Phosphorylation of p27\(^{\text{Kip1}}\) on Serine 10 Is Required for Its Binding to CRM1 and Nuclear Export*

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Phosphorylation of the cyclin-dependent kinase inhibitor p27\(^{\text{Kip1}}\) has been thought to regulate its stability. Ser\(^{10}\) is the major phosphorylation site of p27\(^{\text{Kip1}}\), and phosphorylation of this residue affects protein stability. Phosphorylation of p27\(^{\text{Kip1}}\) on Ser\(^{10}\) has now been shown to be required for the binding of CRM1, a carrier protein for nuclear export. The p27\(^{\text{Kip1}}\) protein was translocated from the nucleus to the cytoplasm at the G\(_0\)-G\(_1\) transition of the cell cycle, and this export was inhibited by leptomycin B, a specific inhibitor of CRM1-dependent nuclear export. The nuclear export and subsequent degradation of p27\(^{\text{Kip1}}\) at the G\(_0\)-G\(_1\) transition were observed in cells lacking Skp2, the F-box protein component of an SCF ubiquitin ligase complex, indicating that these early events are independent of Skp2-mediated proteolysis. Substitution of Ser\(^{10}\) with Ala (S10A) markedly reduced the extent of p27\(^{\text{Kip1}}\) export, whereas substitution of Ser\(^{10}\) with Asp (S10D) or Glu (S10E) promoted export. Co-immunoprecipitation analysis showed that CRM1 preferentially interacted with S10D and S10E but not with S10A, suggesting that the phosphorylation of p27\(^{\text{Kip1}}\) on Ser\(^{10}\) is required for its binding to CRM1 and for its subsequent nuclear export.

The cell cycle of eukaryotic cells is regulated by a series of protein complexes composed of cyclins and cyclin-dependent kinases (CDKs)\(^1\), the activity of which is suppressed by a group of CDK inhibitors (CKIs)\(^1\), 2). Among the CKIs, p27\(^{\text{Kip1}}\) plays a pivotal role in the control of cell proliferation\(^3\)–7). The amount of p27\(^{\text{Kip1}}\) is high during the G\(_1\) phase of the cell cycle in normal cells, but it rapidly decreases on reentry of cells into G\(_1\) phase\(^8\), 9). We and others have shown that mice homozygous for deletion of the p27\(^{\text{Kip1}}\) gene are larger than normal mice and that they exhibit multiple organ hyperplasia as well as a predisposition to cancer\(^10\)–13). These observations support the notion that p27\(^{\text{Kip1}}\) is a key determinant of both body size and the size of organs as a result of its role in the control of cell proliferation and that the loss of p27\(^{\text{Kip1}}\) function may lead to carcinogenesis. Indeed many studies have shown that the expression of p27\(^{\text{Kip1}}\) is deregulated in various human cancers (for review, see Ref. 14).

The function of p27\(^{\text{Kip1}}\) is regulated by changes in its concentration as well as in its localization in the cell. The concentration of p27\(^{\text{Kip1}}\) is thought to be controlled predominantly by the ubiquitin-proteasome pathway\(^15\). Degradation of p27\(^{\text{Kip1}}\) is promoted by its phosphorylation on Thr\(^{187}\) by the cyclin E-CDK2 complex\(^16\), 17), and the phosphorylation of this residue is required for the binding of p27\(^{\text{Kip1}}\) 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 ubiquitination and degradation of p27\(^{\text{Kip1}}\)\(^18\)–22). We have recently shown that the degradation of p27\(^{\text{Kip1}}\) at the G\(_0\)-G\(_1\) transition is independent of Skp2 and occurs in the cytoplasm, whereas the Skp2- and Thr\(^{187}\) phosphorylation-dependent degradation of p27\(^{\text{Kip1}}\) occurs in the nucleus\(^23\). These observations suggest that the nuclear export of p27\(^{\text{Kip1}}\) may be critical for its down-regulation early during reentry of quiescent cells into the cell cycle. Consistent with this notion, Jab1 promotes the translocation of p27\(^{\text{Kip1}}\) from the nucleus to the cytoplasm, decreasing the amount of p27\(^{\text{Kip1}}\) in the cell by accelerating its degradation\(^24\).

We previously identified Ser\(^{10}\) as a major phosphorylation site of p27\(^{\text{Kip1}}\), accounting for ~70% of the total phosphorylation of this protein, and the extent of phosphorylation at this site was 75-fold greater than that at Thr\(^{187}\)\(^25\). The extent of Ser\(^{10}\) phosphorylation was markedly increased in cells in the G\(_0\)-G\(_1\) phase of the cell cycle compared with that apparent for cells in S or M phase. Mutation analysis revealed that phosphorylation of Ser\(^{10}\), like that of Thr\(^{187}\), contributes to regulation of p27\(^{\text{Kip1}}\) stability. We now show that Ser\(^{10}\) phosphorylation is required for the binding of p27\(^{\text{Kip1}}\) to CRM1, a carrier protein for nuclear export, and that the substitution of Ser\(^{10}\) with other residues affects the nuclear export of p27\(^{\text{Kip1}}\). Our data suggest that Ser\(^{10}\) phosphorylation is a key event in regulation of the function of p27\(^{\text{Kip1}}\) at the G\(_0\)-G\(_1\) transition.

**EXPERIMENTAL PROCEDURES**

Cell Culture and Synchronization—Mouse embryonic fibroblasts (MEFs) were prepared from 13.5-day-postcoitum Skp2\(^{-/-}\) and Skp2\(^{-/-}\) embryos as described previously\(^26\). Only nonsenescent MEFs (no other with other residues affects the nuclear export of p27\(^{\text{Kip1}}\). Our data suggest that Ser\(^{10}\) phosphorylation is a key event in regulation of the function of p27\(^{\text{Kip1}}\) at the G\(_0\)-G\(_1\) transition.

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The abbreviations used are: CDK, cyclin-dependent kinase; CKI, CDK inhibitor; MEF, mouse embryonic fibroblast; GFP, green fluores-
Nuclear Export of Phosphorylated p27\textsuperscript{Kip1} by CRM1

When the nuclear export of p27\textsuperscript{Kip1} was monitored by immunoblot analysis of nuclear and cytoplasmic fractions of the cells (Fig. 1B). The amount of p27\textsuperscript{Kip1} in the nuclear fraction thus gradually decreased, whereas that in the cytoplasmic fraction was transiently increased at 7–10.5 h after the onset of serum stimulation. Given that the expression of Skp2 was not detected until 10.5 h after the onset of stimulation and that Skp2 was localized predominantly to the nucleus (28), Skp2 likely does not contribute to the translocation and degradation of p27\textsuperscript{Kip1} during this time period. Treatment of cells with leptomycin B, a specific inhibitor of CRM1-dependent nuclear export (29), blocked the translocation of p27\textsuperscript{Kip1} but did not prevent the decrease in the abundance of this protein (Fig. 1A), suggesting that the nuclear export of p27\textsuperscript{Kip1} is not required for its degradation. Furthermore, the addition of MG132, a rapid-acting inhibitor of the proteasome, to the culture medium together with leptomycin B inhibited the degradation of p27\textsuperscript{Kip1} in the nucleus. These data suggest the existence of two independent pathways for p27\textsuperscript{Kip1} proteolysis, one in the nucleus and one in the cytoplasm.
Our previous report demonstrated that the extent of Ser¹⁰ phosphorylation was markedly increased in cells in the G₀ phase of the cell cycle compared with that apparent for cells in S or M phase (25). We thus examined the relative amounts of two (phosphorylated versus nonphosphorylated) forms of p27^Kip₁ in the nucleus versus cytoplasm (Fig. 1C). Two-dimensional electrophoresis and immunoblot analysis with antibodies against endogenous p27^Kip₁ (upper panels of each set), and the resulting images were superimposed with those of nuclear staining with Hoechst 33258 (lower panels). A, Skp2⁺/⁺ and Skp2⁻/⁻ MEFs synchronized at G₀ phase by serum deprivation for 96 h were restimulated to enter the cell cycle by exposure to 20% serum for the indicated times. Cells were then subjected to immunostaining for endogenous p27^Kip₁ (upper panels) and/or its S10D, S10E, S10A, or T187A mutants. 

Nuclear Export of Phosphorylated p27^Kip₁ by CRM1

The stimulation-induced translocation of the S10A mutant of p27^Kip₁ was markedly inhibited compared with that observed with the wild-type and S10D proteins. Quantitative analysis revealed that wild-type, S10D, and S10A derivatives of p27^Kip₁ were located in the nucleus in the absence of serum stimulation (Fig. 4A), suggesting that Ser¹⁰ phosphorylation is not sufficient for nuclear export. This notion is consistent with our previous observation that p27^Kip₁ was highly phosphorylated at Ser¹⁰ in the nucleus of quiescent cells even though Ser¹⁰ is highly phosphorylated (25).

Finally we examined the ability of the various p27^Kip₁ derivatives to undergo translocation from the nucleus to the cytoplasm. Wild-type and mutant derivatives of p27^Kip₁ were expressed in NIH 3T3 cells with the use of the retroviral Dox-regulated system. The expression of the p27^Kip₁ derivatives was induced by Dox during serum deprivation for 96 h and was then terminated by removal of Dox from the medium at which time serum was added back to the medium to induce the translocation of p27^Kip₁. Immunofluorescence analysis revealed that wild-type, S10D, and S10A derivatives of p27^Kip₁ were located in the nucleus in the absence of serum stimulation (Fig. 4A), suggesting that Ser¹⁰ phosphorylation is not sufficient for nuclear export. This notion is consistent with our previous observation that p27^Kip₁ was highly phosphorylated at Ser¹⁰ in the nucleus of quiescent cells even though Ser¹⁰ is highly phosphorylated (25). The stimulation-induced translocation of the S10A mutant of p27^Kip₁ was marked reduced compared with that observed with the wild-type and S10D proteins. Quantitative analysis indicated that the efficiency of export was greater for the S10D mutant than for wild-type p27^Kip₁ (Fig. 4B). The amount of the S10A mutant remaining in the nucleus was markedly greater than that for p27^Kip₁ (Fig. 4B). The amount of this protein in the nucleus of stimulated cells never achieved the level apparent in quiescent cells (Fig. 4B) suggest that a...
fraction of p27<sup>Kip1</sup> is exported from the nucleus by a mechanism independent of CRM1 and Ser<sup>10</sup> phosphorylation. These results indicate that phosphorylation of Ser<sup>10</sup> of p27<sup>Kip1</sup> is required for the binding of CRM1 and subsequent translocation of p27<sup>Kip1</sup> to the cytoplasm. However, the phosphorylation is not sufficient for the nuclear export, and another factor(s) may be necessary in addition to the phosphorylation of Ser<sup>10</sup>.

Nuclear Export Controlled by Ser<sup>10</sup> Phosphorylation Is Important for p27<sup>Kip1</sup> Down-regulation at the G<sub>S</sub>-G<sub>1</sub> Transition—We have recently shown that the ubiquitin-mediated proteolysis of p27<sup>Kip1</sup> at the G<sub>S</sub>-G<sub>1</sub> transition occurs normally in Skp2<sup>−/−</sup> cells (23), whereas the degradation of this protein during S and G<sub>2</sub> phases is markedly impaired in these cells. Given also that Skp2 is not expressed in the early phase (G<sub>0</sub>-S), whereas the degradation of this protein in the nucleus and are means ± S.E. of values from three independent experiments. WT, wild type.

nuclear export of p27<sup>Kip1</sup>. Although our present results are mostly consistent with those of Rodier et al., these latter researchers demonstrated that endogenous p27<sup>Kip1</sup> phosphorylated on Ser<sup>10</sup> is translocated from nucleus into cytoplasm by using the antibody that is specific for p27<sup>Kip1</sup> phosphorylated on Ser<sup>10</sup>. We added the finding that p27<sup>Kip1</sup> physically associates with the carrier protein CRM1 and that the formation of this complex is controlled by the phosphorylation of p27<sup>Kip1</sup> on Ser<sup>10</sup>. Both reports suggest that the nuclear export of p27<sup>Kip1</sup> is regulated by the phosphorylation on Ser<sup>10</sup> and plays a critical role to decrease the abundance of p27<sup>Kip1</sup> protein below a certain threshold to allow the activation of cyclin-CDK complexes.

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