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Successive Phosphorylation of $p27^{KIP1}$ Protein at Serine-10 and C Terminus Crucially Controls Its Potency to Inactivate Cdk2*[S]

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Atish R. Mohanty[‡], Qiuming Kan[‡], Saumya Srivastava[‡], Baasanjav Uranbileg[‡], Shiho Arakawa-Takeuchi[‡], Naoya Fujita[§], and Hiroto Okayama[‡1]

From the [‡]Department of Biochemistry and Molecular Biology, Graduate School and Faculty of Medicine, The University of Tokyo, Tokyo 113-0033 and [§]Cancer Chemotherapy Center, Japanese Foundation for Cancer Research, Tokyo 135-8550, Japan

Background: $p27^{KIP1}$ Cdk inhibitor undergoes phosphorylation at serine-10 and the C terminus.
Results: C-terminal phosphorylation requires prior serine-10 phosphorylation. When double-phosphorylated, unbound p27 no longer inactivates Cdk2, and Cdk2-bound p27 can be removed by Cdc6 to reactivate the Cdk2.
Conclusion: Phosphorylation at these sites critically controls the ability of p27 to inactivate Cdk2.
Significance: A new mechanism controlling the inhibitor potency of p27 is discovered.

During the G_1-S transition, the activity of Cdk2 is regulated by its association with $p27^{KIP1}$, which in rodent fibroblasts undergoes phosphorylation mainly at serine 10, threonine 187, and C-terminal threonine 197 by KIS, Cdk2, and Pim or ROCK, respectively. Recently Cdc6 the AAA+ ATPase, identified initially to assemble pre-replicative complexes on origins of replication and later to activate $p21^{CIP1}$-inactivated Cdk2, was found also to activate p27-bound Cdk2 but only after the bound p27 is C-terminally phosphorylated. On the other hand, the biological significance of the serine 10 phosphorylation remains elusive aside from its involvement in the stability of p27 itself. We report here that serine 10 phosphorylation is required for efficient C-terminal phosphorylation of its own by PIM and ROCK kinases and critically controls the potency of p27 as a Cdk2 inhibitor. *In vitro*, PIM1 and active ROCK1 efficiently phosphorylated free as well as Cdk2-bound p27 but only when the p27 was phosphorylated at Ser-10 in advance. Consistently, a Ser-10 nonphosphorylatable mutant p27 protein was not phosphorylated at the C terminus *in vivo*. Furthermore, when double-phosphorylated, free p27 was no longer a potent inhibitor of Cdk2, and Cdk2-bound p27 could be removed by Cdc6 to reactivate the Cdk2. Thus, phosphorylation at these two sites crucially controls the potency of this CDK inhibitor in two distinct modes.

The onset of S phase is prepared in advance by the assembly of prereplicative complexes that takes place in late M and G_1 phases (1, 2). Cdc6 the AAA+ ATPase catalyzes this assembly by loading the minichromosome maintenance (MCM) helicase complexes on the origin recognition complex (ORC)-bound origins of replication. Thereafter several factors, some of which require activation by Cdk2, are further loaded on the origins, MCM helicase is then activated by Cdc7 and finally, DNA polymerases are recruited to those origins to initiate DNA replication (3). Cdc6 has an additional function as an activator of $p21^{CIP1}$-inactivated Cdk2 (4, 5).

During the transition from G_1 to S phase, the activity of Cdk2 is tightly regulated by its association with $p27^{KIP1}$ the CDK inhibitor (6). During this transition, p27 undergoes modification by phosphorylation at Ser-10 catalyzed by the KIS and MIRK kinases, at Thr-187 catalyzed by Cdk2, and at C-terminal Thr-197 catalyzed by the PIM kinases in rodent fibroblasts (7–10). Phosphorylation at Ser-10 and Thr-197 occurs early in growth-stimulated cells and facilitates its own stabilization and nuclear exclusion, whereas Cdk2-mediated Thr-187 phosphorylation promotes the SKP2 ubiquitin ligase-mediated degradation of its own (11–14). In addition to these regulations, it was recently discovered that C-terminal Thr-197 phosphorylation of p27 is required for the activation of the p27-bound Cdk2 by Cdc6 the AAA+ ATPase in an entirely new mechanism (15). Furthermore, this C-terminal phosphorylation was found to be exerted not only by PIM kinases but also by ROCK1 kinase that mediates an anchorage signal to promote actin fiber assembly as well as activate mTORC1 signaling (16, 17). Thus, an additional biological role of C-terminal Thr-197 phosphorylation was discovered. On the other hand, the biological significance of the Ser-10 phosphorylation still remains elusive because even its involvement in the nuclear export of p27 itself is controversial (11, 14).

We, therefore, examined the effects of the Ser-10 phosphorylation in combination with the C-terminal Thr-197 phosphorylation of p27 on its ability to inactivate Cdk2 in a free or Cdk2-bound form and found a new biological role for phosphorylation at these sites; that is, the control of the potency of p27 to inhibit Cdk2 during the G_0-S transition.

EXPERIMENTAL PROCEDURES

Antibodies and Chemicals—Anti-Cdk2 (M2)-G, anti-Cdk2(M2), anti-Pim (12H8), anti-p27 (F5), anti-p27 (M197),

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[S] This article contains supplemental Fig. S1.
[1] To whom correspondence should be addressed: E-mail: okayama@m.u-tokyo.ac.jp; okayama-tky@umin.net.

and anti-phospho-p27 (Thr-187) antibodies and other antibodies against Cdk6, cyclin D1, and cyclin D2 were purchased from Santa Cruz Biotechnology; anti-KIS (N-term), anti-Cdk4, anti-cyclin A and anti-β-actin antibodies were from Sigma; anti-cyclin D3 and anti-phospho-Rb (Ser-780) was from MBL; anti-Rb and anti-p70 (S6 kinase 1 (S6K1)) was from BD Biosciences; anti-Cdc6 (DCS180) was from Neomarkers; anti-phospho-p27(Ser-10) was from Epitomics; anti-phospho-p27(Thr-198) was from R&D Systems; anti-phospho-LIMK1 (Thr-508) was from Abcam; anti-phospho-Cdk2 (Thr-160), anti-phospho-S6K1 (Thr-389), anti-phospho-Rb (Ser-807/811), anti-LIMK, and anti-ROCK1 were from Cell Signaling Technology. Anti-Cdk2 (M2)-G conjugated with agarose was obtained from Santa Cruz Biotechnology. Protein G-Sepharose 4 Fast Flow was obtained from GE Healthcare. Protein G-agarose Fast Flow was obtained from Millipore, and glutathione-Sepharose beads were from GE Healthcare. Baculovirus-expressed and affinity-purified active Cdk2-cyclin A complexes were purchase from Millipore.

Cell Culture—Rat embryonic fibroblasts (REF)[2] were maintained in DMEM with 10% fetal calf serum at 35 °C. For methylcellulose semisolid culture, logarithmically proliferating REF and its derivative were harvested by trypsinization and embedded in semisolid 1.17% methylcellulose medium containing DMEM with 10% FCS at a density of 1×10^6 cells per 10 ml in a 50-ml falcon tube or 250 ml of conical tube and incubated at 35 °C.

Cell Construction—REF cells overexpressing PIM1 (REF-PIM1) were constructed with the retroviral expression vector pQCXIN harboring an N-terminally histidine hexamer-tagged human PIM1 c-DNA (GenBank^{TM} accession number NM_002648) as described (10). REF-PIM1 cells inducible for Cdc6 expression were constructed with the pRevTRE response vector and the pRevTet-Off vector harboring a histidine hexamer-tagged Cdc6 cDNA N-terminally as described (4). Cells were maintained in DMEM containing 10% FCS and 1 μg of doxycycline per ml.

Preparation of Histidine Hexamer-tagged Recombinant Proteins—The pQCXIN mammalian expression vector harboring a C-terminally histidine hexamer-tagged rat KIS cDNA was constructed, transfected into logarithmically proliferating REF cells, and incubated for 48 h before cell lysis. The histidine hexamer-tagged KIS protein was purified with Ni-NTA beads and neutralized for EDTA with $MgCl_2$.

N-terminally histidine hexamer-tagged rat wild-type p27 and phosphomimetic mutants p27^{S10D}, p27^{T197D}, and p27$^{S10D/T197D}$ were constructed by polymerase chain reaction with appropriately designed primers and expressed in *Escherichia coli* with the pGEX-5X-1 vector (GE Healthcare). These recombinant p27 proteins were then affinity-purified with the Ni-NTA beads. C-terminally histidine hexamer-tagged PIM1 and active ROCK1 (16) was expressed in *E. coli* and affinity-purified as described above.

Preparation of Recombinant Cdc6—Rat Cdc6 C-terminally tagged with 3×FLAG and a histidine hexamer was expressed in Sf9 cells by using a baculovirus vector and affinity-purified with anti-FLAG (M2) gel (Sigma) and Ni-NTA beads.

In Vitro Phosphorylation of p27—PIM1- and active ROCK1-catalyzed *in vitro* Thr-197 phosphorylation of unbound p27 was performed with p27 and p27^{S10D} as substrate in 30 μl of a reaction mixture containing 10 mM ATP, 30 mM $MgCl_2$, and 50 mM Tris-HCl (pH 7.5). Alternatively, p27 was pretreated with KIS or empty vector preparation in a 30-μl reaction mixture containing 50 mM MES (pH 7.5), 10 mM ATP, 30 mM $MgCl_2$, 10% glycerol, 1 mM inhibitor mixture, and 10 mM β-glycerophosphate. After incubation for 30 min, PIM1 was added to the above reaction mix and incubated further for up to 60 min at 30 °C.

For PIM1- or active ROCK1-catalyzed *in vitro* phosphorylation of Cdk2-bound p27, Cdk2-bound p27 was prepared as follows. Commercially available baculovirus-expressed, affinity-purified active Cdk2-cyclin A complexes (10–20 ng) and *E. coli*-expressed p27 were incubated at 30 °C for 30 min in 50 mM Tris-HCl (pH 7.5) containing 150 mM concentrations each of NaCl and $MgCl_2$, the latter to neutralize the EDTA contained in the p27 preparation. After incubation, Cdk2 was immunoprecipitated with agarose-conjugated Cdk2 (M2)-goat antibody. The beads were washed with 50 mM Tris-HCl (pH 7.5) containing 150 mM NaCl. PIM1- or active ROCK1-catalyzed *in vitro* phosphorylation of Cdk2-bound p27 was performed as above.

Assay for Cdk2 Inhibition by Phosphomimetic or Phosphorylated p27—Active Cdk2-cyclin A complexes (10–20 ng) and a predetermined minimal amount or its equivalent of p27, p27^{S10D}, p27$^{S10D, S197D}$, Ser-10-phosphorylated p27, Ser-10/Thr-197- double phosphorylated p27 or a control empty vector preparation was incubated at 30 °C for 30 min in 30 μl of 50 mM Tris-HCl (pH 7.5) containing 150 mM concentrations each of NaCl and $MgCl_2$, the latter to neutralize the EDTA and immunoprecipitated with agarose-conjugated Cdk2 (M2)-goat antibody. The immunoprecipitated Cdk2 complex was washed twice with 50 mM Tris-HCl (pH 7.5) containing 10 mM $MgCl_2$ and assayed for Cdk2 activity and for the amount of the immunoprecipitated Cdk2. Throughout the experiments, Cdk2 activity was determined by *in vitro* phosphorylation of Rb protein and subsequent immune-detection of Ser-807/811- phosphorylated Rb as described (18).

In Vitro Cdk2 Reactivation Assay—Active Cdk2-cyclin A complexes (10–20 ng) were incubated at 30 °C for 30 min with a minimal amount of predetermined p27 or a control empty vector preparation in 30 μl of a 50 mM Tris-HCl (pH 7.5) buffer containing 150 mM concentrations each of NaCl and $MgCl_2$, the latter to neutralize EDTA and immunoprecipitated with agarose-conjugated Cdk2 (M2)-goat antibody. The p27-bound inactive Cdk2 was washed once with the reaction buffer and incubated at 30 °C in a 30-μl reaction mixture containing 50 mM MES (pH 7.5), 10 mM ATP, 30 mM $MgCl_2$, 10% glycerol, 1 mM Inhibitor mixture, and 10 mM β-glycerophosphate for 15 min with KIS or a control empty vector preparation first and then for 30 min with the addition of PIM1. The bead-bound Cdk2 complexes were recovered by a brief centrifugation,

[2] The abbreviations used are: REF, rat embryonic fibroblast; MC, methylcellulose culture; Ni-NTA, nickel-nitrilotriacetic acid; S6K1, S6 kinase 1; LIMK, Lim kinase; Rock, Rho-associated coiled-coil forming kinase; Pim, proviral integration Moloney virus; KIS, kinase interacting with stathmin; MIRK, mini-brain-related kinase.

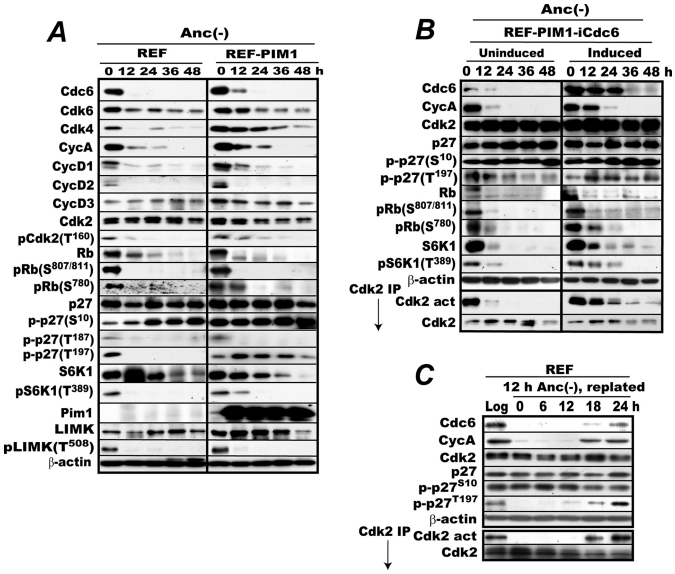

FIGURE 1. **Overexpression of PIM1 leads to continued C-terminal phosphorylation of p27 but not activation of mTORC1.** *A*, logarithmically proliferating REF and REF-Pim1 cells were incubated in methylcellulose semisolid medium (*Anc(−)*) for the indicated times and analyzed by immunoblotting for the levels of Cdc6, Cdk6, Cdk4, three D-type cyclins, Cdk2, its Thr-160-phosphorylated form, Rb and its Ser-807/811 and Ser-780 phosphorylated forms that reflect Cdk2 and Cdk4/Cdk6 activities, respectively, p27, its three phosphorylated forms, S6K1, and its Thr-389 phosphorylated form that reflects mTORC1 activity, Pim1, Lim kinase (LIMK), and its Thr-508 phosphorylated form. *B*, logarithmically proliferating REF-PIM1-iCdc6 cells were induced for Cdc6 protein by withdrawal of doxycycline, then cultured in MC (*Anc(−)*) and determined for the levels of the indicated factors and Cdk2 activity. Cdk2 activity was assayed by *in vitro* phosphorylation of Rb and subsequent immune-detection of Ser-807/811-phosphorylated Rb (18). *C*, logarithmically proliferating REF cells were incubated in MC for 12 h, re-cultured in anchorage-furnished culture dishes for the indicated times, and analyzed for the indicated factors and Cdk2 activity as in *B*. As a positive control, log-phase proliferating REF was similarly analyzed. *IP*, immunoprecipitate. *Anc*, anchorage.

washed with a buffer containing 50 mM Tris-HCl (pH 7.5) and 10 mM $MgCl_2$, and incubated at 30 °C for 30 min in 20 μl of a reactivation buffer containing 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 10 mM ATP, and Cdc6 or a control empty vector preparation. After reactivation, the reaction mixture was split into two parts. One part was used for determining the amounts of the indicated factors in the reactivation mixture. The other part was briefly centrifuged to collect beads, and the bead-bound Cdk2 complexes were washed and assayed for Cdk2 activity and the amounts of bound Cdk2 and Cdc6.

RESULTS

Combined Overexpression of PIM1 and Cdc6 Transiently Activates Cdk2 without Activation of mTORC1 in Absence of Anchorage—We previously showed that C-terminal phosphorylation of p27, which is quickly lost upon anchorage deprivation, is exerted by PIM and ROCK in rodent fibroblasts (10, 15). To confirm PIM1-mediated C-terminal phosphorylation of p27 under anchorage deprivation *in vivo* and the functional distinction between PIM1 and ROCK1, we constructed by retrovirus-mediated gene transfer and analyzed REF-overexpressing PIM1 (REF-PIM1) during culture in anchorage-free methylcellulose medium(MC). As already demonstrated, one effective way to understand molecular events during the onset of cell proliferation is to analyze the cells arresting in G_1 by culturing in MC while furnished with growth factors (15–17). Original REF and REF-PIM1 cells were cultured in MC with cell sampling every 12 h for 48 h and analyzed for phosphorylation of p27 at Thr-197 and S6K1 at Thr-389, the latter a hallmark of activated mTORC1, as well as several cell cycle and related factors (Fig. 1*A*). As anticipated, in the PIM1 overexpressor, p27 Thr-197 continued to be phosphorylated during MC culture. In sharp contrast to in the REF-overexpressing active ROCK1 (16), the phosphorylation of S6K1 at Thr-389 disappeared upon anchorage deprivation just like in original REF even though one of the highest PIM1-overexpressing REF cell clones was used for analysis as shown here. Furthermore, unlike active ROCK1, Pim1 failed to phosphorylate Lim kinase at Thr-508. Its phosphorylation was immediately lost despite continued Pim1 expression. This result establishes that Pim1 and Rock1 do not share phosphorylation targets in general, although they both phosphorylate the C terminus of p27.

We next examined the effects of enforced expression of Cdc6 on Cdk2 activity during MC (Fig. 1*B*). For this purpose, REF-PIM1 cells additionally inducible for Cdc6 (REF-PIM1-iCdc6) by withdrawal of doxycycline were constructed as before (15, 16), induced or uninduced for enforced Cdc6 expression, and analyzed for Cdk2 activity and the levels of Rb Ser-780 phosphorylation and S6K1 Thr-389 phosphorylation, hallmarks of Cdk4/Cdk6 activity and mTORC1 activity, respectively. In this cell, induced Cdc6 protein was far more unstable than in mTORC1-activated cells perhaps because of inactivation of Cdk4/Cdk6 and consequent failed induction of Emi1 the G_1 phase inhibitor of the APC/C^{CDH1} ubiquitin ligase that degrades Cdc6 (17). Consistently, Cdk2 activity markedly diminished at 36–48 h of the MC. Furthermore, unlike in REF overexpressing Cdc6 and active ROCK1 (16), cyclin A protein that is required for mitosis (19) disappeared within 36 h. These *in vivo* data support the recent finding that Cdc6 activates p27-bound Cdk2 in a manner absolutely dependent on C-terminal phosphorylation of the bound p27 (15). However, unlike REF overexpressing both Cdc6 and active ROCK1, which proliferated in anchorage-free soft agar medium (16), REF overexpressing both Cdc6 and PIM1 shown here is unlikely to have such an ability because both Cdc6 and cyclin A required for S phase onset and mitosis, respectively, ceased expression during 48 h of MC.

To further confirm the double dependence of activation of p27-bound Cdk2 on Cdc6 and C-terminal phosphorylation of the bound p27, we carried out anchorage deprivation-and-replate analysis. Proliferating REF cells were incubated in MC for 12 h to inactivate the Cdk2 with Thr-197 dephosphorylation of the p27, re-cultured in anchorage-furnished culture dishes with every 6-h cell sampling until 24 h, and analyzed for the levels of Cdc6, cyclin A, Cdk2, p27, and its Ser-10- and Thr-197-phosphorylated forms and for Cdk2 activity (Fig. 1*C*). Consistently, the p27 in the cells was constantly phosphorylated at Ser-10. By contrast, Thr-197 phosphorylation began to appear at 12 h post-replate and Cdc6 and cyclin A began to appear at 18 h. Coinciding with this timing, Cdk2 started to be active. This observation is consistent with the double dependence mechanism.

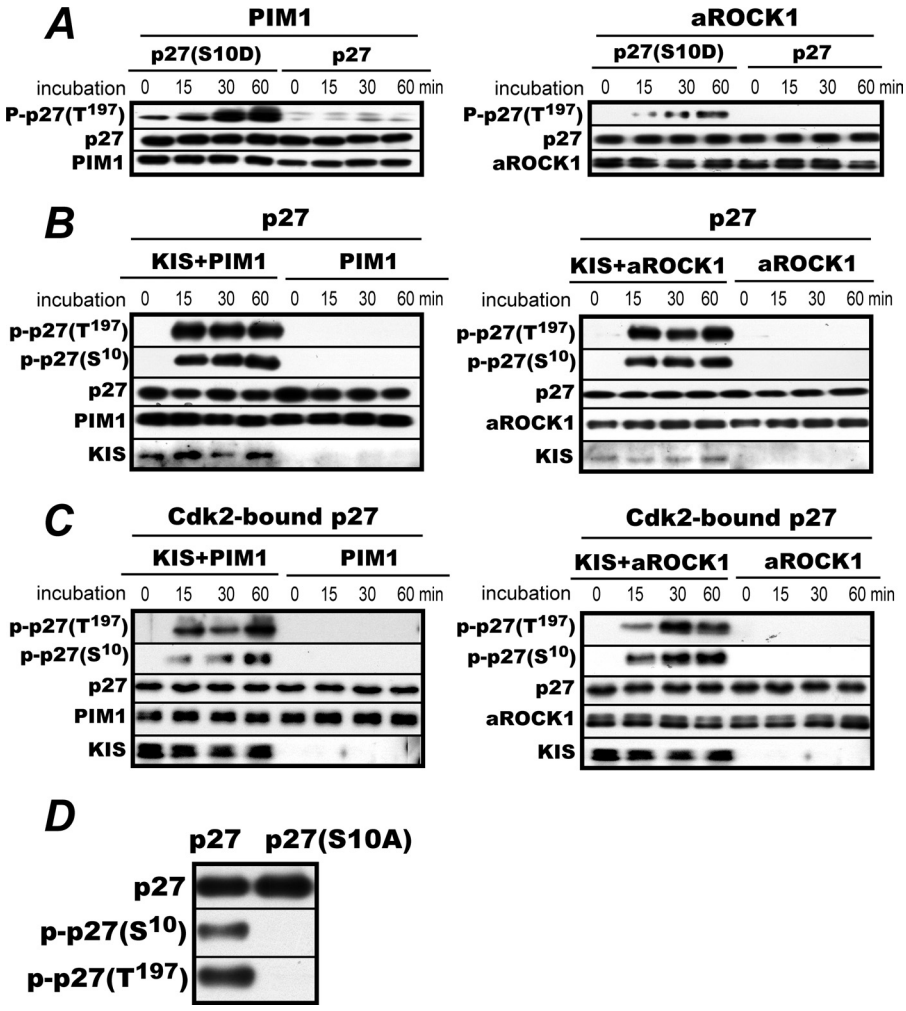

FIGURE 2. **Phosphorylation at Ser-10 is required for efficient C-terminal phosphorylation by PIM1 and active ROCK1.** *A*, the Ser-10 phosphomimetic mutant p27S10D but not wild-type p27 is an efficient substrate for PIM1 and active ROCK1. *E. coli*-expressed affinity-purified p27 and p27S10D were incubated in the reaction mixture with *E. coli*-expressed affinity-purified PIM1 or active ROCK1 and assayed for their C-terminal Thr-197 phosphorylation by immunoblotting. *B*, PIM1 and active ROCK1 phosphorylate the C terminus of p27 only when co-incubated with KIS. The recombinant p27 was incubated with the PIM1 or the active ROCK1 in the presence or absence of REF-expressed affinity-purified KIS and determined for its phosphorylation at Thr-197 and Ser-10. *C*, Cdk2-bound p27 also requires KIS-mediated Ser-10 phosphorylation for its C-terminal phosphorylation by PIM1 and active ROCK1. Cdk2-bound p27, prepared from baculovirus-expressed affinity-purified Cdk2-cyclin A complexes and the p27 as described under "Experimental Procedures," was incubated in the reaction mixture with the PIM1 or the active ROCK1 in the presence or absence of the KIS and determined for its Thr-197 and Ser-10 phosphorylation. *D*, Ser-10 phosphorylation is required for p27 C-terminal phosphorylation *in vivo*. N-terminally His-tagged wild-type p27 and mutant p27S10A were transiently expressed in logarithmically proliferating REF cells, purified with Ni-NTA beads, and determined for their phosphorylation at Ser-10 and Thr-197 by immunoblotting.

Phosphomimetic Mutant p27^{S10D} in Which Ser-10 Is Substituted with Aspartic Acid Is Much Better Substrate for PIM1 and Active ROCK1—Given the results, we next sought to obtain a mechanistic insight into Pim1- and ROCK1-mediated C-terminal phosphorylation of p27 and carried out *in vitro* phosphorylation of *E. coli*-produced recombinant p27 by *E. coli*-produced Pim1 and a similarly produced truncated constitutively active form of ROCK1. As shown in Fig. 1*A*, unlike at C-terminal Thr-197, p27 was highly phosphorylated at Ser-10 no matter whether the cells were under proliferation or arrested in G_1 by anchorage loss. We, therefore, first compared wild-type p27 and a phosphomimetic mutant p27^{S10D} in which Ser-10 was substituted with aspartic acid for the effectiveness as a substrate for these kinases by monitoring time-dependent C-terminal phosphorylation of p27 during reaction. To our surprise, unmodified wild-type p27 was found to be a very poor substrate, and by contrast, the Ser-10-phosphomimetic mutant p27^{S10D} was much more efficiently phosphorylated by these kinases (Fig. 2*A*). In this phosphorylation reaction, PIM1 was more efficient than active ROCK1.

Co-incubation with KIS Kinase That Phosphorylates Ser-10 Greatly Enhances C-terminal Phosphorylation of p27 by PIM1 and Active ROCK1—We, therefore, tested whether or not co-incubation with KIS kinase that phosphorylates p27 at Ser-10 can enhance C-terminal phosphorylation of unmodified p27 by PIM1 and active ROCK1. Rat KIS C-terminally tagged with a histidine hexamer was constructed by manipulating a corresponding cDNA, transiently expressed in rat cells and affinity-purified with nickel beads. We initially tried to express the tagged rat KIS in *E. coli*, but it was extremely unstable in the bacteria with little yield. The purified KIS was incubated with PIM1 or active ROCK1 in the reaction mixture containing

unmodified wild-type p27 (Fig. 2*B*). When KIS was added to the reaction mixture, C-terminal phosphorylation of p27 occurred with both kinases. Interestingly, unlike when the phosphomimetic mutant p27^{S10D} was used, in this reaction active ROCK1 was as efficient as PIM1 in C-terminal phosphorylation, both completing reaction within 15 min of incubation. Confirming the wild-type p27 results in Fig. 2*A*, when KIS was not added to the reaction mixture, both kinases failed to phosphorylate p27 at the C terminus.

The same Ser-10 phosphorylation dependence was also observed with Cdk2-bound p27 (Fig. 2*C*). When baculovirus-produced, affinity-purified recombinant Cdk2-cyclin A complexes were incubated with unmodified p27, immunoprecipitated with anti-Cdk2 antibody-conjugated beads, and used for the reaction, the Cdk2-bound p27 was phosphorylated at the C terminus only when KIS was added to the reaction mixture. KIS itself had no ability to phosphorylate the C-terminal Thr-197 of p27 (supplemental Fig. S1*A*). These *in vitro* reaction data clearly show that PIM1- and ROCK1-mediated C-terminal phosphorylation of p27 was almost absolutely dependent on its own phosphorylation at Ser-10 in advance. The mechanistic basis for this requirement is unknown at present, but the binding of PIM1 to p27 was not significantly influenced by the presence of Ser-10 phosphorylation (supplemental Fig. S1*A*) nor did Ser-10-unphosphorylated p27 interfere with PIM1-mediated C-terminal phosphorylation of p27S10D (supplemental Fig. S 1*B*).

Given the *in vitro* results, we examined whether or not Ser-10 phosphorylation is strictly required for C-terminal phosphorylation of p27 *in vivo*. N-terminally histidine hexamer-tagged p27 and p27^{S10A}, in the latter of which Ser-10 was substituted with non-phosphorylatable alanine, were constructed and transiently expressed in proliferating REF cells. The expressed tagged p27 molecules were affinity-purified and analyzed for the level of their C-terminal phosphorylation. As shown in Fig. 2*D*, unlike the wild-type p27 examined as a positive control, C-terminal phosphorylation of p27^{S10A} could not be detected, confirming the *in vitro* results.

p27 Double-phosphorylated at Ser-10 and C-terminal Thr-197 Cannot Inactivate Cdk2 Effectively—The next obvious question is whether or not phosphorylation at these sites influences the potency of p27 to inactivate Cdk2. We addressed this question as follows. First, the recombinant active Cdk2-cyclin A complexes were incubated with varying amounts of p27, p27^{S10D}, C-terminal phosphomimetic p27^{T197D} in which Thr-197 was substituted with aspartic acid, and double phosphomimetic p27^{S10D,T197D}, immunoprecipitated with anti-Cdk2 antibody, and assayed for Cdk2 activity. As shown in Fig. 3*A*, p27^{S10D} was comparable to unmodified wild-type p27 in the potency to inactivate the Cdk2, whereas p27^{T197D} was significantly reduced in its potency. Unlike wild-type p27, this phosphomimetic mutant failed to inactivate the Cdk2 completely when the lowest amount was tested. The double phosphomimetic mutant was similar to the C-terminal phosphomimetic mutant in this potency assay.

Given the results, we next examined the effects of real phosphorylation at these sites on the potency of p27. The p27 proteins phosphorylated at Ser-10 alone or both Ser-10 and Thr-197 were prepared by treatment with KIS or both KIS and PIM1 and then used for inactivation of Cdk2-cyclin A complexes as in Fig. 3*A*. The p27 protein phosphorylated at C-terminal Thr-197 alone could not be prepared because it was not doable as already shown in Fig. 2. Consistent with the experiment using the phosphomimetic form, Ser-10 phosphorylation did not alter the potency of p27 to inactivate Cdk2. By contrast, the double-phosphorylated p27 failed to inactivate Cdk2 even with the highest amount, whereas roughly ⅕-1/10 the amount was sufficient for unmodified and Ser-10-phosporylated p27 to inactivate Cdk2 completely (see the lowest amount). This result indicates that the double-phosphorylated p27 no longer functions as a potent inhibitor of Cdk2.

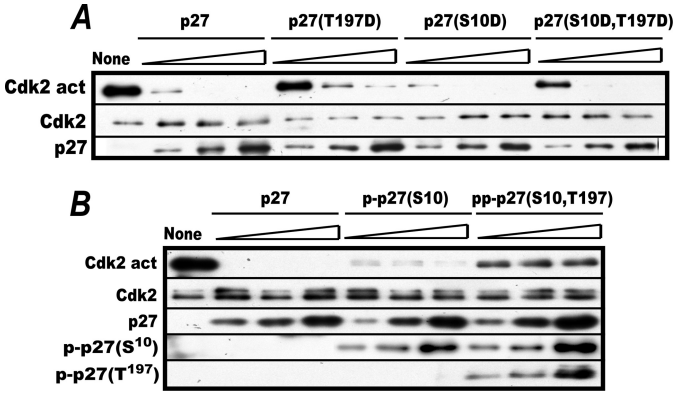

FIGURE 3. **Double phosphorylation at Ser-10 and C-terminal Thr-197 markedly attenuates the potency of p27 to inactivate Cdk2.** *A*, the phosphomimetic mutants p27T197D and p27S10D,T197D are less potent in inactivating Cdk2 than p27 and p27S10D. Varying amounts of the bacterially expressed p27, p27S10D, p27T197D, and p27S10D,T197D were incubated with the recombinant active Cdk2-cyclin A complex and directly assayed for its activity with Rb as a substrate. *B*, Ser-10 and Thr-197 double-phosphorylated p27 is a poor inhibitor for Cdk2. The *E. coli*-expressed p27 was treated with the KIS alone or together with the PIM1. The active Cdk2-cyclin A complex was incubated with each p27 reaction mixture and then assayed for Cdk2 activity as in *A*.

In Vitro Reconstitution of Stepwise Activation of p27-bound Cdk2 by KIS, PIM1, and Cdc6—Finally, we sought to confirm the requirement for Ser-10 phosphorylation in the Cdc6-mediated activation of p27-inactivated Cdk2 in an *in vitro* reconstitution system. To this goal, we first compared the Cdk2s immunoprecipitated from the REF and REF-PIM1 cells that had been cultured in MC for 12 and 14 h, respectively. During this culture, Cdk2 was inactivated, whereas its activating Thr-160 phosphorylation still remained albeit slightly diminished in both cells, but the Thr-197 phosphorylation of p27 persisted only in REF-PIM1 cells (Fig. 1). We previously showed that the Cdk2 immunoprecipitated from the REF cells in MC for 12 h requires C-terminal phosphorylation of the bound p27 for the effective activation of the p27-bound Cdk2 by Cdc6 (15). In the same experiment, we confirmed and extended this previous result. When p27-bound inactive Cdk2 was immunoprecipitated from the REF cells incubated in MC for 12 h and used for the reactivation assay, Cdc6 activated the Cdk2 only when the Cdk2 was co-treated with *E. coli*-produced PIM1 (Fig. 4*A*). Furthermore, when inactive Cdk2 was isolated from the 14-h cultured REF-PIM1 cells (a 2-h longer MC was needed to completely inactivate Cdk2) and used, treatment with Cdc6 alone was sufficient to activate the Cdk2. The activation of Cdk2 was

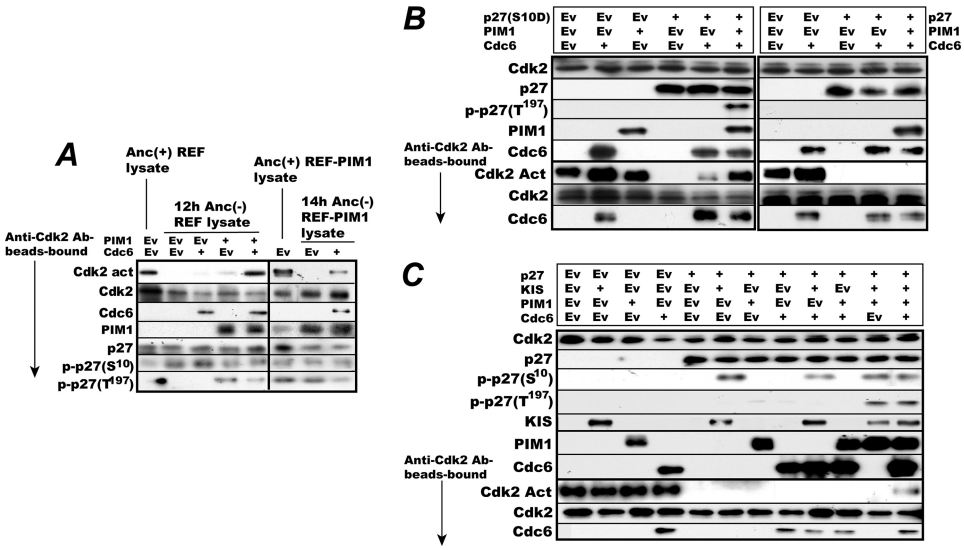

FIGURE 4. **Phosphorylation of the bound p27 at both Ser-10 and Thr-197 is required for Cdc6 to reactivate the p27-bound Cdk2.** *A*, Cdc6 activates cell-derived p27-bound inactive Cdk2 in a PIM1-dependent manner. Rapidly proliferating REF and REF-PIM1 were incubated in MC (*Anc(−)*) for 12 and 14 h, respectively, and lysed. p27-bound inactive Cdk2 molecules were then immunoprecipitated with the agarose bead-conjugated anti-Cdk2 antibody, incubated first with PIM1 or its control empty vector preparation (*Ev*) and then with Cdc6 or Ev, and the bead-bound fraction was determined for Cdk2 activity and the amounts of Cdk2, Cdc6, p27, and its Ser-10- and Thr-197-phosphorylated forms. *Anc*, anchorage. *B*, Cdc6 activates p27S10D-bound Cdk2 only when the inactive Cdk2 is co-treated with PIM1. p27S10D- or p27-bound inactive Cdk2-cyclin A complexes bound to anti-Cdk2 antibody-conjugated agarose beads, prepared as under "Experimental Procedures," were incubated first with PIM1 or Ev and then with Cdc6 or Ev. The reaction mixture was split into halves; one-half was determined for the levels of Cdk2, p27, its Thr-197-phosphorylated form, PIM1, and Cdc6. The beads in the other half were spin down and assayed for Cdk2 activity and the levels of the bound Cdk2 and Cdc6. *C*, Cdc6 activates unmodified p27-bound Cdk2 only after the bound p27 undergoes phosphorylation at Ser-10 and Thr-197 by KIS and PIM1. Baculovirus-produced active Cdk2-cyclin A complexes were incubated with the recombinant p27 or Ev. The Cdk2 complexes were immunoprecipitated with anti-Cdk2 antibody-conjugated agarose beads. The antibody bead-bound Cdk2 complexes were washed and incubated with KIS or Ev first and with PIM1 or Ev next. The bead-bound Cdk2 complexes were spin down, washed, and incubated with Cdc6 or Ev for reactivation of the Cdk2. The reaction mixture was split into two parts, one for the determination of the amounts of Cdk2, p27, its Ser-10-phosphorylated form, its Thr-197-phosphorylated form, KIS, PIM, and Cdc6 and the other for the determination of the levels of bead-bound Cdc6, Cdk2 and its activity as described under "Experimental Procedures."

accompanied by a significant reduction in the amount of the Thr-197-phosphorylated form of the Cdk2-bound p27 with only a marginal if any decrease of the total amount of the bound p27, consistent with the previous report (15).

Given this result, we proceeded to confirm the requirement for Ser-10 phosphorylation of the Cdk2-bound p27 in the Cdc6-mediated Cdk2 activation in an entirely *in vitro* reconstitution system. We first tested the bacterially expressed Ser-10-phosphomimetic mutant p27^{S10D} for PIM1 treatment-dependent reactivation of Cdk2 by the baculovirus-produced Cdc6. As shown in Fig. 4*B*, when p27^{S10D} was used to inactivate the Cdk2, Cdc6 activated the Cdk2 in a PIM1 treatment-dependent manner, with accompanying Thr-197 phosphorylation of the p27. By contrast, when unmodified wild-type p27 was used, PIM1 failed to phosphorylate the Cdk2-bound p27, and consequently Cdc6 could not activate the Cdk2. Finally, we examined the dependence of Cdc6-mediated activation of unmodified p27-bound Cdk2 on treatment with both KIS and PIM1. Active Cdk2-cyclin A complexes were first inactivated with unmodified p27 and immunoprecipitated with agarose bead-conjugated anti-Cdc2 antibody. The antibody bead-bound Cdc2 complexes were then consecutively treated with KIS and PIM1. The bead-bound complexes were collected and treated with Cdc6 for reactivation and assayed for Cdk2 activity. As shown in Fig. 4*C*, only when the unmodified p27-bound Cdk2 was treated with both KIS and PIM1, Cdc6 activated the Cdk2.

DISCUSSION

During the G_0-S phase transition, p27^{KIP1}, a critical inhibitor of Cdk2, undergoes chemical modification by phosphorylation at multiple sites. The major phosphorylation sites in rodent p27 during this phase transition are Ser-10, Thr-187, and Thr-197. Besides the Thr-187 phosphorylation that is mediated by activated Cdk2 and invokes SKP2-dependent ubiquitylation of its own for proteasomal degradation, the biological role of the phosphorylation at Ser-10 and Thr-197 has been elusive. The Ser-10 phosphorylation stabilizes p27 protein with a marked elevation of its intracellular level, yet oddly this phosphorylation promotes proliferation (8, 14). The Ser-10 phosphorylation was reported to also promote nuclear export of the p27, but this finding is controversial as already mentioned (11, 14).

Recently we found that Cdc6 the AAA+ ATPase, known to assemble pre-replicative complexes on origins of replication and activate p21^{CIP1}-bound Cdk2, can also activate p27-bound Cdk2 but only after the Cdk2-bound p27 undergoes Thr-197 phosphorylation (15). Thus, this C-terminal phosphorylation is required for the p27-bound Cdk2 to be activated by the multifunctional AAA+ ATPase. Our current work shows that the PIM- and ROCK-mediated C-terminal phosphorylation of p27 requires its Ser-10 phosphorylation in advance no matter whether it is free or Cdk2-bound and moreover that unbound p27 is no longer a potent Cdk2 inhibitor once double-phosphorylated at Ser-10 and Thr-197, yet its Cdk2-bound form is still active as an inhibitor, and the Cdk2 remains inactivated

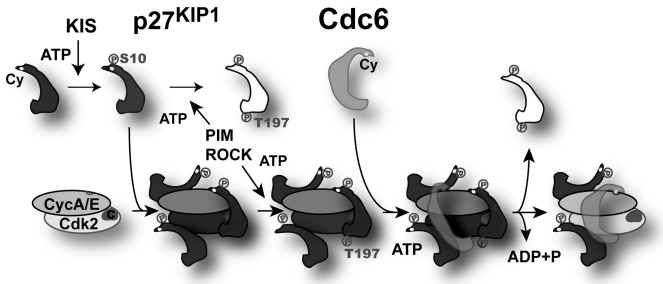

FIGURE 5. **Model for the phosphorylation-mediated regulation of the potency of p27^{KIP1} to inhibit Cdk2.** In G_0-G_1, nascent p27 is stabilized by its Ser-10 phosphorylation by KIS or other kinases including MIRK. This phosphorylated form is ready to bind and inactivate Cdk2-cyclin A/E complexes. In general, several p27 molecules bind to a single molecule of the Cdk2-cyclin complex, but one molecule that properly binds the Cdk2 is responsible for inactivation of the Cdk2 (20–23). When induced or activated by cytokine or anchorage signals, PIM and ROCK phosphorylate the C terminus of free and Cdk2-bound p27 molecules but more efficiently the one that properly binds and inactivates the Cdk2, resulting in attenuation of the free p27 as a Cdk2 inhibitor and facilitation of Cdc6-mediated removal of the bound p27 with concomitant activation of the Cdk2. The removed p27 is no longer a potent inhibitor of Cdk2 because it is double-phosphorylated. *C* stands for the Cdk2 catalytic domain; *Cy* is for cyclin binding motif. A small pit on cyclin A shows a hydrophobic groove to which the cyclin binding motif of both p27 and Cdc6 binds.

until Cdc6 removes the p27 from the Cdk2 in an ATP-dependent manner. Fig. 5 illustrates a model for the phosphorylation-mediated control of the potency of this CDK inhibitor. In G_0 and early G_1 cells, nascent p27 is quickly phosphorylated at Ser-10 by KIS or MIRK to be stabilized. This Ser-10-phosphorylated p27 is a major species in cells and is ready to bind and inactivate Cdk2-cyclin A/E complexes. In general, multiple molecules of p21^{CIP1} and p27^{KIP1} bind to one molecule of Cdk2-cyclin A/E complexes, but only one molecule of these CDK inhibitors that is properly bound to the Cdk2 complex is responsible for the inactivation of the Cdk2 (20–23). When PIM is induced or ROCK is activated via cytokine and/or anchorage signaling, these kinases phosphorylate the C terminus of free and Cdk2-bound p27, resulting in inactivation of the free p27 and facilitation of removal of the properly bound p27 by Cdc6 to reactivate the Cdk2. The removed p27 no longer participates in the inactivation of Cdk2 because it is double-phosphorylated at Ser-10 and Thr-197. As reported previously (15) and shown in Fig. 4*A*, upon treatment with Cdc6, the amount of Cdk2-bound C-terminal-phosphorylated p27 markedly decreased concomitant with Cdk2 activation, whereas there was only a marginal if any reduction in the total amount of Cdk2-bound p27. This indicates that as depicted in Fig. 5, of the several p27 molecules bound to the Cdk2, the one that properly binds and is responsible for inactivation of the Cdk2 is preferentially phosphorylated by the C-terminal kinases.

Although this model needs extensive scrutiny, it can account for the seeming self-contradiction surrounding the cell phenotypes regarding the Ser-10 phosphorylation of p27. As mentioned above, Ser-10 phosphorylation stabilizes the p27, markedly elevating the intracellular level of this potent CDK inhibitor, yet paradoxically this phosphorylation promotes cell proliferation instead of arrest. As shown in this work, Ser-10 phosphorylation makes the p27 susceptible to subsequent C-terminal phosphorylation by PIM or ROCK, and the resulting double-phosphorylated p27 is no longer a potent inhibitor of Cdk2. Furthermore, the Cdk2 bound to Ser-10 phosphorylatable or Ser-10-phosphorylated p27 can be activated by sequential phosphorylation by KIS and PIM/ROCK or PIM/ROCK alone and the subsequent action of Cdc6. By contrast, the mutant p27 non-phosphorylatable for Ser-10 or the p27 in Ser-10 phosphorylation-deficient cells will continue to be a potent Cdk2 inhibitor. Moreover, the Cdk2 bound to such p27 cannot be activated by the Cdc6-dependent mechanism. Consequently, in the cytokine-activated and/or anchorage-provided cells where PIM or ROCK is abundantly induced or highly activated, the Ser-10 phosphorylation of p27 rather facilitates cell proliferation despite the marked increase in its amount. Contrary to expectation, p27^{S10A} knock-in mice develop normally (11, 14). In the knock-in mice, however, the adverse effects of the non-inactivatable p27 might be attenuated by its rapid degradation.

Finally, we would like to briefly comment on the several articles reporting a close association of Cdc6 expression with Cdk2 activation and an elevation of its CDK-activating kinase (CAK)-catalyzed T-loop phosphorylation (24–26). Cdc6 might have an additional independent function to control Thr-160 T-loop phosphorylation of Cdk2, but so far we have no data to support this scenario. Alternatively, the elevated T-loop phosphorylation might be a mere consequence of autocatalytic Thr-160 phosphorylation of the Cdk2 that is activated by Cdc6-mediated removal of the bound p27 as reported (27).

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