We have studied mechanisms of Akt-mediated phosphorylation and regulation of cellular localization of p27. Akt phosphorylates Thr-157 in p27 and retains it in the cytosol. In cells arrested in G1 and then synchronized to enter into S phase, Akt-mediated phosphorylation of Thr-157 in p27 occurred in the cytosol during G1 phase of the cell cycle. Both T157A and S10A p27 mutants localized in the nucleus in all phases of the cell cycle regardless of the expression of active Akt. Thr-157 phosphorylation was undetectable in S10A-p27, suggesting that Ser-10 phosphorylation is required for p27 localization in the cytosol and subsequent phosphorylation at Thr-157. Phosphorylation at Thr-157 interrupted the association of p27 with importin α. A T157A-p27 mutant protein exhibited higher association with importin α than wild-type-p27. Treatment of transfected and endogenous p27 with alkaline phosphatase rescued its association with importin α. Leptomycin B inhibited cytosolic Thr-157 P-p27 staining, implying that CRM1-dependent nuclear export is required for Akt-mediated Thr-157 phosphorylation. Heterokaryon shuttling assays with NIH3T3 (mouse) cells transfected with FLAG-p27 and HeLa (human) cells revealed that both wild type and T157A-p27 shuttled from NIH3T3 to HeLa cell nuclei with similar frequencies. However, S10A-p27 was found only in the NIH3T3 nuclei of NIH3T3-HeLa cell fusions. These results suggest that 1) Ser-10 phosphorylation is required for nuclear export of p27, 2) subsequent Akt-mediated phosphorylation at Thr-157 during G1 phase correals p27 in the cytosol, and 3) Thr-157 phosphorylation inhibits the association of p27 with importin α thus preventing its re-entry into the nucleus.

Cell cycle progression is dependent on the activity of complexes containing cyclins and cyclin-dependent kinases (Cdk). The Cdk inhibitor p27 is an important regulator of the mammalian cell cycle (1). p27 causes G1 arrest by inhibiting the activities of G1 cyclins and Cdkss. An increase in p27 levels results in a delay of cell cycle progression, whereas a decrease in p27 promotes arrested cells to resume proliferation. The expression of p27 is regulated both transcriptionally and post-translationally. Akt-mediated phosphorylation and inhibition of Forkhead transcription factors were reported to decrease p27 gene transcription (2). Cdk2-mediated phosphorylation of p27 at Thr-157 results in complex formation with ubiquitin ligase SCF{sup Skp2} leading to 26 S proteasome-mediated degradation of p27 in proliferating cells (3, 4). During G0/G1 phase of the cell cycle, p27 degradation can also occur independently of Thr-187 phosphorylation and binding to SCF{sup Skp2} (5, 6). Localization of p27 in the nucleus is required to inhibit Cdk activation by Cdk-activating kinase (7). The binding to and inhibition of Cdk in the nucleus by p27 is probably impaired (8) in many human tumor cells where cytosolic redistribution of p27 has been observed (9, 10).

More recent evidence suggests that phosphorylation of p27 is an important determinant of its subcellular localization. Akt-mediated phosphorylation of p27 at Thr-157 results in retention of p27 in the cytosol (11–13). The nuclear localization sequence (NLS) of p27 contains an Akt consensus motif RXRXXT{sup 157D}. Expression of constitutively active mutants of Akt results in cytosolic localization of wild-type (WT) p27. However, a mutant p27 protein in which Thr-157 has been replaced with Ala shows nuclear localization regardless of high cellular Akt activity (11–13). Furthermore, high expression of active (phosphorylated) Akt in primary human breast cancers statistically correlates with localization of p27 in tumor cytosol (12). Ser-10 is another major phosphorylation site in p27 (14), and this modification is necessary for the nuclear export of the protein mediated by the exportin (15, 16). The binding of p27 to CRM1 requires Ser-10 phosphorylation, as an S10A p27 mutant shows reduced association with CRM1, whereas phospho-mimicking p27 mutants in which Ser-10 has been substituted with aspartic acid (S10D) or glutamic acid (S10E) show a markedly enhanced interaction with CRM1 (15, 17). In addition to point mutations in Ser-10, mutations in basic residues within the nuclear export sequence also impair p27 nuclear export, suggesting that export is mediated by the interaction of CRM1 with the atypical nuclear export sequence in p27 (17). The kinase responsible for Ser-10 phosphorylation is human kinase-interacting stathmin (hKIS) (18). The forced expression of hKIS reverses the cell cycle arrest induced by WT-p27 overexpression but not by S10A-p27, indicating that hKIS modulates cell cycle progression through Ser-10 phosphorylation and nuclear export (18).

The macromolecular cargo transport between the nucleus and the cytosol is mediated by nuclear pore complexes. Mammalian nuclei typically contain several thousand nuclear pore complexes (19) each composed of proteins termed nucleoporins, which form a complex of around <125 MDa (20). Soluble transport receptors (karyopherins) that bind either the NLS or nu-
clear export sequence in cargo proteins can be classified into exporters and importins. The most widely characterized exporter is CRM1, which binds basic amino acid residues in the nuclear export sequence. Importin α recognizes NLS, and the association of the importin α-NLS complex with the nuclear pore complex is mediated by importin β (19, 21). The C terminus of p27 contains a classical bipartite NLS consisting of an N-terminal cluster of three basic residues and a C-terminal cluster of two basic residues separated from each other by 10 amino acids (22). This NLS is thought to be responsible for p27 import into the nucleus (23). The binding of NLS to importin α might be mediated by the ionic interaction between basic amino acid residues in the NLS and acidic residues in importin α (19).

We report herein that Akt-mediated phosphorylation of p27 at Thr-157 occurs in the cytosol during the G1 phase of the cell cycle. Blockade of nuclear export with the CRM1 inhibitor leptomycin B eliminated detectable levels of cytosolic Thr-157 P-p27, implying that CRM1-dependent nuclear export is required for Akt-mediated Thr-157 phosphorylation in the cytosol. A T157A mutant protein exhibited high association with importin α and exclusive nuclear localization, whereas p27 phosphorylation at this site prevented the association with importin α. The binding with importin α was dependent on Thr-157 dephosphorylation, because treatment with calf intestine alkaline phosphatases (CIAP) restored the association of both transfected and endogenous p27 with importin α, although there was no effect of CIAP on the association between T157A-p27 and importin α. Co-expression of a vector encoding active Akt abrogated the association of WT-p27 with importin α. These results along with previous reports (15–18) suggest that Akt-mediated phosphorylation of p27 at Thr-157 prevents nuclear re-entry by inhibiting the association of p27 with importin α.

MATERIALS AND METHODS

Cell Culture and Vectors—BT-474, 293T, NIH3T3 (mouse), and HeLa (human) cells were all from the American Type Culture Collection (ATCC, Manassas, VA). BT-474 cells were maintained in improved minimal essential medium, 10% FBS (Invitrogen). 293T, NIH3T3, and HeLa cells were passaged in Dulbecco’s modified Eagle’s medium, 10% FBS. The WT and S10A human p27 genes each in a pET vector were expressed in E. coli (MDH) and purified to homogeneity. The T157A mutant was described previously (12). The binding with importin α was dependent on Thr-157 dephosphorylation, because treatment with calf intestine alkaline phosphatases (CIAP) restored the association of both transfected and endogenous p27 with importin α, although there was no effect of CIAP on the association between T157A-p27 and importin α. Co-expression of a vector encoding active Akt abrogated the association of WT-p27 with importin α. These results along with previous reports (15–18) suggest that Akt-mediated phosphorylation of p27 at Thr-157 prevents nuclear re-entry by inhibiting the association of p27 with importin α.

RESULTS

Akt Phosphorylates Cytosolic p27 on Thr-157 during G1 Phase—We first analyzed the spatial and temporal regulation of Akt-mediated phosphorylation of p27 in G1-arrested and cycling 293T cells (Fig. 1). Serum starvation for 48 h increased the 293T G1 fraction from 40 to 70%. Akt was predominantly localized in the cytosol throughout the cell cycle. At short intervals (5–30 min) after the addition of serum to G1-arrested...
cells, total Akt and Ser-473 P-Akt were detectable in 293T nuclear fractions. Ser-473 P-Akt was undetectable in serum-starved cells but was maximal 3–6 h after serum addition. p27 was found both in cytosol and the nucleus in asynchronous cultures, whereas serum-starved quiescent cells exhibited high p27 levels only in the nucleus. p27 translocated to the cytosol 1 h after serum addition reaching its maximal level in this compartment 6 h later in mid G1. Three to six hours after serum addition and when (cytosolic) P-Akt levels were maximal, levels of Thr(P)-157 p27 were also maximal. Phosphorylation of p27 in Thr-157 was only detectable in cytosolic fractions of 293T cells. Twenty-four hours after serum stimulation when cells have entered S phase, p27 was degraded consistent with reports of proteolytic degradation of p27 in cycling cells (29).

Similar results were obtained with erbB2-overexpressing BT-474 human breast cancer cells induced into quiescence by serum starvation and treatment with the erbB tyrosine kinase inhibitor erlotinib. P-Akt and Thr(P)-157 p27 were detectable in asynchronous cell cultures but undetectable in quiescent cells (Fig. 2A). Upon serum addition and release from G1, P-Akt and Thr(P)-157 p27 content as monitored by immunohistochemistry increased with similar kinetics achieving a maximum in mid-to-late G1 phase of the cell cycle. Immunofluorescence staining of synchronized cultures showed minimal P-Akt and Thr(P)-157 p27 staining of quiescent BT-474 cells and a time-dependent increase upon serum addition at 5 h (Fig. 2B). These data suggest that temporal and spatial phosphorylation of p27 in Thr-157 correlate with the cellular distribution of Akt and its activation.

Ser-10 Phosphorylation Is Required for p27 Cytosolic Localization and Its Phosphorylation at Thr-157—To further document that phosphorylation of p27 in Thr-157 occurs in the cytosol, FLAG-WT-p27, FLAG-T157A-p27, and FLAG-S10A-p27 were expressed in 293T cells and examined during cell cycle progression. WT-p27 localized in both cytosol and nuclei of asynchronous 293T cells. In quiescent cells WT-p27 was almost exclusively localized in the nucleus with cytosolic FLAG-WT-p27 levels markedly increasing 6 h after the addition of serum (Fig. 3A, lane 3). Co-transfection of activated Akt markedly increased FLAG-WT-p27 cytosolic levels throughout the cell cycle (Fig. 3A, lanes 5–8). Both FLAG-T157A-p27 and FLAG-S10A-p27 were exclusively nuclear in 293T cells in the presence or absence of transfected active Akt, suggesting that phosphorylation at Ser-10 is also required for cytosolic localization of p27 and phosphorylation at Thr-157. Consistent with this possibility, Thr-157 phosphorylation was detectable in transfected wild-type p27 but it was almost undetectable in pull downs of the S10A p27 mutant (Fig. 3B). Finally, treatment of BT-474 cells with leptomycin B, an inhibitor of CRM1-dependent nuclear export, abrogated cellular levels of Thr(P)-157 p27 (Fig. 3C), implying that nuclear export of p27 is required for Thr-157 phosphorylation.

The results shown in Fig. 3A were confirmed by immunolocalization studies. 293T cells were transfected with FLAG-WT-p27, FLAG-T157A-p27, and FLAG-S10A-p27, each with or without constitutively active HA-AktDD and subjected to staining with FLAG antibodies. FLAG staining was observed in the cytosol and nuclei of asynchronous 293T cells transfected with FLAG-WT-p27. Serum starvation resulted in nuclear localization of WT-p27, which translocated to the cytosol 6 h after the addition of serum (Fig. 4A). At 24 h, there was a 70% reduction in FLAG-positive cells (data not shown) consistent with p27 degradation during S phase. Expression of HA-AktDD resulted in cytosolic localization of WT-p27 in serum-starved cells and in cycling cells at 6- and 24-h post-serum addition. Staining with an HA antibody indicated that the active Akt mutant was predominantly localized in the cytosol. This result agrees with the localization of endogenous activated Akt in 293T cell fractions (shown in Fig. 1A). Both T157A-p27 and S10A-p27 were exclusively nuclear throughout the cell cycle (Fig. 4, A and B), and this localization was not altered by HA-AktDD expression. These results suggest that 1) both Thr-157 and Ser-10 phosphorylation are required for the cytosolic localization of p27, and 2) Akt activation and translocation of p27 to the cytosol correlate temporally.
Phosphorylation of p27 at Thr-157 Inhibits Its Association with Importin—Phosphorylation of p27 at Ser-10 has been reported to be required for association of p27 with CRM1 and subsequent nuclear export (15, 16) explaining the constitutively nuclear localization of the S10A p27 mutant (Figs. 3A and 4C). However, the exclusive nuclear localization of the T157A mutant is less clear and suggests the possibility of defective nuclear export (as for the S10A mutant) and/or enhanced nuclear import. Very recently, Sekimoto et al. (30) reported that 14-3-3 proteins sequester the NLS of p27 from interacting with importin in vitro. Therefore, to determine whether phosphorylation at these sites regulates nuclear import via association with karyopherins, we studied the interaction in vivo of p27 with importin α, a molecule that binds to the NLS of proteins thus promoting nuclear entry. As shown in Fig. 5A, endogenous importin α was detected in FLAG precipitates from cells transfected with FLAG-tagged WT-, S10A-, and T157A-p27 and FLAG immunoblots confirmed there was no significant cross-contamination between cytosolic and nuclear fractions. B, 293T cells were transfected with FLAG-tagged WT-, T157A- or S10A-p27 for 48 h. FLAG-p27 was precipitated from 500 μg of whole cell lysates and subjected to Thr(P)-157 p27 and FLAG immunoblot analyses. C, BT-474 cells on coverslips were treated with LMB at a concentration of 10 ng/ml for 2.5 h, fixed, and stained with a Thr(P)-157 p27 antibody or Hoechst dye.

Phosphorylation of p27 at Thr-157 Inhibits Its Association with Importin α—Phosphorylation of p27 at Ser-10 has been reported to be required for association of p27 with CRM1 and

FIG. 2. Time course of Akt and p27 phosphorylation in BT-474 cells. A, BT-474 cells were synchronized by serum starvation and incubation with 3 μM erlotinib for 48 h. At this time, improved minimal essential medium, 10% FBS was added. Cells were then harvested at the indicated times and subjected to flow cytometry as indicated under “Materials and Methods.” The percentage of cells in G1, S, and G2/M phases of the cell cycle at different times following serum addition are indicated on top. Cell lysates (30 μg/lane) were subjected to immunoblot analyses with the antibodies indicated on the right of each panel. B, simultaneously, BT-474 cells on coverslips were induced to undergo cell cycle arrest (as in Fig. 2). At 48 h, 10% FBS was added to induce the progression into S phase. At the indicated times following serum addition, the cells were fixed and stained with P-Akt and Thr(P)-157 p27 antibodies as well as Hoechst nuclear dye.

FIG. 3. Phosphorylation of p27 on Thr-157 occurs in the cytosol. A, 293T cells were transfected with FLAG-WT-p27, FLAG-T157A-p27, or FLAG-S10A-p27 either with or without HA-AktDD. Sixteen hours after transfection, the cells were serum-starved (ss) for 48 h. To release cells into S phase, synchronized cells were treated with 10% FBS and harvested 6 or 24 h after as indicated. Both cytosolic and nuclear fractions were subjected to FLAG immunoblot analysis. Tubulin and c-jun (cytosolic and nuclear markers, respectively) immunoblots confirmed there was no significant cross-contamination between cytosolic and nuclear fractions. B, 293T cells were transfected with FLAG-tagged WT-, T157A- or S10A-p27 for 48 h. FLAG-p27 was precipitated from 500 μg of whole cell lysates and subjected to Thr(P)-157 p27 and FLAG immunoblot analyses. C, BT-474 cells on coverslips were treated with LMB at a concentration of 10 ng/ml for 2.5 h, fixed, and stained with a Thr(P)-157 p27 antibody or Hoechst dye.
sociation of WT- and S10A-p27 with importin α to the same level as that seen with the T157A mutant (Fig. 5A, lanes 2, 4, and 6), suggesting that Thr-157 phosphorylation reduces the p27-importin α association. This was further implied by the experiment shown in Fig. 5B. Importin α coprecipitated with FLAG-WT-p27, but a minimal level of Thr(P)-157 p27 was detected in the importin α-associated p27. However, we were unable to detect 14-3-3 in immunoblots of WT-p27 pull downs (data not shown). Finally, cotransfection with HA-AktDD markedly increased Thr-157 phosphorylation and eliminated the association with importin α.

The physical interaction between p27 and importin α was modulated by cell cycle progression. Serum starvation reduced Thr-157 phosphorylation and the association of p27 with importin α in quiescent 293T cells (Fig. 5C). Six and 24 h after serum addition, Thr-157 phosphorylation was increased (6 > 24 h), whereas the association with importin α was markedly abrogated (lanes 3 and 4). Total levels of FLAG-p27 were reduced 24 h after serum addition, suggestive of p27 degradation during the S phase. Importin α also coprecipitated with endogenous p27 in asynchronous 293T and BT-474 cells. This association was markedly increased by dephosphorylation of cell lysates with CIAP prior to precipitation with p27 antibodies (Fig. 5D), further suggesting that phosphorylation of p27 at Thr-157 impedes its association with importin α. These results also imply that the nuclear localization of T157A mutant p27 (Figs. 3A and 4B) is the result of enhanced binding to importin α and subsequent nuclear import.

Nucleus to Cytosol Shuttling Is Impaired in S10A-p27 Not in T157A-p27—To determine whether the impaired cytosolic localization of Thr-157-p27 and S10A-p27 were because of impaired nuclear export, we performed an interspecies heterokaryon shuttling assay. NIH3T3 cells seeded on glass coverslips were transfected with FLAG-WT-p27, FLAG-T157A-p27, or FLAG-S10A-p27 (Fig. 6). HeLa cells were plated on top of the transfected NIH3T3 cells, and cell fusion was induced by polyethylene glycol. FLAG-p27 was monitored by indirect immuno-
munofluorescence, and nuclei were counterstained with Hoechst 33342. NIH3T3 nuclei were identified by their punctate staining pattern. In the heterokaryons, where NIH3T3 and HeLa cells fused as evidenced by their shared cytosol in differential interference contrast image, the shuttling frequency was calculated by dividing the number of heterokaryons showing FLAG-staining in HeLa nuclei by the total number of transfected heterokaryons. In the heterokaryons transfected with WT-FLAG-p27, 12.5% of them showed FLAG staining in both transfected NIH3T3 nuclei and fused HeLa cell nuclei. The addition of LMB inhibited FLAG-WT-p27 shuttling between mouse and human nuclei suggesting that nuclear export of FLAG-WT-p27 was indeed mediated by a CRM1 exportin-dependent mechanism. Interestingly, FLAG-T157A-p27 was found in HeLa nuclei at the same frequency as FLAG-WT-p27 and was also inhibited by LMB (12.8 versus 0%). In contrast, FLAG-S10A-p27 was not observed in the HeLa nuclei of the heterokaryons. This result suggests that FLAG-S10A-p27 was not exported from NIH3T3 cell nuclei, whereas FLAG-T157A-p27 was still able to undergo nuclear export. Thus, we surmised that the nuclear localization of FLAG-T157A-p27 results from the enhanced nuclear import by preferred association with importin α and not by impaired nuclear export.

**DISCUSSION**

We report herein that p27 is phosphorylated by cytosolic Akt in the G1 phase of the cell cycle and that this modification impairs the association of p27 with importin α, thus preventing its re-entry into the nucleus. In cultured cells, Akt is found mainly in the cytosol (31), but stimulation by growth factors results in its activation in the cell membrane and nuclear localization (32). Consistent with this, asynchronous 293T cells showed mainly cytosolic Akt, whereas the addition of serum to quiescent cells induced transient presence of Akt in the nucleus (Fig. 1A). This transient localization in the nucleus temporally correlated with evidence of Akt phosphorylation in Ser-473. One hour after serum addition, both total and Ser-473 P-Akt were only detectable in 293T cell cytosol with active Akt and Thr-157 P-p27 peaking at 3–6 h when cells were still in the G1 phase (Fig. 1B). In addition, an S10A p27 mutant localized exclusively in the nucleus and failed to exhibit phosphorylation at Thr-157 (Fig. 3B). One hour after serum addition, both total and Ser-473 P-Akt were only detectable in 293T cell cytosol with active Akt and Thr-157 P-p27 peaking at 3–6 h when cells were still in the G1 phase (Fig. 1A). This transient localization in the nucleus temporally correlated with evidence of Akt phosphorylation in Ser-473.

**Fig. 5.** p27 associates with importin α. A, 293T cells were transfected with FLAG-WT-p27, FLAG-S10A-p27, or FLAG-T157A-p27. After 48 h, the cells were lysed in CIAP buffer as indicated under "Materials and Methods." Where indicated, the cell lysates were treated with CIAP (200 units) at 37 °C for 1 h. p27 was precipitated from whole cell lysates with a FLAG antibody, and immune complexes were subjected to importin α and FLAG immunoblot procedures. B, FLAG-WT-p27 was co-expressed with HA-AktDD in 293T cells. The association of importin α with total and Thr(P)-157 p27 was monitored by the indicated immunoblot analyses (left side of panels) of FLAG precipitates from 500 μg of total cell lysates. AktDD expression was monitored by HA immunoblot of total cell lysates. C, 293T cells were transfected with FLAG WT-p27 and serum-starved (ss) for 48 h. FBS (10%) was added to the G1-arrested cells, which were harvested and lysed 6 or 24 h later as indicated. The association of importin α with total p27 and Thr(P)-157 p27 was measured as in B. D, endogenous p27 was precipitated from 293T and BT-474 cells. Where indicated, cell lysates were treated with CIAP in vitro prior to the immunoprecipitation (IP) as in A. The association of p27 and importin α was monitored by immunoblot with an importin α antibody.
Phosphorylation of p27Kip1 at Thr-157 (33), requires additional investigation. Although we detected Thr-308 phosphorylation in cytosolic Akt, we were unable to detect this modification in nuclear Akt (data not shown). Thus, we cannot rule out the possibility that nuclear Akt activity does not reach a threshold required to phosphorylate nuclear p27. It can also be speculated that p27 needs some other cytosolic bridging factor to associate with Akt. Indeed, we have shown that the association of p27 with Akt is independent of Thr-157 p27 phosphorylation (12), suggesting that Akt may bind a domain other than the Akt consensus in p27 and/or that another cytosolic factor mediates this protein-protein interaction.

Like in p27, phosphorylation of residues within the NLS of nuclear proteins has been shown to inhibit nuclear import. SW15, a yeast nuclear protein involved in mating type switching, is excluded from the nucleus upon phosphorylation by the Cdk CDC28 (38). Similarly, nuclear transport of lamin B2 is inhibited by protein kinase C-mediated phosphorylation at Ser-410 and Ser-411 near its NLS (39). Heat shock- or dimethyl sulfoxide-induced dephosphorylation of the actin-binding protein coflin on its Ca2+/calmodulin-dependent protein kinase consensus site results in its translocation to the nucleus (40). The Ca2+/calmodulin-dependent protein kinase site Ser-24 is located adjacent to the NLS of coflin. Conversely, phosphorylation-enhanced, NLS-dependent nuclear transport has also been observed. Casein kinase II-induced phosphorylation at Ser-111 and Ser-112 in SV40 T-antigen promotes its nuclear transport (41). Protein kinase A-mediated phosphorylation of dorsal (42) and the c-rel oncogene (43) enhances their nuclear localization.

It is interesting to note that phosphorylation-dependent inhibition of NLS function is generally mediated by phosphorylation on a residue(s) very close to or within the NLS. One of the CDC28 sites in SW15 is located within the spacer region of its bipartite NLS (38). The protein kinase C phosphorylation sites Ser-410 and Ser-411 in lamin B2 are adjacent to the N terminus of its NLS (39). Ser-24 in coflin is separated by only four amino acids from its NLS (40). Ser-248 in v-jun is adjacent to its NLS and phosphorylation at this residue inhibits the nuclear translocation of v-jun (44). However, the CK II sites in SV40 T-antigen are located 13 amino acids from the N terminus of its NLS (41) and the protein kinase A sites in dorsal (42) and c-rel (43) are separated by 22 amino acids from their NLS. These findings suggest the possibility that phosphorylation on a residue(s) very close to or within the NLS abrogates the ionic interaction between basic amino acids in the NLS and acidic residues in the NLS binding domain of importin α (19) resulting in disassembly of the importin α complex and impaired nuclear import. We have shown here that phosphorylation on Thr-157 in the NLS of p27 abrogated the p27-importin α interaction in and inhibited nuclear re-entry of p27. Using a heterokaryon shuttling assay (Fig. 5), we also showed that Ser-10 phosphorylation is required for nuclear export of p27 and that Thr-157 phosphorylation prevents nuclear re-entry. Furthermore, Thr-157 phosphorylation does not promote nuclear export as evidenced by the same shuttling frequency of WT p27 and a T157A p27 mutant. This result is in agreement with a report by Liang et al. (11) in which a T157A p27 mutant exhibited a faster rate of nuclear import compared with WT p27. In addition, preincubation with purified Akt enzyme impairs nuclear import of WT-p27 in digitonin-permeabilized MCF-7 cells (11).

The schema shown in Fig. 7 suggests a model for the regulation of p27 transport. As suggested by Boehm et al. (18), p27 is phosphorylated on Ser-10 by hKIS in G1. This modification promotes the binding of p27 to exportin CRM1 in an LMB-dependent manner. In mid-G1, cytosolic Akt phosphorylates p27 on Thr-157, and this modification in the NLS of p27 inhibits co-localized with HA-Akt1DD in proliferating cells, further suggesting that Akt-mediated phosphorylation of p27 at Thr-157 occurs in the cytosol during G1 phase of the cell cycle.

It is unclear why nuclear Akt could not phosphorylate p27 at Thr-157 shortly after serum addition when Ser-473 phosphorylation of nuclear Akt was detectable (Fig. 1A). It is possible that in early G1, the majority of nuclear p27 is associated with Cdk2 and cyclin E, and this association may sterically hinder the interaction of p27 with Akt or, as recently suggested (33), nuclear protein phosphatase PP2A may down-regulate nuclear Akt activity after stimulation with growth factors in serum. In this report, nuclear Akt activity as monitored by immunoblot with a Thr-308 P-Akt antibody was diminished as early as 45 min after treatment with nerve growth factor and pretreatment with inhibitors of PP2A restored Akt activity (33). Akt is phosphorylated on both Ser-473 and Thr-308. It has been reported that Thr-308 phosphorylation is obligatory for Akt activation and that Ser-473 phosphorylation may be dispensable for platelet-derived growth factor-stimulated Akt activity in NIH3T3 cells (34, 35). Despite its minor role in Akt activation as compared with Thr-308 phosphorylation (36, 37), phosphorylation of Akt at Ser-473 is easier to detect and commonly used to monitor cellular Akt activity. Whether phosphorylation at Thr-308 is more transient and therefore harder to detect, perhaps because of PP2A activity as reported by Borgatti et al.
complex formation with importin $\alpha$ leading to the retention of p27 in the cytosol. However, we cannot rule out the presence of a docking protein disrupting this complex formation. Indeed, Sekimoto et al. (30) recently reported that 14-3-3 can disrupt the association between importin $\alpha$ and the NLS of recombinant p27 in a cell-free reconstituted system. Nonetheless, we were unable to detect 14-3-3 in WT-p27 pull downs in our studies. When cytosolic Akt activity is not high enough to phosphorylate p27, importin $\alpha$ rapidly forms a complex with p27 and transports it back to the nucleus. The nuclear re-entry may occur extremely rapidly as a T157A mutant of p27 predominantly localized in the nucleus just as the export-deficient S10A mutant did.

Recent reports also suggest that Akt- and p90 ribosomal protein S6 kinase-mediated phosphorylation of p27 at Thr-198 promotes p27 binding to 14-3-3 proteins, leading to cytosolic localization of p27 (45, 46). Two different groups reported that p27 interacts with Nup50/Npap50 (47, 48), a component of nuclear pore complexes, which plays a role in nuclear export and binds directly to CRM1 (49). Recently, Nup50/Npap50 was also shown to act as a cofactor for importin $\alpha$/importin $\beta$-mediated nuclear import (50). The association of p27 with Nup50/Npap50 was reported to be involved in p27 import into the nucleus where phosphorylation by Cdk2 and subsequent ubiquitylation-dependent degradation occur (47). The complex modulation of the nuclear transport of p27 by phosphorylation and/or association of importin/exportins represents a novel mechanism for regulating nuclear function of Cdk inhibitors such as p27.

REFERENCES
1. Sherr, C. J., and Roberts, J. M. (1999) Genes Dev. 13, 1501–1512
2. Medema, R. H., Kops, G. J., Bos, J. L., and Burgering, B. M. (2000) Nature 404, 782–787
3. Montagnoli, A., Fiore, F., Eytan, E., Carrano, A. C., Draetta, G. F., Hershko, A., and Pagano, M. (1999) Genes Dev. 13, 1181–1189
4. Carrano, A. C., Eytan, E., Hershko, A., and Pagano, M. (1999) Nat. Cell Biol. 1, 193–199
5. Hara, T., Kamura, T., Nakayama, K., Oshikawa, K., Hatakeyama, S., and Nakayama, K. (2001) J. Biol. Chem. 276, 48937–48941
6. Malek, N. P., Sundberg, H., McGrew, S., Nakayama, K., Kyriakides, T. R., Roberts, J. M., and Kyriakides, T. R. (2001) Nature 413, 323–327
7. Darbon, J. M., Devault, A., Taviaux, S., Fesquet, D., Martinez, A. M., Galas, S., Cavadore, J. C., Duree, M., and Blanchard, J. M. (1994) Oncogene 9, 3127–3138
8. Orend, G., Hunter, T., and Ruoslahti, E. (1998) Oncogene 16, 2575–2583
9. Giapponi, M., Yamamoto, H., Yao, Y., Spangato, A., Cattoretti, G., Tomita, N., Munden, T., Rotterdam, H., and Weinstein, I. B. (1998) Cancer Res. 58, 114–122
10. Singh, S. P., Lipman, J., Goldman, H., Ellia, F. H., Jr., Aizenman, L., Carra, M. G., Signoretti, S., Chiaro, D. S., Pagano, M., and Loda, M. (1998) Cancer Res. 58, 1730–1735
11. Liang, J., Zubovitz, J., Petrocelli, T., Kotchetkov, R., Connor, M. K., Han, K., Lee, J. H., Ciarallo, S., Catzavelos, C., Beniston, R., Franssen, E., and Slingerland, J. M. (2002) Nat. Med. 8, 1145–1152
12. Vishlott, G., Matti, M. L., Bruni, P., Melillo, R. M., D’Alessio, A., Calianno, D., Vinci, F., Chiaipetta, G., Tisch, P., Bellacosa, A., Fusco, A., and Santoro, N. (2002) Nat. Med. 8, 1136–1144
13. Ishida, N., Kitagawa, M., Hatakeyama, S., and Nakayama, K. (2000) J. Biol. Chem. 275, 25146–25154
14. Ishida, N., Hara, T., Kamura, T., Yoshida, M., Nakayama, K., and Nakayama, K. I. (2002) J. Biol. Chem. 277, 14355–14358
15. Rodier, G., Montagnoli, A. I., Marcello, L., Coulombe, P., Draetta, G. F., Pagano, M., and Meloche, S. (2001) EMBO J. 20, 6672–6682
16. Connor, M. K., Kotchetkov, R., Carro, S., Resch, A., Lepri, R., Beniston, R. G., Melchior, P., Hengst, L., and Slingerland, J. M. (2003) Mol. Biol. Cell.
18. Boehm, M., Yoshimoto, T., Crook, M. F., Nallamshetty, S., True, A., Nabel, G. J., and Nabel, E. G. (2002) *EMBO J.* **21**, 3390–3401
19. Macara, I. G. (2001)  *Microbiol. Mol. Biol. Rev.* **65**, 570–594
20. Doye, V., and Hurt, E. (1997)  *Curr. Opin. Cell Biol.* **9**, 401–411
21. Gorlich, D., Vogel, F., Mills, A. D., Hartmann, E., and Laskey, R. A. (1995)  *Nature* **377**, 246–248
22. Gorlich, D., and Mattaj, I. W. (1996)  *Science* **271**, 1513–1518
23. Zeng, Y., Hirano, K., Hirano, M., Nishimura, J., and Kanaide, H. (2000)  *Biochem. Biophys. Res. Commun.* **274**, 37–42
24. Hutchinson, J., Jin, J., Cardiff, R. D., Woodgett, J. R., and Muller, W. J. (2001)  *Mol. Cell. Biol.* **21**, 2203–2212
25. Lenferink, A. E., Buser, D., Flanagan, W. M., Yakes, F. M., and Arteaga, C. L. (2001)  *Cancer Res.* **61**, 6583–6591
26. Hidalgo, M., Siu, L. L., Nemunaitis, J., Rizzo, J., Hammond, L. A., Takimoto, C., Eckhardt, S. G., Tolcher, A., Britten, C. D., Denis, L., Ferrante, K., Von Hoff, D. D., Silberman, S., and Rowinsky, E. K. (2001)  *J Clin. Oncol.* **19**, 3267–3279
27. Hernan, R., Fasheh, R., Calabrese, C., Frank, A. J., Maclean, K. H., Allard, D., Barraclough, R., and Gilbertson, R. J. (2003)  *Cancer Res.* **63**, 140–148
28. Alt, J. R., Cleveland, J. L., Hannink, M., and Diehl, J. A. (2000)  *Genes Dev.* **14**, 3102–3114
29. Sutterluty, H., Chatelain, E., Marti, A., Wirbelauer, C., Senften, M., Muller, U., and Krek, W. (1999)  *Nat. Cell Biol.* **1**, 207–214
30. Sekimoto, T., Fukumoto, M., and Yoneda, Y. (2004)  *EMBO J.* **23**, 1934–1942
31. Ahmed, N. N., Frank, T. F., Bellacosa, A., Datta, K., Gonzalez-Portal, M. E., Taguchi, T., Testa, J. R., and Tsichlis, P. N. (1993)  *Oncogene* **8**, 1557–1563
32. Andjelkovic, M., Alesi, D. R., Meier, R., Fernandez, A., Lamb, N. J., Freich, M., Cron, P., Cohen, P., Lucoq, J. M., and Hemmings, B. A. (1997)  *J. Biol. Chem.* **272**, 31515–31524
33. Borgatti, P., Martelli, A. M., Tabellini, G., Bellacosa, A., Capitani, S., and Neri, L. M. (2003)  *J. Cell. Physiol.* **196**, 79–88
34. Alessi, D. R., Andjelkovic, M., Caudwell, B., Cron, P., Morrice, N., Cohen, P., and Hemmings, B. A. (1996)  *EMBO J.* **15**, 6541–6551
35. Bellacosa, A., Chan, T. O., Ahmed, N. N., Datta, K., Malstrom, S., Stokoe, D., McCormick, F., Feng, J., and Tsichlis, P. (1998)  *Oncogene* **17**, 313–325
36. Chan, T. O., Rittenhouse, S. E., and Tsichlis, P. N. (1999)  *Annu. Rev. Biochem.* **68**, 965–1014
37. Chen, T. O., and Tsichlis, P. N. (2001)  *Science’s STKE* http://stke. sciencemag.org/cgi/content/full/sigtrans;2001/66/pe1
38. Moll, T., Tebb, G., Surana, U., Robitsch, H., and Nasmyth, K. (1991)  *Cell* **66**, 743–758
39. Hennekens, H., Peter, M., Weber, K., and Nigg, E. A. (1993)  *J. Cell Biol.* **120**, 1293–1304
40. Obia, Y., Nishida, E., Sakai, H., and Miyamoto, E. (1989)  *J. Biol. Chem.* **264**, 16143–16148
41. Jans, D. A., and Jans, P. (1994)  *Oncogene* **9**, 2961–2966
42. Norris, J. L., and Manley, J. L. (1992)  *Genes Dev.* **6**, 1654–1667
43. Moll, T., Tebb, G., Carbia, A. J., Lasserson, R. A., and Gilmore, T. D. (1991)  *J. Cell. Biol.* **11**, 5867–5877
44. Sutterluty, H., Chatelain, E., Marti, A., Wirbelauer, C., Senften, M., Muller, U., and Krek, W. (1999)  *Nat. Cell Biol.* **1**, 207–214
45. Ahmed, N. N., Frank, T. F., Bellacosa, A., Datta, K., Gonzalez-Portal, M. E., Taguchi, T., Testa, J. R., and Tsichlis, P. N. (1993)  *Oncogene* **8**, 1557–1563
46. Andjelkovic, M., Alesi, D. R., Meier, R., Fernandez, A., Lamb, N. J., Freich, M., Cron, P., Cohen, P., Lucoq, J. M., and Hemmings, B. A. (1997)  *J. Biol. Chem.* **272**, 31515–31524
47. Borgatti, P., Martelli, A. M., Tabellini, G., Bellacosa, A., Capitani, S., and Neri, L. M. (2003)  *J. Cell. Physiol.* **196**, 79–88
48. Alessi, D. R., Andjelkovic, M., Caudwell, B., Cron, P., Morrice, N., Cohen, P., and Hemmings, B. A. (1996)  *EMBO J.* **15**, 6541–6551
49. Bellacosa, A., Chan, T. O., Ahmed, N. N., Datta, K., Malstrom, S., Stokoe, D., McCormick, F., Feng, J., and Tsichlis, P. (1998)  *Oncogene* **17**, 313–325
50. Chan, T. O., Rittenhouse, S. E., and Tsichlis, P. N. (1999)  *Annu. Rev. Biochem.* **68**, 965–1014
51. Chen, T. O., and Tsichlis, P. N. (2001)  *Science’s STKE* http://stke. sciencemag.org/cgi/content/full/sigtrans;2001/66/pe1
52. Moll, T., Tebb, G., Surana, U., Robitsch, H., and Nasmyth, K. (1991)  *Cell* **66**, 743–758
53. Hennekens, H., Peter, M., Weber, K., and Nigg, E. A. (1993)  *J. Cell Biol.* **120**, 1293–1304
54. Obia, Y., Nishida, E., Sakai, H., and Miyamoto, E. (1989)  *J. Biol. Chem.* **264**, 16143–16148
55. Jans, D. A., and Jans, P. (1994)  *Oncogene* **9**, 2961–2966
56. Norris, J. L., and Manley, J. L. (1992)  *Genes Dev.* **6**, 1654–1667
57. Mostalos, G., Hamer, P., Capobianco, A. J., Lasserson, R. A., and Gilmore, T. D. (1991)  *J. Cell. Biol.* **11**, 5867–5877
58. Tagawa, T., Kuroki, T., Varg, P. K., and Chida, K. (1995)  *J. Cell Biol.* **130**, 255–263
59. Fujita, N., Sato, S., Katayama, K., and Tsuruo, T. (2002)  *J. Biol. Chem.* **277**, 28706–28713
60. Fujita, N., Sato, S., and Tsuruo, T. (2003)  *J. Biol. Chem.* **278**, 49254–49260
61. Muller, D., Thiele, K., Burgin, A., Dickmanns, A., and Elers, M. (2000)  *EMBO J.* **19**, 2168–2180
62. Smithman, M., Lee, K., Swaner, J., Kapur, R., and Clurman, B. E. (2000)  *J. Biol. Chem.* **275**, 5631–5642
63. Guan, T., Kehlenbach, R. H., Schirmer, E. C., Kehlenbach, A., Fan, F., Clurman, B. E., Arnheim, N., and Gerace, L. (2000)  *J. Biol. Chem.* **275**, 5631–5630
64. Lindsay, M. E., Pfaffler, K., Smith, A. E., Clurman, B. E., and Macara, I. G. (2002)  *Cell* **110**, 349–360
Phosphorylation of p27Kip1 at Thr-157 Interferes with Its Association with Importin α during G1 and Prevents Nuclear Re-entry
Incheol Shin, Jeremy Rotty, Frederick Y. Wu and Carlos L. Arteaga

J. Biol. Chem. 2005, 280:6055-6063.
doi: 10.1074/jbc.M412367200 originally published online December 3, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M412367200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 49 references, 28 of which can be accessed free at http://www.jbc.org/content/280/7/6055.full.html#ref-list-1