunoprecipitation assay buffer containing 0.4 μM Na~2~PO~4~, 0.4 mM EDTA, and 1 mM antipain, pepstatin, chymostatin, leupeptin, and phenylmethylsulfonyl fluoride, each at a concentration of 10 μg/ml. The lysates were incubated on ice for 15 min and then centrifuged at 20,000 × g. After determining protein concentration with the Bradford assay, the supernatant was diluted 1:5 with PBS and lysed by sonication, and then subjected to centrifugation at 20,000 × g. The resulting supernatant, and the mixture was rotated for 4 hr at 4°C, was removed by centrifugation for 20 min at 13,000 × g, containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM EDTA, and 10 μg/ml glycogen synthase kinase-3β (Santa Cruz Biotechnology), to cyclin E (Santa Cruz Biotechnology), to Skp2 (Zymed Laboratories Inc.), or to glycogen synthase kinase-3β (GSK-3β) (Transduction Laboratories). Immune complexes were detected with appropriate horseradish peroxidase-conjugated secondary antibodies and either SuperSignal West Pico or SuperSignal West Dura chemiluminescence reagents (Pierce).

**Cell Cycle Analysis by Flow Cytometry**—Single-cell suspensions of lymphocytes were cultured as described above. Cells were exposed to 10 μM MG132 (Peptide Institute) for 30 min before lysis and were then subjected to fixation overnight in 70% ethanol at −20°C followed by denaturation 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-conjugated antibodies to BrdUrd (Becton Dickinson) and propidium iodide (5 μg/ml). The cells were then analyzed with a FACSCalibur flow cytometer and Cell Quest software (Becton Dickinson).

**Reverse Transcription (RT)-PCR Analysis of Immediate-early Gene Expression**—Total RNA (1 μg) extracted from lymph node cells (2.0 × 10^6) with the use of Isogen (Wako) was subjected to RT with ReverTra Dash (Toyobo) in a final volume of 20 μl. The resulting cDNA (1 μl) was amplified by the polymerase chain reaction (PCR) in a final volume of 50 μl of PCR reaction mix. The respective sequences of sense and antisense primers specific for the target genes were as follows: c-Myc, 5′-ATG GCA TTG ATC CCT CAG TGG TCT CTT CCA A-3′ and 5′-CAG CTC GTT CCT CTC AGG TTC CAA GCC GTT-3′; c-Fos, 5′-GAG CTT CAG ACA GAT CCA TCA CCA GCCG-3′ and 5′-CAG TCT GCT GCA TAG AAG GAA CCG-3′; c-Jun, 5′-GCA TGA GCA GGC GTA CTA CCA ATA GCC CAA ATC GCC GGC T-3′; and 5′-CAG GTC GGT GCC TAC CAT-3′. The sense and antisense primers targeted to the gene for glyceraldehyde-3-phosphate dehydrogenase (G3PDH) as the internal standard were 5′-ACC ACA GTC CAT GCC ATC ACC-3′ and 5′-TCC ACC ACC CTG TTG CTA-3′, respectively. Products separated by electrophoresis on a 1.5% agarose gel and visualized by ethidium bromide staining.

**Production of Recombinant Proteins in Bacteria—Glutathione S-transferase (GST) fusion proteins of mouse p27Kip1 and the T187A mutant of p27Kip1 (each of which was tagged at its COOH terminus with the hexahistidine (Hi6) epitope) as well as a GST fusion protein of mouse Csk1 were expressed in Escherichia coli strain XL1-blue (Stratagene); the bacteria were cultured in the presence of 0.1 mM isopropyl-β-D-thiogalactopyranoside. The recombinant proteins were purified with the use of ProBond resin (Invitrogen) or glutathione-Sepharose CL-4B. After dialysis against a solution containing 40 mM Hepes-NaOH (pH 7.6), 60 mM NaCl, 1 mM EDTA, and 10% glycerol, the proteins were stored at −80°C.

**Baculovirus Expression System**—Baculoviruses were generated in Sf9 cells with the use of BacPAK6 virus DNA and the pBacPAK-GST vector (CLONTECH) containing either the cDNA for a GST fusion protein of human cyclin E tagged at its NH2 terminus with the Myc epitope or the cDNA for human cyclin D2 tagged at its NH2 terminus with the hemagglutinin epitope. The GST-cyclin E-CDK2 recombinant protein complex was purified (according to the protocol described above) for purification of GST fusion proteins expressed in bacteria) from a lysate of Sf9 cells coinfected with the two viruses. Only the purified recombinant proteins were detected by electrophoresis and Coomassie Blue staining.

**In Vitro Ubiquitination Assay**—Primary mouse embryonic fibroblasts (MEFs) were derived from 13.5-day-postcoitum Skp2−/− or Skp2+/− embryos and cultured as described previously (11). In all experiments of this study, we used nensesenes MEFs (no more than passage 2). In some experiments, the cell cycle of MEFs was synchronized either at G1-G0 by serum deprivation for 96h in medium supplemented with 0.1% fetal bovine serum, or at G0-G1, by arresting the G1-S boundary induced by incubation with aphidicolin (1 μg/ml) for 14 hr, followed by culture for 3 hr in aphidicolin-free medium. The cells (1.0 × 10^6) were washed with PBS and then suspended in 500 μl of buffer A (40 mM Hepes-NaOH (pH 7.6), 1 mM DTT, and protease inhibitor mixture (antipain, pepstatin, chymostatin, leupeptin, and phenylmethylsulfonyl fluoride, each at 10 μg/ml)) containing 60 μg/ml NaCl. For preparation of nuclear and cytoplasmic extracts, the cell suspension was frozen, thawed, and then subjected to centrifugation at 2000 × g for 5 min at 4°C.

The resulting nuclear pellet was resuspended in 300 μl of ice-cold buffer A containing 300 mM NaCl, rotated for 30 min at 4°C, and centrifuged at 100,000 × g for 4 hr at 4°C. The new supernatant was dialyzed against buffer A containing 60 μg/ml NaCl, centrifuged for 10 min at 20,000 × g to remove debris, and then mixed with 800 μl of DE52 resin (Whatman) that had been equilibrated with buffer A containing 60 μg/ml NaCl. After incubation for 30 min at room temperature, the slurry was transferred to a 2-ml-diameter column and washed with buffer A. Proteins were eluted from the column in a stepwise manner with buffer A containing 150 and 300 mM NaCl, and 160-μl fractions were collected.

The cytoplasmic supernatant resulting from the centrifugation of the MEF lysate at 2000 × g was subjected to further centrifugation at 100,000 × g for 4 hr at 4°C. The new supernatant was mixed with 800 μl of DE52 that had been equilibrated with buffer A containing 60 μg/ml NaCl, and then subjected to column chromatography as described above.

For preparation of whole-cell extracts, MEFs were suspended in buffer A containing 300 μg/ml KCl, frozen and thawed, rotated for 30 min at 4°C, and then centrifuged at 100,000 × g for 4 hr at 4°C. The resulting supernatant was dialyzed against buffer A containing 60 μg/ml NaCl, centrifuged for 10 min at 20,000 × g to remove debris, and then mixed with 800 μl of DE52 (that had been equilibrated with buffer A containing 60 μg/ml NaCl). Column chromatography was then performed as described above.

DE52 column fractions (3 μl) were mixed with 50 ng of Uba1, 100 ng of Ubc5 or Ubc3, 3 μg of GST-ubiquitin, and 50 ng of wild-type p27Kip1 or the T187A mutant in a 10-μl reaction mixture containing 40 μg Hepes-NaOH (pH 7.6), 60 mM NaCl, 2 mM DTT, 5 mM MgCl2, 0.5 mM DTT, 10% glycerol, and 1.5 μM ATP. In some experiments, 100 ng of GST-cyclin E-CDK2 complex or 100 ng of Csk1 were added to the reaction mixture. After incubation for 30 min at 26°C, the reaction mixtures were subjected to SDS-PAGE and immunoblot analysis with a monoclonal antibody to p27Kip1.

**RESULTS**

**Normal Degradation of p27Kip1 at the G0-G1 Transition in Skp2−/− Cells**—Analysis of Skp2−/− mice revealed that phenotypic characteristics as such cellular accumulation of p27Kip1 were less evident in lymphocytes than in liver, kidney, and lung (Ref. 31 and data not shown). With the use of immunoblot analysis, we examined the mitogen-induced down-regulation of p27Kip1 in mature lymphocytes, almost all of which reside in...
the G₀ phase of the cell cycle in the absence of stimulation (6).
Mitogenic stimulation with the combination of PDBo and ionomycin resulted in the almost complete disappearance of p27kip1 from wild-type lymphocytes within 12 h (Fig. 1A). Unexpectedly, Skp2⁺/⁻ lymphocytes exhibited similar kinetics of p27kip1 down-regulation, suggesting that the decrease in the abundance of p27kip1 in the early phase (up to 12 h) of mitogenic stimulation occurs independently of Skp2-mediated proteolysis. However, the abundance of p27kip1 increased again to substantial levels by 36-48 h in the Skp2⁻/⁻ cells, whereas the amount of this protein remained low in wild-type lymphocytes (Fig. 1B).

The abundance of cyclin D, cyclin E, and Skp2 was also examined in wild-type lymphocytes in the early (Fig. 1C) and late (Fig. 1D) phases of mitogenic stimulation. Consistent with previous results (32), cyclin D1 was not detected in the lymphocytes (data not shown). Cyclin D2 was detected as early as 3 h after stimulation, whereas the amount of cyclin E (which is expressed in late G₁ phase and degraded in S phase) increased 18-24 h after stimulation. The expression of Skp2 was first evident 18-24 h after stimulation and was maximal at 30-36 h.

The cell cycle profile did not differ substantially between Skp2⁺/⁻ and Skp2⁻/⁻ cells for up to 48 h after stimulation (Fig. 1E). In both instances, cells began to enter S phase 18-24 h after stimulation and to enter G₂ phase at 36 h. Given that neither Skp2 nor cyclin E was expressed in the early phase (G₀-G₁) of p27kip1 degradation, and that the down-regulation of p27kip1 occurred in Skp2⁻/⁻ cells, this process appears to require neither Skp2 nor cyclin E-mediated phosphorylation of p27kip1. In contrast, Skp2 is indispensable for the late phase (S-G₂) of p27kip1 degradation.

Prevention of p27kip1 Degradation at G₀-G₁ by a Proteasome Inhibitor—We next examined whether the early phase of p27kip1 degradation induced by mitogenic stimulation is dependent on the ubiquitin-proteasome pathway. We incubated wild-type lymphocytes in the presence of lactacystin, a specific inhibitor of the proteasome, during mitogenic stimulation. The down-regulation of p27kip1 was markedly inhibited by lactacystin (Fig. 2A). Short-term inhibition of the proteasome by incubation of cells with the quick-acting compound MG132 for the 2 h before cell harvest yielded similar results. MG132 thus markedly inhibited the further degradation of p27kip1 after its addition to the culture (Fig. 2B). To exclude the possibility that proteasome inhibition affects transduction of the mitogenic signal, we also examined the expression of the immediate-early genes c-myc, c-jun, and c-fos by RT-PCR analysis (Fig. 3). The levels and kinetics of expression of these genes were not substantially affected by treatment of the wild-type cells with lactacystin, indicating that mitogenic signaling was intact in lymphocytes exposed to this agent. These data suggest that the early phase of degradation of p27kip1 at the G₀-G₁ transition is mediated by the ubiquitin-proteasome pathway.
Skp2-independent Degradation of p27<sup>Kip1</sup>

S-G<sub>2</sub> phases of the first cycle (caused by the absence of Skp2) results in G<sub>1</sub> arrest during the second cell cycle. The putative machinery responsible for p27<sup>Kip1</sup> degradation at the G<sub>1</sub>-S transition of the first cell cycle thus does not appear to function at the G<sub>1</sub>-S transition of subsequent cycles (see Fig. 8).

**Skp2-dependent and -independent Polyubiquitination of p27<sup>Kip1</sup>**—We fractionated total extracts of Skp2<sup>+/+</sup> and Skp2<sup>−/−</sup> MEFs by DE52 ion-exchange chromatography with stepwise elution with KCl. We then examined the in vitro ubiquitination of p27<sup>Kip1</sup> in the presence of the ubiquitin-activating enzyme Uba1, the ubiquitin-conjugating enzyme Ubc5a, and GST-ubiquitin (Fig. 6). The Skp2<sup>+/+</sup> cell extract yielded a pronounced peak of polyubiquitination activity encompassing fractions 4 and 7, whereas the corresponding activity peak derived from Skp2<sup>−/−</sup> cells spanned fractions 6–9. To determine whether ubiquitination of p27<sup>Kip1</sup> was dependent on phosphorylation of Thr<sup>187</sup> at the COOH terminus of the protein, we subjected a p27<sup>Kip1</sup> mutant in which Thr<sup>187</sup> was replaced by Ala (T187A) to the in vitro ubiquitination assay (Fig. 5B). A large peak of polyubiquitination activity was detected mostly in fractions 5–8 and a smaller peak of activity was apparent in fractions 14 and 15 derived from both Skp2<sup>+/+</sup> and Skp2<sup>−/−</sup> cells. These data suggest that a mediator of p27<sup>Kip1</sup> polyubiquitination is present in the Skp2<sup>−/−</sup> cells, and that the polyubiquitination activity conferred by this factor is independent of phosphorylation of p27<sup>Kip1</sup> on Thr<sup>187</sup>.

We previously showed that Skp2 is predominantly localized to the nucleus (33, 34). We thus also subjected nuclear extracts prepared from Skp2<sup>+/+</sup> and Skp2<sup>−/−</sup> MEFs to ion-exchange chromatography. Skp2 was detected mostly in fractions 5 and 6 of the column eluate (Fig. 6A). Fraction 5 of the nuclear extracts of Skp2<sup>+/+</sup> and Skp2<sup>−/−</sup> cells was then included in the in vitro ubiquitination assay with wild-type p27<sup>Kip1</sup>, ubiquitin-activating enzyme, the ubiquitin-conjugating enzymes Ubc3 or Ubc5a, and GST-ubiquitin (Fig. 6B). With the fraction from Skp2<sup>+/+</sup> cells, polyubiquitination of p27<sup>Kip1</sup> was evident in the additional presence of cyclin E and CDK2 and was further enhanced by Cks1. In contrast, polyubiquitinated species of p27<sup>Kip1</sup> were not detected with fraction 5 from Skp2<sup>−/−</sup> cells.

To confirm that the polyubiquitination of p27<sup>Kip1</sup> mediated by Skp2 is dependent on phosphorylation of p27<sup>Kip1</sup> on Thr<sup>187</sup>, we subjected the T187A mutant of p27<sup>Kip1</sup> to the in vitro ubiquitination reaction in the presence of fraction 5 of the nuclear extracts of Skp2<sup>+/+</sup> and Skp2<sup>−/−</sup> cells (Fig. 6C). In contrast to wild-type p27<sup>Kip1</sup> (which underwent polyubiquitination in the presence of fraction 5 from wild-type cells), the T187A mutant did not undergo polyubiquitination in the presence of fraction 5 from either Skp2<sup>+/+</sup> or Skp2<sup>−/−</sup> cells. These data suggest that polyubiquitination activity in the nu-
the T187A mutant in the absence or presence of DE52 column fraction.

ubiquitination assays were performed with wild-type p27 Kip1 in vitro column, and portions of the resulting fractions 5 were subjected to the MEFS were fractionated by stepwise elution with KCl from a DE52

E-CDK2. Portions of the resulting fractions were subjected to immunoblot anal-

mediate p27 Kip1 column eluate of the nuclear extracts were assayed for their ability to

were detected by immunoblot analysis with antibodies to p27 Kip1 panel or Ubc5a (right panel).

FIG. 6 .

Kip1

A

C

B

D

Fig. 6. Skp2-dependent and -independent ubiquitination of p27Kip1. A, nuclear extracts prepared from Skp2+/+ and Skp2−/− MEFs were fractionated by stepwise elution with KCl from a DE52 column. Portions of the resulting fractions were subjected to immunoblot analysis with antibodies to Skp2. B, portions of fraction 5 from the DE52 column eluate of the nuclear extracts were assayed for their ability to mediate p27Kip1 ubiquitination in the absence or presence of cyclin E-CDK2 or of Cks1. Reaction mixtures also included Uba1, Ubc3 (left panel) or Ubc5a (right panel), and GST-ubiquitin. Reaction products were detected by immunoblot analysis with antibodies to Skp2. C, in vitro ubiquitination assays were performed with wild-type p27Kip1 or the T187A mutant in the absence or presence of DE52 column fraction 5 from nuclear extract of Skp2−/− or Skp2+/+ MEFs, Cks1, cyclin E-CDK2, and the combination (Reaction) of Uba1, Ubc5a, and GST-ubiquitin, as indicated. D, cytoplasmic extracts from Skp2+/+ and Skp2−/− MEFs were fractionated by stepwise elution with KCl from a DE52 column, and portions of the resulting fractions 5 were subjected to the in vitro ubiquitination assay as in C in the absence of Cks1 and cyclin E-CDK2.

cleus requires Skp2, and that phosphorylation of p27Kip1 on Thr187 by the cyclin E-CDK2 complex is necessary for Skp2-mediated polyubiquitination.

Given that p27Kip1 is translocated from the nucleus to the cytoplasm by Jab1-mediated nuclear export (35), we next examined whether molecular machinery for polyubiquitination of p27Kip1 is present in the cytoplasm. Cytoplasmic extracts were prepared from Skp2+/+ and Skp2−/− cells and were fractionated by ion-exchange chromatography. The resulting fractions 5 were then included in the in vitro ubiquitination assay with wild-type p27Kip1 or the T187A mutant as the substrate. Similar extents of polyubiquitination were apparent with the fractions derived from Skp2+/+ and Skp2−/− cells as well as with wild-type p27Kip1 and the T187A mutant (Fig. 6D). Thus, this cytoplasmic polyubiquitination activity is independent both of Skp2 and of phosphorylation of p27Kip1 on Thr187. The putative ubiquitin ligase responsible for this Skp2-independent polyubiquitination appears to be located predominantly in the cytoplasm, given that the corresponding fraction derived from nuclear extracts did not contain such an activity. Together, our results suggest the existence of two pathways of p27Kip1 ubiquitination: a Skp2- and Thr187 phosphorylation-dependent pathway in the nucleus, and a Skp2- and Thr187 phosphorylation-independent pathway in the cytoplasm.

Finally, we examined the cell cycle dependence of the polyubiquitination activities in the nucleus and cytoplasm. Nuclear and cytoplasmic extracts were prepared from Skp2+/+ and Skp2−/− MEFs synchronized at G0 phase (Fig. 7A) or at S-G2 phases (Fig. 7B), and were subjected to the in vitro ubiquitination assay. The polyubiquitination activity in the nuclear extract of Skp2+/+ cells was greater at S-G2 than at G0. In contrast, the activity in the cytoplasmic extracts of both Skp2+/+ and Skp2−/− cells at G0 was similar to that at S-G2. Thus, the Skp2- and Thr187 phosphorylation-dependent pathway of p27Kip1 polyubiquitination in the nucleus is active when cells enter successive rounds of the cell cycle. In contrast, the cytoplasmic polyubiquitination pathway is active at both G0 and S-G2 phases. Given that signal-dependent nuclear export of p27Kip1 occurs at the G0-G1 transition,2 exported p27Kip1 may be polyubiquitinated by the constantly active ubiquitination machinery in the cytoplasm.

DISCUSSION

The amount of p27Kip1 is relatively high in quiescent (G0) cells, decreases on entry of cells into the cell cycle, and is controlled predominantly by the rate of p27Kip1 degradation (16, 17). This CKI undergoes ubiquitination and is degraded in a proteasome-dependent manner (5). Phosphorylation of p27Kip1 on Thr187 is mediated by the cyclin E-CDK2 complex and has been shown to be required for ubiquitination of p27Kip1 (23–25). Previous studies indicate that Skp2 specifically interacts with the extreme COOH terminus of p27Kip1 (26–28). This association of Skp2 with p27Kip1 results in recruitment of the latter to the SCF core complex, thereby promoting its ubiquitination and degradation. This ubiquitination process was recently shown to be markedly enhanced by Cks1 associated with Skp2 (29, 30).

In parallel with Skp2-mediated ubiquitination, p27Kip1 is exported from the nucleus and degraded. This export step appears to be dependent on Jab1, a component of the 450-kDa COP9 (signalsome) complex (35). Our unpublished data indicate, however, that the Jab1-mediated export of p27Kip1 from the nucleus at the G0-G1 transition occurs in a Skp2-independent manner.2 Furthermore, given that Skp2 is localized pre-

2. N. Ishida and K.-I. Nakayama, manuscript in preparation.
The Skp2-dependent mechanism, mediated by the SCFSkp2, targets this protein to the degradation machinery in the cytoplasm.

The stability of p27Kip1 is markedly increased in cells in G0 phase of the cell cycle compared with that apparent in cells in S or M phase. The stability of p27Kip1, therefore propose that p27Kip1 may be a determinant of the nuclear export of p27Kip1 or of the ubiquitination of this protein in the cytoplasm.

Dominantly to the nucleus, the ubiquitination of p27Kip1 at this stage of the cell cycle is likely mediated by a ubiquitin ligase (other than SCFSkp2) that is located in the cytoplasm. We therefore propose that p27Kip1 is degraded by at least two distinct mechanisms, one of which is dependent on Skp2 and one of which is not (Fig. 8). The Skp2-independent mechanism operates in the cytoplasm and degrades p27Kip1 at the G0-G1 transition. Jab1-mediated nuclear export of p27Kip1 may transfer this protein to the degradation machinery in the cytoplasm. The Skp2-dependent mechanism, mediated by the SCFSkp2 ubiquitin ligase, serves to degrade p27Kip1 that is phosphorylated on Thr187 in the nucleus during S-G2 phases in successive rounds of the cell cycle.

In addition to Thr187, phosphorylation of other residues of p27Kip1 may be important in the control of its stability. We recently showed that phosphorylation of Ser10 accounted for ~70% of the total phosphorylation of p27Kip1, and that the extent of phosphorylation at this site was ~75-fold greater than that at Thr187 (36). The extent of Ser10 phosphorylation is markedly increased in cells in G0 phase of the cell cycle compared with that apparent in cells in S or M phase. The stability of p27Kip1 phosphorylated on Ser10 is also substantially greater than that of the unphosphorylated form of the protein. These observations suggest that Ser10 is the major site of phosphorylation of p27Kip1, and that phosphorylation at this site (like that at Thr187) contributes to the regulation of p27Kip1 stability (36). The increased stability of the Ser10-phosphorylated form of p27Kip1 suggests that dephosphorylation of this residue might play an important role in progression of the cell cycle from G0 to G1. It is possible that phosphorylation of Ser10 may be a determinant of the nuclear export of p27Kip1 or of the ubiquitination of this protein in the cytoplasm.

The most obvious cellular phenotype of Skp2−/− mice is the presence of markedly enlarged, polyploid nuclei and multiple centrosomes (31). However, there is substantial tissue variability in the penetrance of this phenotype. It is most prominent in liver, kidney, lung, and testis, whereas hematopoietic cells and neurons appear normal. Although the reason for such tissue differences has been unclear, they might result from variability in dependence on the two distinct mechanisms of p27Kip1 degradation. Purification of the activity responsible for the ubiquitination of p27Kip1 in the cytoplasm and molecular identification of the corresponding putative ubiquitin ligase should provide insight into these issues.

Mutations in the p27Kip1 gene appear to be rare in human cancers. However, a reduced abundance of p27Kip1 in a subset of colon and breast cancers correlates well with poor prognosis (37−40). Furthermore, the loss of p27Kip1 alleles in mice increases the sensitivity of these animals to cancer-inducing agents (14). Identification of components of the protein degradation machinery that determine the turnover rate of p27Kip1 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 (41), suggesting that p27Kip1 degradation mediated by Skp2 may be related to carcinogenesis. It is thus possible that the putative ubiquitin ligase responsible for the Skp2-independent pathway of p27Kip1 degradation is also deregulated in cancer cells.

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Addendum—During revision of this manuscript, Malek et al. (42) demonstrated the existence of two pathways of p27Kip1 degradation, one dependent on Thr187 phosphorylation and one not, with the use of a mouse "knock-in" model. Although our present results are mostly consistent with those of Malek et al., these latter researchers suggested that the degradation of p27Kip1 at the G0-G1 transition is dependent on Skp2. This discrepancy might be attributable to a difference in the number of passages of the MEFs studied. We have thus observed that the Skp2-independent ubiquitination activity apparent at the G0-G1 transition is prominent in lymphocytes and in MEFs subjected to a small number of passages, whereas senescent MEFs are prone to loss of this activity with successive passages.2

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FIG. 7. Constant activity of the Skp2-independent p27Kip1 polyubiquitination pathway in the cytoplasm. Nuclear and cytoplasmic extracts were prepared from Skp2−/− and Skp2+/− MEFs synchronized either at G0 phase (A) or at S-G2 phases (B). The extracts were fractionated by stepwise elution with KCl from a DE52 column, and the resulting fractions were subjected to in vitro ubiquitination assays with wild-type p27Kip1 or the T187A mutant in the presence of Csk1, cyclin E-CDK2, and the combination (Reaction 2) of Uba1, Ubc5a, and GST-ubiquitin, as indicated. Reaction products were detected by immunoblot analysis with antibodies to p27Kip1.

FIG. 8. Model for cell cycle regulation through p27Kip1 degradation by two distinct mechanisms. The degradation of p27Kip1 in the nucleus during successive phases (G0-S-G1-M) of the cell cycle appears to be regulated by the SCFSkp2 ubiquitin ligase, which targets p27Kip1-phosphorylated on Thr187 (T187-P). In contrast, p27Kip1 appears to be ubiquitinated in the cytoplasm at the G0-G1 transition by an as-yet-unidentified ubiquitin ligase that functions independently both of Skp2 and of phosphorylation of p27Kip1 on Thr187.
Degradation of p27 Kip1 at the G0-G1 Transition Mediated by a Skp2-independent Ubiquitination Pathway

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