cdk6 Can Shorten G1 Phase Dependent upon the N-terminal INK4 Interaction Domain*

Martha J. Grossel§, Gregory L. Baker, and Philip W. Hinds§

From the Department of Pathology, Harvard Medical School, Boston, Massachusetts 02115

Deregulated activity of cdk4 or cdk6 can lead to inappropriate cellular proliferation and tumorigenesis accompanied by unchecked inactivation of the retinoblastoma tumor suppressor protein. Certain tumor types preferentially activate either cdk4 or cdk6, suggesting that these kinases may not be equivalently oncogenic in all cell types. Although it is clear that cdk4 can act as an oncogene at least in part by evading inhibition by p16INK4a, the role of cdk6 in tumorigenesis is less well understood. To investigate the consequences of aberrant expression of cdk6, the requirements for proliferation caused by cdk6 overexpression were studied. cdk6-transfected U2OS cells displayed an accelerated progression through G1 phase that was dependent on kinase activity and that did not correlate with p27 binding. Furthermore, a mutation that prevents cdk6 interaction with INK4 proteins (cdk6R31C) was found to inactivate the proliferative effect of cdk6 and increase cytoplasmic localization, despite the fact that this mutant could phosphorylate the retinoblastoma protein in vitro. Together, these data suggest a role for the cdk6 INK4 interaction domain in the generation of functional, nuclear cdk6 complexes and demonstrate the importance of elevated cdk6 kinase activity in G1 acceleration.

In mammalian cells, the regulation of cell division is tightly controlled through a series of checkpoints within the cell cycle, including the restriction point in late G1 phase, a checkpoint that determines commitment to DNA replication. The restriction point may be viewed as the culmination of activation of G1 cyclin-dependent kinases, enzymes that govern cell cycle progression through phosphorylation of key regulatory substrates. Specifically, the cyclin D proteins and their associated kinases, cyclin-dependent kinase (cdk)3 and cdk6, function early in G1 phase of the cell cycle to link growth regulatory signals to the control of cell division. Both cdk4 and cdk6 can be activated by all three D-type cyclins (cyclins D1, D2, and D3) and are thought to function as positive effectors of G1 progression (1–4). Activation of cdk4 and cdk6 allows progression from G1 phase to the start of DNA synthesis in normal eukaryotic cells by phosphorylating and inactivating the retinoblastoma protein (pRb). This initial modification of pRb by cdk4/cdk6-dependent phosphorylation may be followed by further phosphorylation by cyclin E/cdk2 complexes and ultimately relieves repression of E2F-dependent promoters, allowing the transcription of S phase genes and the onset of DNA replication (for review, see Ref. 5).

Because the cyclin D-dependent kinases play a pivotal role linking growth regulatory signals to cell division, activity of these kinases is very tightly controlled. Kinase activity is regulated by the periodic synthesis and destruction of the cyclin subunits, by phosphorylation and dephosphorylation of the kinase subunit, and through complex formation with two families of cyclin-dependent kinase inhibitors (CKIs) (6). The CIP/KIP family of inhibitors includes p21, p27, and p57, which associate with several different cyclin/cdk complexes (7–11). These proteins may act as stimulators of cdk activity as well as inhibitors because p21 has been shown to both activate and inactivate cyclin/cdk complexes, perhaps dependent on stoichiometry (12, 13). Indeed, the ability of p21 and p27 to stabilize D cyclin/cdk4 (6) complexes may be required for the proper formation of these complexes (14). In contrast to the CIP/KIP family, the INK4 family of CKIs (p15INK4b, p16INK4a, p18INK4a, and p19INK4a) specifically inhibits the activities of cdk4 and cdk6 by binding directly to the kinase subunit, disallowing association with the activating cyclin D subunit (Refs. 15 and 16; for review, see Ref. 17).

Aberrant cell proliferation and tumorigenesis can result from deregulated activity of cdk4 and/or cdk6 with subsequent, inappropriate inactivation of pRb in several tissue types. This increased kinase activity can result from overexpression of the regulatory subunit, cyclin D1, and also from amplification of the kinase-encoding gene. In addition, deletion or inactivation of the gene encoding p16INK4a frequently leads to dysregulated cdk4/cdk6 activity in human tumors, as do mutations in cdk4 that prevent its association with p16INK4a (18–25, 29). In most cases, tumors containing hyperactivated cdk4 or cdk6 retain intact RB alleles, suggesting that such kinase activations render pRb unable to control proliferation. These findings indicate that deregulated cdk4 and cdk6 activity can substitute for RB mutations and define the "pRb pathway" of genetic events that have the identical phenotypic consequence of pRb inactivation and inappropriate proliferation. A further oncogenic consequence of excess cyclin D/cdk4(6) expression is sequestration of p21 and p27 with consequent elevated activity of cyclin E/cdk2, a function that may be an extension of a physiological role of cyclin D/cdk4 (6) complexes (14, 26–28).

To better understand the consequences of dysregulated cdk6 expression as is seen in some tumor types (30, 31), we began by studying the requirements for proliferation caused by ectopic cdk6 expression. cdk6-transfected cells demonstrated accelerated transit through the G1 phase of the cell cycle. This effect of cdk6 on cell cycle progression was dependent on the INK4 family...
cdk6 Acceleration of G1 Phase

binding domain, because a cdk6 mutant (R31C) unable to associate with INK4a proteins did not show G1 acceleration. In addition, this mutant protein failed to accumulate in the nucleus, suggesting that nuclear localization and function of cdk6 is dependent on the INK4 interaction domain. Furthermore, catalytic activity of cdk6 was required for G1 acceleration in this assay, because a catalytically inactive NFG mutant slowed S phase entry rather than accelerated it, despite an ability to form complexes with cyclin D1 and p27. Thus, cdk6 activity is limiting for G1-to-S phase progression, even in tumor cells such as U2OS, which lacks p16INK4a, strongly supporting a role for cdk6-specific phosphorylation events in G1 progression.

EXPERIMENTAL PROCEDURES

Expression Vectors, Transfection Procedures, and Cell Lines—The kinase expression plasmids pCMVcdk6 and HA-tagged cdk6, pCMVcdk6HA, pCMVcdk6NFG, the vector pCMVneo, and the CD20-encoding plasmid pCMVCD20 were kindly provided by Dr. Sander van den Heuvel (32). pCMVBamNeo, the vector containing the cDNAs, has been described previously (33). U2OS cells were transiently transfected with 15 μg of kinase-expressing plasmid plus 5 μg of pCMV-CD20 (where appropriate) and sheared herring sperm DNA to a total of 60 μg by calcium phosphate precipitation essentially as described by Chen et al. (16). DNA precipitates were pelleted on the cell monolayer for 17 h, and cells were harvested 24 h after removal of DNA precipitates unless otherwise noted. U2OS cells were maintained in 10% fetal calf serum at 5% CO2.

Analysis of Cell Cycle Distribution—For fluorescence-activated cell sorting (FACS) experiments, transfected U2OS cells were harvested in PBS with 0.1% EDTA at 24 h after removal of DNA precipitates. Cells were then stained with fluorescein isothiocyanate (FITC)-conjugated antibody to human CD20 (Pharmingen) and ethanol fixed and stained with propidium iodide for DNA content. Cell cycle distribution was analyzed by flow cytometry of CD20-positive (FITC-positive) cells using a Coulter cytometer and Multicycle DNA analysis. In FACS studies of nocodazole-treated cells, nocodazole was added at 24 h after removal of DNA precipitates to a final concentration of 100 ng/ml for 18 h. In FACS studies following mitotic shake, mitotic fractions were harvested by gentle pipetting followed by centrifugation at 1000 rpm at 25 °C for 5 min. Cells were washed three times with media to remove nocodazole and replated in 10% fetal calf serum media. Cells were harvested at time points indicated and prepared for FACS as described above.

BrdUrd and Immunofluorescence—5-bromo-2'-deoxyuridine (BrdUrd) incorporation experiments nocodazole was added to 100 ng/ml approximately 5 h after removal of DNA precipitates and remained on cells 18 h. BrdUrd was added to a final concentration of 10 μM at the time points indicated. Coverslips from BrdUrd experiments were fixed in 70% ethanol, 50 mM glycine, pH 2.0, and incubated with BrdUrd monoclonal antibody (Roche Molecular Biochemicals) and polyclonal peptide antibodies to cdk6 (Santa Cruz Biotechnology, C-21) for 60 min at 37 °C. Secondary antibodies rhodamine-conjugated donkey anti-rabbit (Jackson ImmunoResearch, West Grove, PA) and fluorescein-conjugated anti-mouse (Roche Molecular Biochemicals) were incubated 30 min at 37 °C. Coverslips were mounted in Fluoromount. For time courses, mitotic shake was performed as described above, and coverslips were fixed as printed at the indicated time points. For immunofluorescence without BrdUrd, coverslips were stained in methanol followed by acetone. Immunofluorescence was performed with antibodies indicated above, as well as cdk6 polyclonal sera of Meyerson (4) and cdk6 monoclonal sera Ab-3 (Neomarkers, Fremont, CA) for 60 min at 37 °C. Secondary antibody staining was performed as described above or with fluorescein-conjugated donkey anti-mouse antibody (Jackson ImmunoResearch). In relevant cases, cells were counterstained by Hoechst stain. All photography was performed on a Leica microscope with Sony digital imaging.

Biochemical Assays—For immunoblot and immunoprecipitation experiments, 1.25 × 105 U2OS cells were transfected as described above, harvested 24 h after removal of DNA precipitates, washed twice with phosphate-buffered saline (PBS) and harvested in 1 ml of E1A lysis buffer (50 mM NaCl, 50 mM Hepes, pH 7.0, 5 mM EDTA, 0.1% Nonidet P-40). Extracts were incubated for 20 min on ice with mixing and centrifugation for 20 min at 4 °C. Proteins were separated on polyacrylamide denaturing gels, transferred to supported nitrocellulose (Life Technologies, Inc.) and blotted using antisera as noted.

Immunoprecipitations were performed with 2 μg of polyclonal cdk6 antisera C-21. Transfected U2OS cells were lysed in E1A lysis buffer as described above, and 200 μg (cyclin D1) or 400 μg (p18, p27) of extract was immunoprecipitated for 60 min at 4 °C with mixing. 35 μl of swollen protein A-Sepharose beads were added for an additional 30 min, washed four times with 1 ml of E1A lysis buffer, and separated on denaturing acrylamide gels. Antibodies used were p18 polyclonal (1:3000) (Santa Cruz), p27 monoclonal antibody (1:2500) (Transduction Laboratories), and cyclin D1 monoclonal DCS-6 antibody (1:200) (Neomarkers).

For kinase assays, SAOS-2 cells at 80% confluence were transfected with 10 ng of pCMVD1 and 10 ng of pCMVcdk6HA or cdk6mutantHA in the pCMV vector by calcium phosphate as described above. DNA precipitates remained on cells for 10 h and were harvested 36 h after removal of DNA precipitates. Cells were harvested in D-IP kinase buffer (50 mM Hepes, pH 7.5; 150 mM NaCl; 1 mM EDTA; 2.5 mM EGTA; 0.1% Tween 20; 10% glycerol with the protease inhibitors apronin, leupeptin, and Pefabloc and phosphatase inhibitors sodium orthovanadate (100 μM), sodium fluoride (10 μM), and betaglycophosphate (10 μM)) and incubated on ice for 20 min with gentle mixing. Lysates were clarified by centrifugation at 4 °C for 10 min. 100 μl of 12CA5 antibody was preincubated with 30 μl of swollen protein A-Sepharose beads for at least 1 h at 4 °C with mixing. 100 μg of cell lysate was added for an additional 1 h at 4 °C with mixing. Beads were washed three times with D-IP buffer and three times with kinase reaction buffer (250 mM Hepes, pH 7.2, 50 mM MgCl2, 25 mM MnCl2, 1 mM DTT, 10% glycerol) and aliquots were removed for 10 μM ATP, 10 μCi of [γ-32P]ATP and 0.5 μg of C-terminal GST-Rb (amino acids 769–921) (Santa-Cruz Biotechnology) as substrate. Reactions were stopped by addition of protein sample buffer with 10% beta-mercaptoethanol and placed on ice. Samples were boiled and separated on 12.5% denaturing acrylamide gel, Coomassie Brilliant Blue-stained to ensure equal loading and addition of Rb substrate, and exposed to film overnight.

RESULTS

cdk6 Causes Increased S Phase of Transfected Cell Populations—The effect of ectopic expression of cdk6 on cell cycle progression was determined by FACS studies in transfected U2OS cells. These human osteosarcoma cells produce wild-type pRb but lack p16INK4a, a defect that is thought to allow constitutive phosphorylation and inactivation of pRb. In light of this, it was surprising to observe that cells transfected with plasmid encoding cdk6 consistently showed a higher percentage of S phase cells than did vector-transfected cells in the same experiment (Fig. 1). To confirm that this increase in DNA content measured by FACS truly reflected an increase in S phase cells, transfected cell cultures were also analyzed using BrdUrd incorporation as a measure of S phase. U2OS cells transfected with pCMVcdk6 or pCMVvector were pulsed with BrdUrd, fixed, and subjected to indirect immunofluorescence using both anti-BrdUrd and anti-cdk6 antibodies. Transfected cells were identified as those that demonstrated intense fluorescence with anti-cdk6 antibody and were scored as either BrdUrd-positive or BrdUrd-negative. The results of at least two independent transfections (≥700 kinase-positive cells counted) demonstrated that 34% of vector-transfected cells were BrdUrd-positive, whereas in the same experiment, 50% of cdk6-transfected cells were BrdUrd-positive (Fig. 1B). These data closely match the results of FACS analysis shown in Fig. 1A and indicate that cells transfected with cdk6 showed a statistically significant (p < 0.05) increase in the percentage of S phase population as compared with vector-transfected cells.

cdk6 Shortens the G1 Interval of Transfected U2OS Cells—The observed increase in S phase cells conferred by cdk6 could result from either an S phase cell cycle block or from decreased transit time through G1→S→G2/M. To distinguish between these possibilities, transfected cells were treated with the mitotic inhibitor nocodazole. In the presence of nocodazole, an S phase delay would reduce the number of cells able to enter G2/M. However, if the increased S phase population was due to a shortening of G1 phase (or G2/M phase) cells would arrest in mitosis under nocodazole treatment. At 24 h after removal of...
DNA precipitates, parallel sets of transfected U2OS cells were either harvested or treated with nocodazole for 18 h. A p16 control for nocodazole arrest demonstrated that p16 transfected cells maintained a G1 phase peak as expected with a G1-arresting inhibitor (Fig. 2A). In the same experiment, cdk6-transfected cells accumulated in mitosis in the presence of nocodazole, indicating that the S phase increase seen by FACS and BrdUrd incorporation studies (Fig. 1) was not due to a profound S phase delay but was more likely a result of a decreased G1 or G2/M transit time.

To determine whether ectopic cdk6 expression decreased G1 phase transit time, FACS analysis was performed on synchronized cell populations. Transfected U2OS cells were treated with nocodazole for 18 h followed by shaking and replating in nocodazole-free medium. The cells were harvested at 4 and 8 h after mitotic shake, and DNA profiles of CD20-positive cells were obtained by FACS as shown in Fig. 2B. At 4 h after mitotic shake, cdk6-transfected cells showed a synchronized DNA profile indistinguishable from that of vector transfected cells. Interestingly, at 8 h post-mitotic shake, cdk6-transfected cells showed a shift toward S phase (increased DNA content) as compared with vector-transfected cells. Intersecting these experiments indicate that cdk6-transfected U2OS cells pass through G1 phase faster than vector-transfected cells, demonstrating a cdk6-dependent acceleration of G1 transit in U2OS cells. Also shown in Fig. 2B are DNA profiles of a mutant form of cdk6, cdk6R31C. cdk6R31C contains a mutation of the arginine residue corresponding to Arg-24 in cdk4. Mutation of this residue (Arg-24 to Cys) in cdk4 was identified in a human melanoma and prevents binding to the kinase inhibitor, p16 (24). Interestingly, cdk6R31C did not demonstrate the shift toward S phase seen with the wild-type cdk6, suggesting a role for the INK4 binding domain in the G1 acceleration function of cdk6. The lack of G1 acceleration exhibited by cdk6R31C could be due to an unexpected loss of catalytic activity or to the disruption of another property of cdk6 required for this G1 acceleration function in U2OS cells.

Biochemical Characterization of cdk6 Mutants—The acceleration of G1 phase caused by ectopic expression of cdk6 could be the result of direct catalytic activity of the introduced kinase subunit phosphorylating substrates such as pRb to shorten G1 phase. Alternatively, excess kinase subunits could titrate inhibitory proteins to allow activation of other cdks and concomitant cell cycle advance. Titration of inhibitory proteins has been observed to occur upon introduction of both functional and nonfunctional kinases in another system, apparently through titration of p21 (28), and cyclin D/cdk4(6) complexes have been suggested to sequester p27 in the absence of anti-mitogenic signals (26, 27). In an effort to determine the properties of cdk6 required to accelerate G1 progression in U2OS cells, a series of cdk6 mutants compromised in their ability to bind to INK4 protein (cdk6R31C), hydrolyze ATP (cdk6NFG) (32), or both (cdk6R31CNFG) were used in cell cycle analyses. The biochemical characterization of these mutant proteins is presented in Fig. 3. All cdk6 mutants consistently showed approximately equal protein levels in transfected U2OS lysates (Fig. 3A). Consistent with the predicted result, we found that the R31C mutation prevented interaction with p16INK4 (data not shown) and p18INK4c in transfected U2OS lysates (Fig. 3B), as has also been observed in breast cancer cell lines (35). Disruption of INK4 binding occurred whether the mutation was present alone (cdk6R31C) or in combination (cdk6R31CNFG) with the catalytically inactive mutation (Fig. 3B). Importantly, the R31C mutation does not disrupt the ability of cdk6 to bind cyclin D1 or p27 in immunoprecipitations of transfected U2OS extracts (Fig. 3, C and D). In these experiments, immunoblots were stripped and reprobed with anti-cdk6 antibody to ensure that equivalent
levels of cdk6 protein were compared in binding studies, and control immunoblots of the lysates demonstrated that cyclin D1 or p27 levels were equivalent in extract of transfected cells (data not shown). Thus, the cdk6R31C mutation that corresponds to the tumor-derived cdk4R24C mutation (24) specifically disrupts cdk6 binding to INK4 proteins without altering interaction with other known cdk6 partners.

To ensure that the R31C mutant form of cdk6 retained catalytic activity, the cdk6 mutants were also examined for kinase activity in transfected SAOS-2 cells (used in these assays because they contain low-levels of endogenous cyclin D1 and cdk6 activity). As shown in Fig. 3E, when co-transfected with cyclin D1 and immunoprecipitated with antibody to the HA tag, HAcdk6 phosphorylated the C-terminal GST-Rb substrate. Conversely, cdk6NFG containing the kinase-inactivating mutation showed no kinase activity above vector-transfected background. Significantly, cdk6R31C-transfected extracts reproducibly showed in vitro kinase activity greater than that observed with wild-type cdk6 extracts, as expected for a mutant that can evade the p16INK4a present at high level in SAOS-2 cells. Anti-HA immunoblots of these extracts confirmed that the level of cdk6R31C was at or below the level of wild-type cdk6 protein (not shown). Interestingly, the double mutant cdk6R31CNFG had a slightly elevated activity relative to the inactive NFG mutant. This result suggests that a low level cdk6NFG activity is unmasked in cdk6R31CNFG by the disruption of INK binding.

Thus, the cdk6R31C mutation disrupts INK4 protein binding but did not disrupt intrinsic catalytic activity of this cdk6 protein, similar to studies demonstrating retention of kinase activity by the p16INK4a binding-defective mutant of cdk4, cdk4R24C (24). Importantly, these binding studies and kinase assays also indicate that these mutations are not causing gross structural alterations in the cdk6 protein. These reagents are thus ideal for assessing the potential roles of catalytic activity and INK4 titration in the cdk6-mediated acceleration of G1 phase in transfected U2OS cells.

cdk6 Mutants Do Not Accelerate G1 Phase—The cdk6 mutants described above were used to further examine cdk6 function in G1, acceleration of U2OS cells. To test the ability of mutant forms of cdk6 to decrease G1 transit time, BrdUrd incorporation was used to measure S phase entry by the cdk6 mutants. In these experiments BrdUrd was added to transfected U2OS cells 4 h after mitotic shake and BrdUrd incorporation was measured at 5, 7, and 10 h after mitotic shake. The results of these experiments in which cumulative BrdUrd incorporation was measured are presented in Fig. 4. These BrdUrd studies indicated that the kinase inactive mutant, cdk6NFG, showed a slower entry into S phase as compared with the vector-transfected control. In fact, cdk6NFG showed a greatly decreased percent of cells in S phase at all time points measured (27% at 10 h). FACS analysis after nocodazole treatment suggested that this decrease in S phase entry was due to a G1 delay because a significant G1 fraction persisted in nocodazole-arrested cells (not shown). A similar delay to S phase entry has been observed with cdk4NFG (36). Thus, catalytic activity in addition to inhibitor titration appears to be required for the G1 acceleration observed with wild-type cdk6 because cdk6NFG was incapable of increasing the S phase fraction of transfected cells but is fully capable of inhibitor interaction.

Because the result above suggests that kinase activity intrinsic to cdk6 is key to accelerating G1 phase in transfected U2OS cells, the cdk6R31C mutant initially was expected to give an increase in S phase cells equal to or greater than that conferred by wild-type cdk6, given the ability of cdk6R31C to give an increase in S phase cells equal to or greater than that observed with wild-type cdk6 extracts, as expected for a mutant that can evade the p16INK4a present at high level in SAOS-2 cells. Anti-HA immunoblots of these extracts confirmed that the level of cdk6R31C was at or below the level of wild-type cdk6 protein (not shown). Interestingly, the double mutant cdk6R31CNFG had a slightly elevated activity relative to the inactive NFG mutant. This result suggests that a low level cdk6NFG activity is unmasked in cdk6R31CNFG by the disruption of INK binding.

FIG. 2. Cell cycle profiles of transfected cells. Cells were incubated with FITC-conjugated CD20 antibody to distinguish transfected populations, fixed, and stained with propidium iodine for DNA content. A, FACS profiles of p16- and cdk6-transfected U2OS cells either untreated or nocodazole-treated, as indicated. B, DNA profiles of nocodazole-synchronized cells transfected with vector (solid line), cdk6 (dotted line), or cdk6R31C (boldface line) and harvested at 4 and 8 h following mitotic shake and replating.
in S phase population caused by cdk6wt because cdk6R31C, which has demonstrated in vitro kinase activity, cannot accelerate G1 phase. Furthermore, the R31C mutation in cdk6R31CNFG nullifies the S phase inhibitory effect seen after introduction of cdk6NFG, suggesting a critical role for the R31 residue in cdk6 function.

**Cell Cycle Effects Correlate with Nuclear Localization**—Previous studies have shown that the subcellular localization of cdk4 may influence its interaction with the CIP and INK families of inhibitors (27). In addition, both cdk4 and cdk6 have been observed to localize to the cytoplasm in a variety of cell types (37–39). Thus, we wished to test the hypothesis that the inability of the cdk6 mutants to accelerate G1 phase may be in part due to differential localization within the cell. Transfected U2OS cells were synchronized using nocodazole and analyzed by indirect immunofluorescence for cdk6. Repeatedly, the mutant forms of cdk6 that failed to bind INK4 proteins (cdk6R31C and cdk6R31CNFG) demonstrated greatly decreased nuclear staining as compared with cdk6wt and cdk6NFG at 8–10 h after mitotic shake (Fig. 5). These results were repeated in at least three separate transfections and with two distinct staining methods using both polyclonal and monoclonal antibodies (Fig. 5).

The decrease in nuclear staining observed in cdk6R31C and cdk6R31CNFG transfectants was also observed in asynchronous populations at 24 h after removal of DNA precipitates, but in these populations the percentage of cdk6R31C and cdk6R31CNFG mutants with predominantly cytoplasmic staining was lower than the percentage seen in a synchronous population (shown in Fig. 5), suggesting that the localization of these kinases is cell cycle-regulated.

Together, these studies demonstrate that the R31C mutation affects compartmentalization of cdk6, as well as the ability to interact with INK4 proteins. R31C mutants showed a remarkable decrease in nuclear staining particularly at time points predicted to be at or near the G1/S boundary. This difference in compartmentalization directly correlated with the inability of the same mutants to accelerate G1 phase of the cell cycle and suggested a role for INK4 protein binding domain in the generation of functional, nuclear cdk6 complexes.

**DISCUSSION**

The D-cyclin-dependent kinases cdk4 and cdk6 share pRb as their only proven physiological substrate, and both can act as oncogenes in human tumors that retain pRb. Previous experiments have identified numerous, tumor-derived cdk4 mutants that fail to interact with p16INK4a, suggesting that this kinase acts as an oncogene by evading inhibition by INK4 proteins. This may in turn result in direct modification of substrates by high levels of kinase activity or may produce an indirect effect through increased p21/p27 titration. The results presented here demonstrate that cdk6 can accelerate G1 phase transit when ectopically expressed in U2OS cells even though these cells do not express p16INK4a.
Expression of cdk6 resulted in increased S phase as measured by FACS and BrdUrd incorporation. Nocodazole and mitotic release studies indicated that the observed increase in S phase cells resulted at least in part from decreased transit time through G1 phase of the cell cycle. Although we cannot rule out an accompanying lengthening of S phase, nocodazole-treated, cdk6-transfected cells did accumulate in M phase, suggesting that any effect of S phase transit time was not large. Surprisingly, the accelerated G1 phase did not require co-transfection of the kinase-activating partner, cyclin D. G1 acceleration by cdk6 in the absence of increased cyclin D is consistent with a model in which the supply of cyclin D is not the only rate-limiting step in kinase activation. Indeed, cyclin D1 levels are quite stable across the cell cycle in many proliferating cells, unlike cyclins A, E, and B. Thus, whereas cyclin D complex formation is obviously a critical step in kinase activation, it may not be the rate-limiting step in cultures of proliferating cells.

The availability of the kinase may be particularly limiting for cyclin D/cdk6 complexes, because the INK4 family of inhibitors act as competitors with cyclin D for cdk6 binding. In light of this, the ability of cdk6 to accelerate G1 in U2OS cells is somewhat surprising, given that U2OS cells lack p16INK4a and thus are thought to be able to phosphorylate and inactivate pRb without hindrance of cdk4 or cdk6 activity. Nevertheless, our results suggest that cdk6 is limiting for cell cycle progression despite the absence of p16INK4a. This is consistent with the observed ability of excess cdk4 to increase the proliferation of astrocyte cell lines lacking p16INK4a (40). One possibility arising from such observations is that cdk6 can act “noncatalytically” in this system by sequestering p21 or p27 from cyclin D/cdk6 complexes, because INK4 proteins can compete with cdc37/hsp90 for binding to cdk6 under certain circumstances, because INK4 proteins can compete with cdc37/hsp90 for binding to cdk4 and cdk6 (44). The precise role of the cdk6 N terminus in cell cycle regulation is currently under investigation, but whatever the mechanism, maintenance of the cdk6R31C mutant in the cdk37 complex predicts a persistence of cytoplasmic localization and a functionally inactive kinase. In fact, this is precisely the phenotype observed with cdk6R31C: an increased cytoplasmic retention (Fig. 5) and a loss of function in either G1 acceleration (cdk6wt) or G1 retardation (cdk6NFG) (Fig. 4), despite greater than wild-type catalytic activity of cdk6R31C in in vitro kinase assays (Fig. 3).

The requirement for cdk6 nuclear localization in G1 acceleration, its likely regulation in the cell cycle, and the novel role of the N terminus in this localization raise the possibility of the existence of a cdk6 regulatory pathway that may result in differential activity of cdk4 and cdk6 in the same cell. Indeed,

\[ G_1 \text{ acceleration in U2OS cells. cdk6NFG was completely incapable of shortening G}_1 \text{ phase in these experiments, and indeed it detectably delayed S phase entry as determined by BrdUrd incorporation following release from nocodazole (Fig. 4). These results strongly argue that catalytic activity of cdk6 is required for the observed effects on cell cycle progression in U2OS cells and inhibitor sequestration is not sufficient for this effect.} \]

It is possible that kinase inhibition by other members of the INK4 family limit proliferation in cultured cells lacking p16INK4a. For example, G1 length may be partly determined by the ratio of endogenous cdk6 to p15INK4b (43), which is expressed in U2OS cells, and excess cdk6 would thus result in a shortened G1 phase. This model predicts that elimination of INK4c binding by the R31C mutation, which is analogous to the oncogenic R24C mutation in cdk4, would enhance the G1 acceleration function of cdk6. However, our results using cdk6R31C stand in direct contradiction to this, because R31C is completely unable to alter G1 phase in transfected U2OS cells. Because this mutation, like the NFG mutation, does not disturb properties of cdk6 such as cyclin D1 or p27 binding, this result further argues against a role for inhibitor titration in the G1 acceleration function of cdk6. Indeed, because cdk6R31C can be activated by cyclin D1 in cotransfected cells, these results suggest that the N terminus of cdk6 may be involved in the proper function of cdk6 within cells (but not in vitro), perhaps at the level of substrate recognition or compartmentalization.

Published reports indicate that both cdk4 and cdk6 are indeed regulated at the level of subcellular localization (12, 27, 38). Consistent with these studies, data shown here demonstrate that cdk6 localizes to both the nucleus and the cytoplasm, whereas cdk6R31C preferentially localizes to the cytoplasm. Synchronized U2OS cells show a striking lack of cdk6R31C and cdk6R31C-NFG protein in the nucleus in late G1 phase. Recently, it has been shown that cytoplasmic cdk6 exists primarily in inactive complexes with cdc37 and hsp90 (or in T cells) with p19INK4d (37, 39). In light of this, the localization pattern of the cdk6R31C protein presents an apparent paradox. Why is an INK4 binding-defective cdk6 protein preferentially localized in the cytoplasm if the major function of the cytoplasmically localized INK4 protein (in this case, specifically p18INK4d) is to anchor kinases in an inactive state? We suggest that the N terminus of cdk6 is critically involved in the dissolution of cytoplasmic complexes and may be a binding site for proteins that serve to promote translocation of cdk6 to the nucleus. It is possible that such proteins resemble INK4 proteins, or it may even be the case that INK4 proteins themselves could promote nuclear entry of cdk6 under certain circumstances, because INK4 proteins can compete with cdc37/hsp90 for binding to cdk4 and cdk6 (44). The precise role of the cdk6 N terminus in subcellular localization is currently under investigation, but whatever the mechanism, maintenance of the cdk6R31C mutant in the cdk37 complex predicts a persistence of cytoplasmic localization and a functionally inactive kinase.

The role of cyclin D/cdk4(6) complexes as “sinks” for p21 and p27 will suggest that catalytic activity of cdk6 would be dispensable for G1 acceleration if titration alone were sufficient to shorten G1. Our results using cdk6NFG, which is catalytically inactive yet still able to bind D cyclins and p27, argue that such p21 and p27 titration is not responsible for cdk6-mediated G1 acceleration in U2OS cells. cdk6NFG was completely incapable of shortening G1 phase in these experiments, and indeed it detectably delayed S phase entry as determined by BrdUrd incorporation following release from nocodazole (Fig. 4). These results strongly argue that catalytic activity of cdk6 is required for the observed effects on cell cycle progression in U2OS cells and inhibitor sequestration is not sufficient for this effect.

It is possible that kinase inhibition by other members of the INK4 family limit proliferation in cultured cells lacking p16INK4a. For example, G1 length may be partly determined by the ratio of endogenous cdk6 to p15INK4b (43), which is expressed in U2OS cells, and excess cdk6 would thus result in a shortened G1 phase. This model predicts that elimination of INK4c binding by the R31C mutation, which is analogous to the oncogenic R24C mutation in cdk4, would enhance the G1 acceleration function of cdk6. However, our results using cdk6R31C stand in direct contradiction to this, because R31C is completely unable to alter G1 phase in transfected U2OS cells. Because this mutation, like the NFG mutation, does not disturb properties of cdk6 such as cyclin D1 or p27 binding, this result further argues against a role for inhibitor titration in the G1 acceleration function of cdk6. Indeed, because cdk6R31C can be activated by cyclin D1 in cotransfected cells, these results suggest that the N terminus of cdk6 may be involved in the proper function of cdk6 within cells (but not in vitro), perhaps at the level of substrate recognition or compartmentalization.

Published reports indicate that both cdk4 and cdk6 are indeed regulated at the level of subcellular localization (13, 27, 38). Consistent with these studies, data shown here demonstrate that cdk6 localizes to both the nucleus and the cytoplasm, whereas cdk6R31C preferentially localizes to the cytoplasm. Synchronized U2OS cells show a striking lack of cdk6R31C and cdk6R31C-NFG protein in the nucleus in late G1 phase. Recently, it has been shown that cytoplasmic cdk6 exists primarily in inactive complexes with cdc37 and hsp90 (or in T cells) with p19INK4d (37, 39). In light of this, the localization pattern of the cdk6R31C protein presents an apparent paradox. Why is an INK4 binding-defective cdk6 protein preferentially localized in the cytoplasm if the major function of the cytoplasmically localized INK4 protein (in this case, specifically p18INK4d) is to anchor kinases in an inactive state? We suggest that the N terminus of cdk6 is critically involved in the dissolution of cytoplasmic complexes and may be a binding site for proteins that serve to promote translocation of cdk6 to the nucleus. It is possible that such proteins resemble INK4 proteins, or it may even be the case that INK4 proteins themselves could promote nuclear entry of cdk6 under certain circumstances, because INK4 proteins can compete with cdc37/hsp90 for binding to cdk4 and cdk6 (44). The precise role of the cdk6 N terminus in subcellular localization is currently under investigation, but whatever the mechanism, maintenance of the cdk6R31C mutant in the cdk37 complex predicts a persistence of cytoplasmic localization and a functionally inactive kinase. In fact, this is precisely the phenotype observed with cdk6R31C: an increased cytoplasmic retention (Fig. 5) and a loss of function in either G1 acceleration (cdk6wt) or G1 retardation (cdk6NFG) (Fig. 4), despite greater than wild-type catalytic activity of cdk6R31C in in vitro kinase assays (Fig. 3).

The requirement for cdk6 nuclear localization in G1 acceleration, its likely regulation in the cell cycle, and the novel role of the N terminus in this localization raise the possibility of the existence of a cdk6 regulatory pathway that may result in differential activity of cdk4 and cdk6 in the same cell. Indeed,

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the fact that cdk4R24C has been reported to be hyperactive but cdk6R31C is inactive in cell cycle progression suggests that these kinases may be subject to discrete activation programs. In the case of cdk6, production of active complexes may require the presence of a factor that interacts with the N terminus and that is itself subject to cell cycle regulation. If this factor is not required by cdk4, the two kinases could respond differently to extracellular signals, and this in turn could favor activation of one kinase versus another in tumor cells. Clearly, a better understanding of the role of the cdk6 N terminus in functional regulation and the identification of putative activating factors that interact with this domain are required to fully understand the role of cdk6 in cancer cells.

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