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Cyclin D1 Splice Variants

DIFFERENTIAL EFFECTS ON LOCALIZATION, RB PHOSPHORYLATION, AND CELLULAR TRANSFORMATION*

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Cyclin D1 is a proto-oncogene that functions by inactivation of the retinoblastoma tumor suppressor protein, RB. A common polymorphism in the cyclin D1 gene is associated with the production of an alternate transcript of cyclin D1, termed cyclin D1b. Both the polymorphism and the variant transcript are associated with increased risk for multiple cancers and the severity of a given cancer; however, the underlying activities of cyclin D1b have not been elucidated relative to the canonical cyclin D1a. Because cyclin D1b does not possess the threonine 286 phosphorylation site required for nuclear export and regulated degradation, it has been hypothesized to encode a stable nuclear protein that would constitutively inactivate the RB pathway. Surprisingly, we find that cyclin D1b protein does not inappropriately accumulate in cells and exhibits stability comparable to cyclin D1a. As expected, the cyclin D1b protein was constitutively localized in the nucleus, whereas cyclin D1a was exported to the cytoplasm in S-phase. Despite enhanced nuclear localization, we find that cyclin D1b is a poor catalyst of RB phosphorylation/inactivation. However, cyclin D1b potently induced cellular transformation in contrast to cyclin D1a. In summary, we demonstrate that cyclin D1b specifically disrupts contact inhibition in a manner distinct from cyclin D1a. These data reveal novel roles for D-type cyclins in tumorigenesis.

Cyclin D1 is an essential regulator of cell cycle progression, and aberrant induction of cyclin D1 activity is well established in human tumorigenesis (1–7). Initially, cyclin D1 was identified as the PRAD1 oncogene by mapping the sites of amplification in parathyroid adenomas to 11q13 (1, 8). Subsequently, deregulation of cyclin D1 has been observed to occur in a variety of cancer types (9). For example, the BCL-1 translocation in centrocytic lymphoma deregulates the expression of cyclin D1 (8). In animal models, cyclin D1 has also been shown to exhibit oncogenic activity when overexpressed in specific tissues (10). Lastly, cyclin D1 activity is critical for tumor formation induced by other oncogenes (*e.g.* Ras), as mice deficient in cyclin D1 are resistant to tumorigenesis (11, 12). Because of the intimate connections between cyclin D1 and oncogenesis, extensive analyses have focused on the mechanisms through which cyclin D1 contributes to human cancer.

Cyclin D1 exerts its effects on cellular proliferation by integrating external signals (*e.g.* mitogens) with the cell cycle machinery (3–7, 13–15). Given this critical role, cyclin D1 action is highly regulated. Cyclin D1 transcription is stimulated as a delayed early response to mitogenic signaling cascades (4, 16). Additionally, protein stability is regulated through the glycogen synthase kinase 3β signal transduction pathway to coordinately enhance accumulation of cyclin D1 protein (17, 18). Once synthesized, cyclin D1 interacts with and activates the G_1 cyclin-dependent kinases (CDK),[1] CDK4 and CDK6 (19, 20). This interaction is assisted through the action of both mitogen-activated protein kinase-mediated signaling and p21Cip1/p27Kip1 proteins (21, 22). CDK4/6-cyclin D1 complexes are both rate-limiting and required for cell cycle progression in most cells studied. For example, ectopic expression of cyclin D1 shortens the G_1 interval, whereas inactivation of cyclin D1 arrests cells in G_1 (23–26). Thus, intricate regulation of cyclin D1 expression and activity plays a critical role in ordered progression through the cell cycle.

The principal substrate for cyclin D1-associated kinase activity is the retinoblastoma tumor suppressor (RB) (26–30). Initially identified via bialleleic inactivation in retinoblastomas, RB has been found to be inactivated through a myriad of mechanisms in the majority of human cancers (2, 3, 6, 31). RB functions as a transcriptional repressor that attenuates the expression of multiple gene products required for cell cycle progression (32–34). As such, active RB potently inhibits cell cycle progression. Cyclin D1 initiates the phosphorylation of RB that results in disruption of RB-mediated transcriptional repression (33–35). Thus, it is viewed that RB represents the critical target of cyclin D1 in cell cycle control. Consistent with this model, cyclin D1 activity is not required for cell cycle progression in cells that are deficient in RB (26, 36–38). Genetic analysis further links RB and cyclin D1. Specifically, those tumors harboring cyclin D1 amplifications rarely target RB for inactivation, whereas those tumors lacking RB exhibit down-regulation of cyclin D1 (3, 4, 6, 37, 39). As such, it has long been held that cyclin D1 acts upstream of RB in a linear pathway.

In human populations cyclin D1 exhibits a common polymorphism that has been associated with human cancers (40–45). The A/G polymorphism is present at nucleotide 870 and shows an allele frequency of 48 and 52%, respectively, in European

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[1] The abbreviations used are: CDK, cyclin-dependent kinase; HA, hemagglutinin; GFP, green fluorescent protein; FRAP, fluorescence recovery after photobleaching.

populations (46). Several studies have indicated that individuals harboring the A/A genotype are substantially pre-disposed to specific cancers and have a poor prognosis as compared with those harboring A/G or G/G genotypes. Such analyses have been carried out in a multitude of tumor types, including squamous cell carcinoma of head and neck, non-small cell lung cancer, sporadic human pituitary tumors, hepatocellular carcinoma, esophageal carcinomas, laryngeal and pharyngeal squamous cell carcinomas, prostate carcinoma, and bladder cancer (40–43, 45). Together, these studies suggest that the A-allele has a central role in increasing cancer risk.

The cyclin D1 A-allele does not influence the amino acid encoded by the affected codon (Pro-242). However, the A-allele is associated with the production of an aberrant splicing product termed cyclin D1 variant b (cyclin D1b) (40). The mature cyclin D1b transcript is composed of both exons 1–4 and intron 4 of the cyclin D1 gene (40). This contrasts with the commonly studied cyclin D1 transcript encoded by exons 1–5 (cyclin D1a). Thus, cyclin D1b is completely homologous to cyclin D1a in the first four exons but encodes unique C-terminal sequence. The cyclin D1b transcript was first recognized in 1991; however, the vast majority of functional studies associated with cyclin D1 have focused specifically on cyclin D1a (46). Strikingly little is known regarding the behavior of cyclin D1b relative to cyclin D1a. Analysis of the primary sequence of cyclin D1b indicates that it is clearly lacking two regulatory motifs encoded within exon 5, the PEST domain implicated in destabilizing cyclin D1 and the threonine 286 phosphorylation site for glycogen synthase kinase 3β. Phosphorylation at this site has been shown to promote nuclear export of cyclin D1 and protein turnover (17, 18, 47). Therefore, it has been hypothesized that the action of cyclin D1b may be attributed to increased stability relative to cyclin D1a. Here we specifically analyzed the action of cyclin D1b relative to cyclin D1a in cell cycle control and cellular transformation. As predicted, we show that cyclin D1b encodes a constitutively nuclear protein, present in the nucleus of actively replicating cells. Surprisingly, we find that cyclin D1b is not significantly more stable than cyclin D1a and fails to accumulate to excessive levels. Interestingly, the cyclin D1b protein is a poor effector of RB phosphorylation/inactivation but proved to potently overcome contact inhibition of growth, as compared with cyclin D1a.

EXPERIMENTAL PROCEDURES

Cell Culture—C33A, NIH-3T3, and SAOS-2 cells were maintained in Dulbecco's modified Eagle's medium (Mediatech) supplemented with 10% fetal bovine serum (Hyclone), 100 units/ml penicillin-streptomycin, and 2 mM L-glutamine at 37 °C in 5% CO_2. Cell transfections were carried out either with calcium phosphate precipitation or using FuGENE 6 reagent (Roche Applied Science) (51, 61). Selection of stable cell lines was carried out with either 2.5 μg/ml puromycin or 200 μg/ml G418 (Calbiochem).

Plasmids—Cyclin D1b was cloned by PCR, and the correct nucleotide sequence was confirmed by sequencing. The HA epitope tag was added to both the cyclin D1a and cyclin D1b cDNA by PCR, and the HA-tagged cDNA was expressed from the pCDNA3.1 vector (Invitrogen). The GFP-cyclin D1a and D1b fusion proteins were produced by subcloning into the pEGFP-C1 vector (Clontech) linking an N-terminal GFP moiety to cyclin D1. The RB, CDK4, and H2B-GFP expression plasmids have been described previously (62). The −608 cyclin A-luciferase and cytomegalovirus β-galactosidase reporter plasmids have been described previously (51). For puromycin selection, the pBABE-PURO plasmid was utilized (63).

Flow Cytometry—Cells were co-transfected with the indicated expression plasmids and H2B-GFP expression plasmid (64). 48 h post-transfection cells were harvested, fixed with ethanol, and stained with propidium iodide. For the analysis of specifically transfected cells, gates were established that encompass specifically those cells positive for GFP fluorescence. This GFP-positive population was then analyzed for propidium iodide staining. Cell cycle distribution was determined using mod-fit software.

Immunoblotting and Immunoprecipitation—All immunoblotting was performed following standard biochemical techniques. For the detection of untagged cyclin D1 proteins, we employed antibodies against the N terminus of cyclin D1 (DCS-6; Neomarkers) and the C terminus of cyclin D1 (Ab-3; Neomarkers). The intron 4-specific antibody was developed using a peptide derived from C-terminal cyclin D1b-specific sequences to immunize rabbits. For the detection of HA epitope, we employed the sc-805 antibody (Santa Cruz Biotechnology, Inc.). For the detection of GFP-tagged proteins, we employed the sc-9996 antibody (Santa Cruz Biotechnology, Inc.). For the RB phosphorylation assays, cells were transfected with RB, CDK4, and/or cyclin D1 expression plasmids at a 1:1:1 ratio. RB was detected with either G3–245 (Pharmingen) or phosphoserine 780 (Cell Signaling) antibodies. For the co-immunoprecipitation of cyclin D1 with CDK4, C33A cells were transfected with cyclin D1 and CDK4 expression plasmids at a 1:1 ratio. Cells were lysed in NET-N and subjected to immunoprecipitation with anti-GFP antibody and protein A-Sepharose. Immunoprecipitates were recovered by centrifugation and washed extensively with NET-N. Immune complexes were disrupted in Laemmli buffer and resolved by SDS-PAGE.

Transcriptional Reporter Assays—SAOS-2 cells were co-transfected with cytomegalovirus β-galactosidase reporter plasmid, −608 cyclin A-luciferase reporter plasmid, RB, cyclin D1, and CDK4 at a 0.5:1:2:2:2 ratio. 48 h post-transfection, cells were harvested, and luciferase activity was determined and normalized against β-galactosidase activity for transfection efficiency. Data were collected from three independent experiments.

Focus Formation Assays—Stable cell lines were selected with 2.5 μg/ml of puromycin and maintained on a passage protocol wherein 9×10^5 cells were passaged per 6-cm dish every third day. Early and late passage NIH-3T3 selected cells were plated at 3×10^5 cells per 6-cm dish. Foci were visualized after 15 days with 1% crystal violet stain. Three independent experiments were performed. To count foci, plates were divided into quadrants, and the number of foci per 3×10^5 cells plated was determined. Photomicrographs were taken with a 4 × objective on a Nikon microscope.

Live Cell Imaging and FRAP—NIH-3T3 and SAOS-2 cells stably expressing GFP-cyclin D1 were seeded on 25-mm coverslips. For imaging, coverslips were transferred to live-cell imaging chambers (Atto) in a water-jacketed stage incubator at 37 °C. FRAP was performed on a Zeiss LSM510 laser scanning confocal unit mated to a Zeiss Axiovert inverted microscope equipped with a C-Apochromat 63 × 1.4 numerical aperture objective. A 2.9-μm × 2.9-μm area of the nucleus was photobleached for 20 iterations (0.42 s) with 100% transmission of 488-nm light from an argon laser running at 6.25 milliwatts. Fluorescent intensity values of the bleached area and of a distal unbleached area of the nucleus of equal size were measured every 50 ms for the indicated time lengths following photobleaching. These values were compared to produce a relative fluorescent intensity to normalize for pre-bleach intensity. Data presented were collected from the indicated number of nuclei per condition.

RESULTS

Cloning and Expression of Cyclin D1b—Although cyclin D1 exists as two distinct protein products, little is known regarding the relative activity of these proteins. To investigate the activity of cyclin D1b, we initially cloned the cyclin D1b cDNA. Sequencing confirmed the identity of cyclin D1b with the exon/intron structure depicted in Fig. 1*A*. To investigate expression of the protein, cyclin D1a and D1b encoding expression plasmids were transfected into SAOS-2 cells, and resultant protein production was monitored by immunoblotting (Fig. 1*B*). Because of mutation of RB, these cells express little endogenous cyclin D1 protein (*lane 1*) (37). As predicted, cyclin D1b exhibited a molecular mass of ~30 kDa, 3 kDa smaller than cyclin D1a (*top panel*, *lanes 2* and *3*). The cyclin D1b polypeptide was detected by cyclin D1 antibodies directed against the N terminus but could not be detected by C-terminal-specific antibodies that recognize exon 5-encoded sequences (*bottom panel*, *lanes 2* and *3*). To enable the specific analysis of either cyclin D1a- or D1b-encoded proteins in any cell type, plasmids encoding N-terminal HA- or GFP-tagged proteins were constructed (Fig. 1*C*). The resulting fusion proteins were expressed and could be detected with the appropriate antibodies (Fig. 1*D*). Addition-

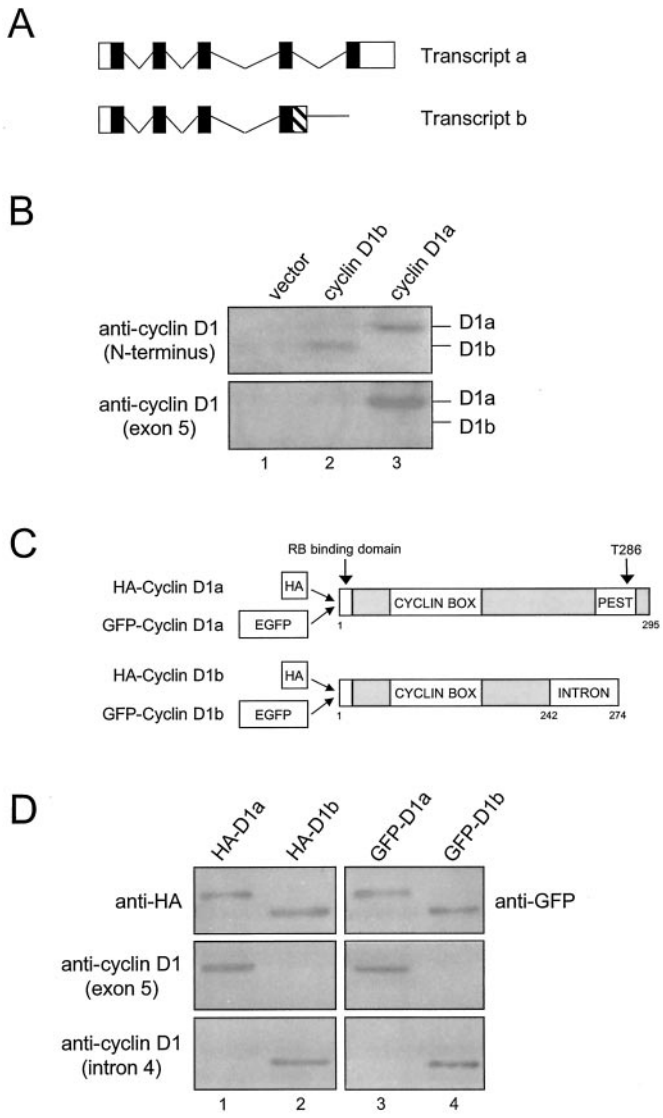


FIG. 1. **Cloning and expression of the cyclin D1 alternative transcript, cyclin D1b.** *A*, diagram depicting the exon/intron structure of the cyclin D1 gene. Cyclin D1a is encoded by five exons, whereas cyclin D1b is the product of four exons and sequences from intron four. *B*, SAOS-2 cells were transfected with vector (*lane 1*), cyclin D1b (*lane 2*), or cyclin D1a (*lane 3*) expression plasmids. Cell lysates were prepared, and total protein was resolved by SDS-PAGE. Cyclin D1 proteins were then detected by immunoblotting with antibodies directed against either the N terminus of cyclin D1 (*top panel*) or specific to exon 5-encoded sequences (*bottom panel*). *C*, sequences encoding the HA epitope or GFP were cloned on to the 5′ end of the cyclin D1a and cyclin D1b cDNA. *D*, SAOS-2 cells were transfected with HA-cyclin D1a (*lane 1*), HA-cyclin D1b (*lane 2*), GFP-cyclin D1a (*lane 3*), or GFP-cyclin D1b (*lane 4*). Cell lysates were prepared, and total protein was resolved by SDS-PAGE. The encoded proteins were then detected by immunoblotting with either GFP or HA antibodies (*top panel*). To confirm the disparate nature of cyclin D1a and cyclin D1b, immunoblotting was also performed with antibodies against exon 5- (*middle panel*) or intron 4 (*bottom panel*)-encoded sequences.

ally, an antibody raised against the C-terminal 15 amino acids of cyclin D1b specifically recognized cyclin D1b (Fig. 1*D*). Together, these results indicated that full-length cyclin D1b protein can be expressed and detected in cells.

Absence of Regulatory Domains in Cyclin D1b Has Minimal Impact on Protein Stability—Through virtue of the C-terminal PEST domain and threonine 286, cyclin D1a is known to be tightly regulated at the level of protein stability (18, 48). Because cyclin D1b lacks both these motifs, it was predicted that this cyclin D1 variant would encode a more stable protein,

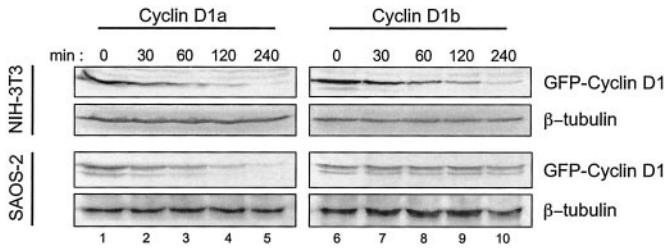


FIG. 2. **Cyclin D1b stability is comparable with cyclin D1a.** Polyclonal SAOS-2 and NIH-3T3 cell populations stably expressing GFP-cyclin D1a (*lanes 1-5*) and D1b (*lanes 6-10*) were cultured in 50 μg/ml cycloheximide and harvested at the indicated time points. Total cell lysate was resolved by SDS-PAGE, and GFP-cyclin D1 proteins were detected by immunoblotting with a GFP antibody. Equal loading was confirmed by β-tubulin immunoblot.

perhaps underlying its role in tumorigenesis. To determine the relative stability of cyclin D1a *versus* D1b, NIH-3T3 and SAOS-2 cells were generated to stably express GFP-cyclin D1a or D1b. Polyclonal populations of stable cell lines were utilized to circumvent difficulties associated with clonal variation and the heterogeneous expression of cyclin D1 that is observed in a transiently transfected population. Cells were treated with cycloheximide to inhibit protein synthesis and specifically monitor protein stability at the times indicated. As can be seen in Fig. 2, cyclin D1b exhibited a stability similar to cyclin D1a in NIH-3T3 cells (*top panel*, compare *lanes 1–5 versus 6–10*). The calculated half-lives of cyclin D1a and D1b were 49 and 54 min, respectively. The relative stability of cyclin D1b was slightly enhanced in SAOS-2 cells (*bottom panel*), indicating that cyclin D1b turnover may be regulated differently in immortal *versus* tumorigenic cell lines. Additionally, these results are largely consistent with the failure of cyclin D1b to accumulate to higher levels than cyclin D1a in our studies (Fig. 1). This surprising finding suggests that cyclin D1b is regulated by additional mechanisms and does not necessarily represent a stabilized form of cyclin D1a.

Cyclin D1b Protein Is Constitutively Nuclear—In addition to protein stability, the C-terminal region of cyclin D1a is important for subcellular localization. Specifically, phosphorylation on threonine 286 stimulates association with CRM1 and nuclear export (18, 47). Absence of this residue suggests that cyclin D1b would demonstrate enhanced nuclear localization. Transient high level expression of cyclin D1a resulted in diffuse cytosolic staining, whereas cyclin D1b exhibited punctate perinuclear staining (not shown). Therefore, we utilized cells stably expressing low, near physiological levels of GFP-cyclin D1. Three distinct cell populations were analyzed: (i) asynchronous (untreated), (ii) mid-S phase (hydroxyurea-treated), and (iii) early S-phase (aphidicolin-treated). For each treatment, representative photomicrographs are shown (Fig. 3*A*), and greater than 200 individual cells were scored from three independent experiments for quantitation (shown in Fig. 3*B*). As expected, GFP-cyclin D1a exhibited both nuclear and cytosolic distributions in asynchronous populations but was principally cytosolic in S-phase through the use of aphidicolin or hydroxyurea (Fig. 3, *A* and *B*, compare asynchronous *versus* +*HU* and +*APH*). These data confirm that GFP-cyclin D1a acts in a manner consistent with endogenous cyclin D1a protein (18). Analysis of GFP-cyclin D1b revealed that the protein was present in the nucleus, with few cells exhibiting nuclear exclusion in an asynchronously proliferating population (Fig. 3, *A* and *B*). Additionally, the GFP-cyclin D1b protein was retained in the nucleus when cells were arrested in S-phase (Fig. 3, *A* and *B*). To unequivocally determine whether cyclin D1b was present in the nucleus of replicating cells, the localization of cyclin D1 was visualized in cells actively incorporating BrdUrd. Consistent

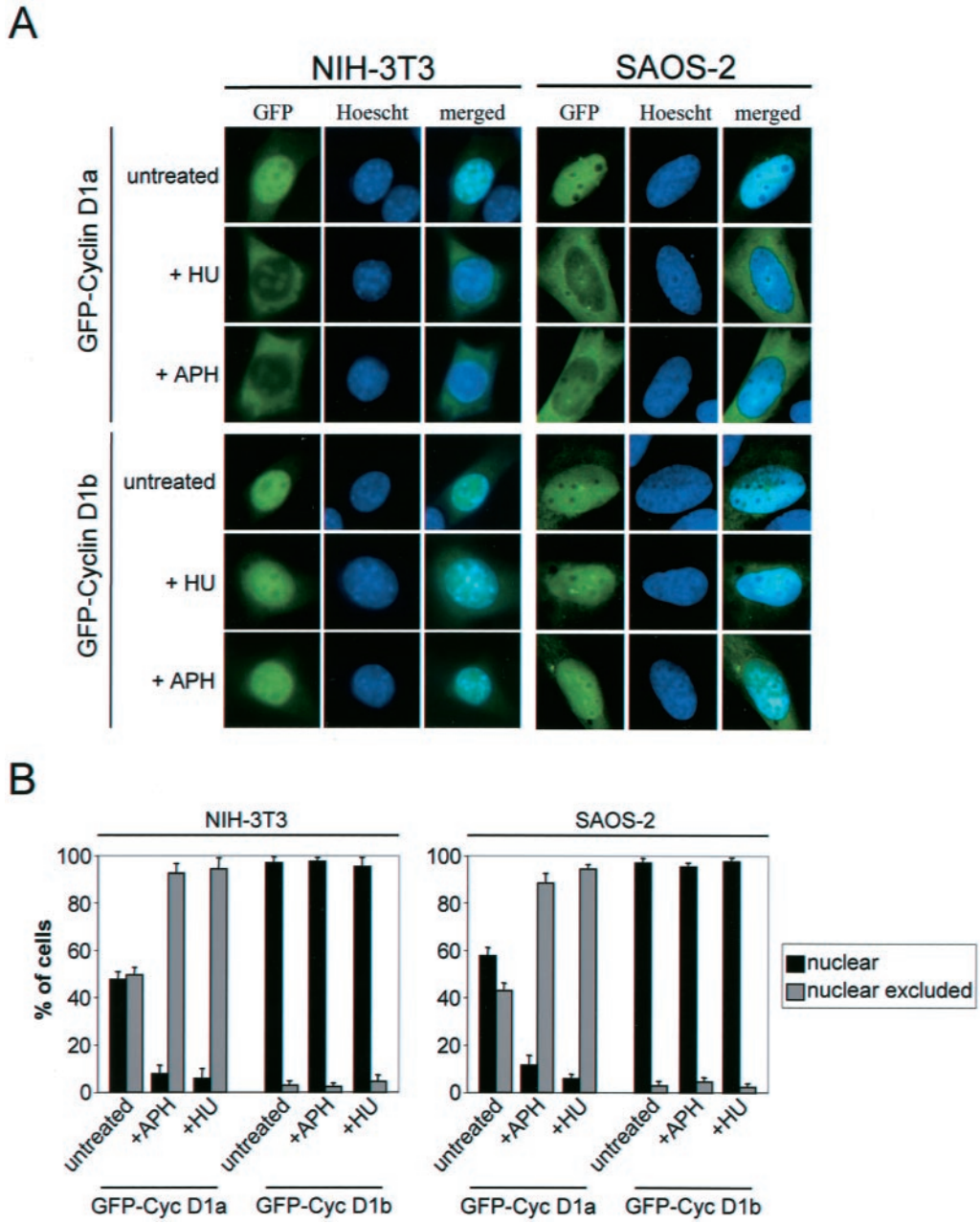


FIG. 3. **Cyclin D1b is a constitutively nuclear protein present in the nucleus during active DNA replication.** *A*, representative photomicrographs of NIH-3T3 and SAOS-2 polyclonal populations stably expressing GFP-cyclin D1a and D1b following 18 h of culture in vehicle, 1 mM hydroxyurea (*HU*), or 2 μg/ml aphidicolin (*APH*). *B*, quantitation of cells in *A* from three independent experiments with greater than 200 cells scored. *C*, SAOS-2 cell populations stably expressing GFP-cyclin D1a and GFP-cyclin D1b were pulse-labeled with BrdUrd for 15 min and fixed. Cells were stained for BrdUrd incorporation. Shown is a representative photomicrograph of S-phase SAOS-2 cells. *D*, quantitation of the percent of BrdUrd-positive cells, either total in the population or exhibiting nuclear GFP-cyclin D1 localization. Data are from two experiments with at least 200 cells scored.

with the prior data we failed to detect nuclear GFP-cyclin D1a in those cells that were BrdUrd-positive, whereas cyclin D1b was present in the nucleus of those cells undergoing DNA replication (Fig. 3, *C* and *D*). Together, these data demonstrate that cyclin D1b is constitutively nuclear.

To analyze the dynamic behavior of cyclin D1 proteins in living cells, FRAP analysis was utilized. In this approach, a small region of the nucleus of cells stably expressing GFP-cyclin D1 proteins was photobleached (Fig. 4). The rate of recovery of this bleached area is directly proportional to the diffusion of GFP-cyclin D1 within the unit area. Nuclear FRAP analysis demonstrated that GFP-cyclin D1a is surprisingly immobile, as it exhibits relatively slow recovery in both cells types analyzed (NIH-3T3 and SAOS-2) (Fig. 4, *A* and *B*) (not shown). Interestingly, GFP-cyclin D1b behaved in two distinct manners in asynchronous cells. In one population of cells, GFP-cyclin D1b was largely immobile, with dynamics comparable with GFP-cyclin D1a (Fig. 4, *A* and *B*). In contrast, approximately one-third of cells exhibited enhanced nuclear mobility of GFP-cyclin D1b (Fig. 4, *A*, *B*, and *C*). This result suggested that the enhanced mobility may represent the behavior of nuclear cyclin D1b in cells that had progressed out of G_1. Consistent with this model, when cells were synchronized with hydroxyurea or aphidicolin, nuclear GFP-cyclin D1b was highly mobile in the majority of cells (Fig. 4*D*). Therefore, cyclin D1b is retained in the nucleus of S-phase cells, wherein it behaves in a manner distinct from cyclin D1a and D1b in G_1 cells. Given that the key substrate of cyclin D1 is a nuclear protein

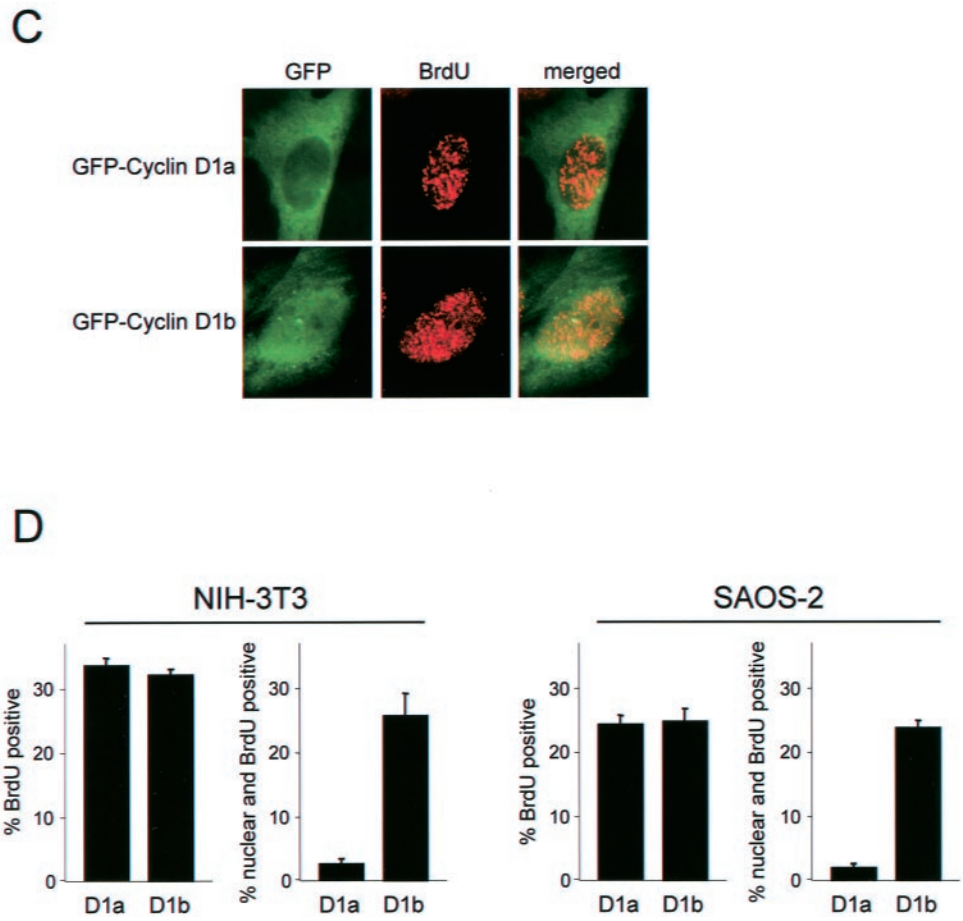
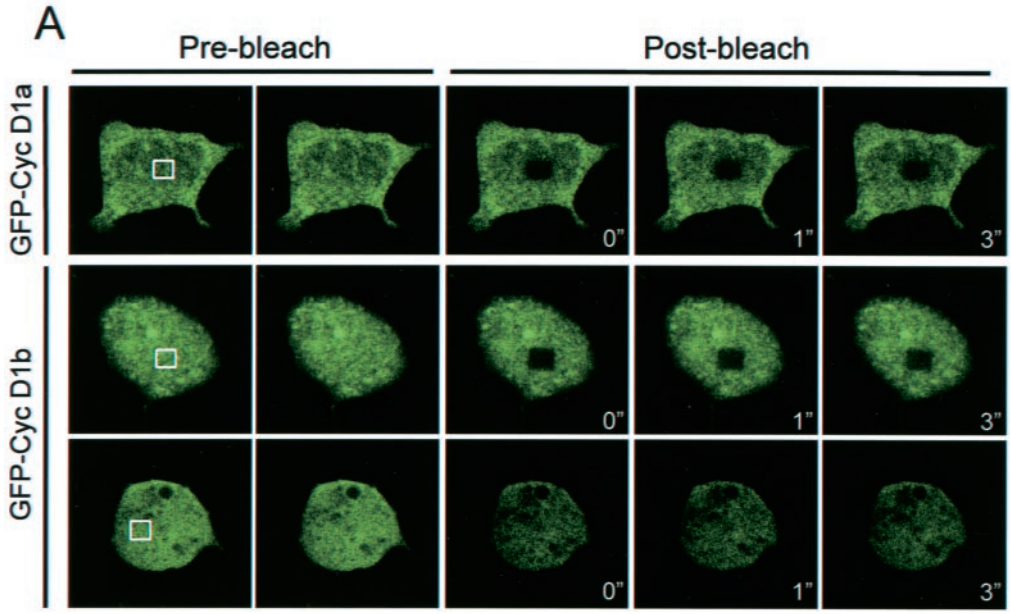


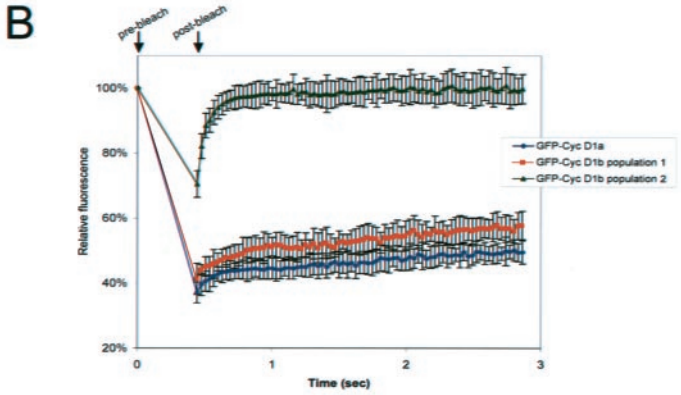
FIG. 3—*continued*

(*i.e.* RB), this result suggested that highly efficient disruption of the RB pathway could underlie the action of cyclin D1b in tumorigenesis.

Cyclin D1b Is a Poor Catalyst of RB Inactivation and Cell Cycle Progression—Prior analyses have demonstrated that RB is the critical target of cyclin D1 in cell cycle progression (26, 37). In these studies both cyclin D1a and cyclin D1b were neutralized; thus the action of cyclin D1b on RB phosphorylation/inactivation has not been addressed. Initially, we monitored the relative activity of cyclin D1a and D1b in interacting with CDK4 (Fig. 5*A*). By co-immunoprecipitation, we observed that both GFP-cyclin D1a and D1b efficiently co-immunoprecipitated CDK4 (Fig. 5*A*). To investigate the ability of these complexes to impact the RB pathway, we monitored the ability of GFP- and HA-tagged cyclin D1 proteins to promote RB phosphorylation in the SAOS-2 cell line (Fig. 5, *B* and *C*). SAOS-2 cells are deficient in endogenous RB, and ectopically expressed protein remains unphosphorylated (30, 49). Therefore, we utilized these cells to assess the activity of CDK4/cyclin D1a or D1b complexes in mediating RB phosphorylation. Both GFP-cyclin D1a and HA-cyclin D1a efficiently promoted RB phosphorylation and stoichiometrically converted RB to the hyperphosphorylated form (Fig. 5, *B* (*lane 4*) and *C* (*lane 3*)). This phosphorylation was confirmed by enhanced reactivity with antibodies specific for phosphoserine 780, which is a site of CDK4-mediated phosphorylation on RB (Fig. 5*C*) (50). In contrast with cyclin D1a, cyclin D1b weakly catalyzed RB phosphorylation, as a significant fraction of total RB remained unphosphorylated and reacted with the phosphoserine 780-specific antibody with reduced affinity (Fig. 5, *B* and *C*). This event was not because of lack of cyclin D1b expression, as cyclin D1a and D1b were expressed at similar levels. Similar results were observed with untagged cyclin D1a and D1b proteins (not shown). Therefore, these results demonstrate that cyclin D1b is a poor mediator of RB phosphorylation.

To specifically evaluate the action of cyclin D1b in disrupting RB activity, we first determined the influence of cyclin D1 on RB-mediated transcriptional repression. We have shown previously (51) that the cyclin A promoter is a critical target of RB and that this repression event can be easily monitored through reporter assay. To test the action of cyclin D1b, SAOS-2 cells were transfected with a reporter construct for the cyclin A promoter and the indicated expression plasmids. As shown in Fig. 5*C*, RB potently repressed cyclin A promoter activity in the absence of ectopically expressed D-type cyclins (greater than 80% repression). This repression was completely alleviated through the action of ectopically expressed cyclin D1a and CDK4 proteins. However, cyclin D1b was less competent for promoting the disruption of RB-mediated transcriptional repression (~40% repression). Thus, these results substantiate the findings that cyclin D1b weakly stimulates RB phosphorylation and suggest that cyclin D1b is impaired in its ability to overcome RB-mediated cell cycle arrest. To test this hypothesis, SAOS-2 cells were transfected with the minimal growth suppressing region of RB (large pocket), in the presence or absence of ectopic CDK4 and/or cyclin D1a or D1b. As shown in Fig. 5*D*, vector transfected cells exhibit a standard cell cycle distribution. The expression of wild-type large pocket markedly inhibited progression through S-phase (74.8% G_1 content). Ectopic expression of either cyclin D1a or D1b alone had minimal effect on RB-mediated cell cycle arrest, whereas cyclin D1a co-expressed with CDK4 efficiently reversed cell cycle inhibi-





C

asynchronous	cells analyzed	% cells immobile	% cells highly mobile
GFP-Cyc D1a	26	100%	0%
GFP-Cyc D1b	41	68%	32%

D

GFP-Cyc D1b	cells analyzed	% cells immobile	% cells highly mobile
0.1% serum	23	96%	4%
+ HU	14	14%	86%
+ APH	14	21%	79%

FIG. 4. **Nuclear FRAP analysis reveals unique dynamic behaviors of cyclin D1a and cyclin D1b.** *A*, nuclear FRAP of SAOS-2 cells stably expressing GFP-cyclin D1a and D1b. The photobleached area is *boxed* in *white* and measures 2.9 μm × 2.9 μm. Images were recorded pre-bleach or 0, 1, and 3 s post-bleach. *B*, quantitation of fluorescence recovery into the photobleached area of nuclei as was determined as described under "Experimental Procedures." *C*, nuclei exhibiting either mobile or immobile behavior of GFP-cyclin D1 was determined in asynchronous populations by FRAP analysis. *D*, nuclei exhibiting either mobile or immobile behavior of GFP-cyclin D1b in cells synchronized by serum starvation or with aphidicolin (*APH*) or hydroxyurea (*HU*).

tion (48.6% G_1 content). By contrast, cyclin D1b when co-expressed with CDK4 only partially reversed RB-mediated cell cycle inhibition (62.5% G_1 content). Therefore, although cyclin D1b is associated with tumorigenesis, it paradoxically had less activity in stimulating cell cycle progression.

Cyclin D1b Elicits Cellular Transformation—Prior studies (47) have demonstrated that constitutively nuclear cyclin D1a proteins have the capacity to transform NIH-3T3 cells, following passage on a 3T9 protocol. Because RB is thought to be the main target of cyclin D1 action with regard to both cell cycle control and tumorigenesis, our data suggested that cyclin D1b would exhibit weakened transforming activity. To analyze the relative activity of cyclin D1b in cellular transformation, we analyzed the action of cyclin D1a, cyclin D1b, and Ras in promoting focus formation in NIH-3T3 cells. To perform these studies NIH-3T3 cells were co-transfected with a puromycin selectable marker plasmid (pBABE-PURO) and vector, cyclin D1a, cyclin D1b, or V12Ras encoding plasmids. Cells were selected with puromycin, and focus formation was monitored on selected polyclonal populations at four passages post-selection (Fig. 6). At these early stages, only V12Ras exhibited strong focus forming activity (Fig. 6, *A* and *B*). However, by passage 12 cyclin D1b transfected cells were capable of forming foci (Fig. 6, *A* and *B*). Under these same conditions, cyclin D1a or vector control transfected cells, even at passage 20, failed to form foci (Fig. 6) (not shown). Thus, these data demonstrate

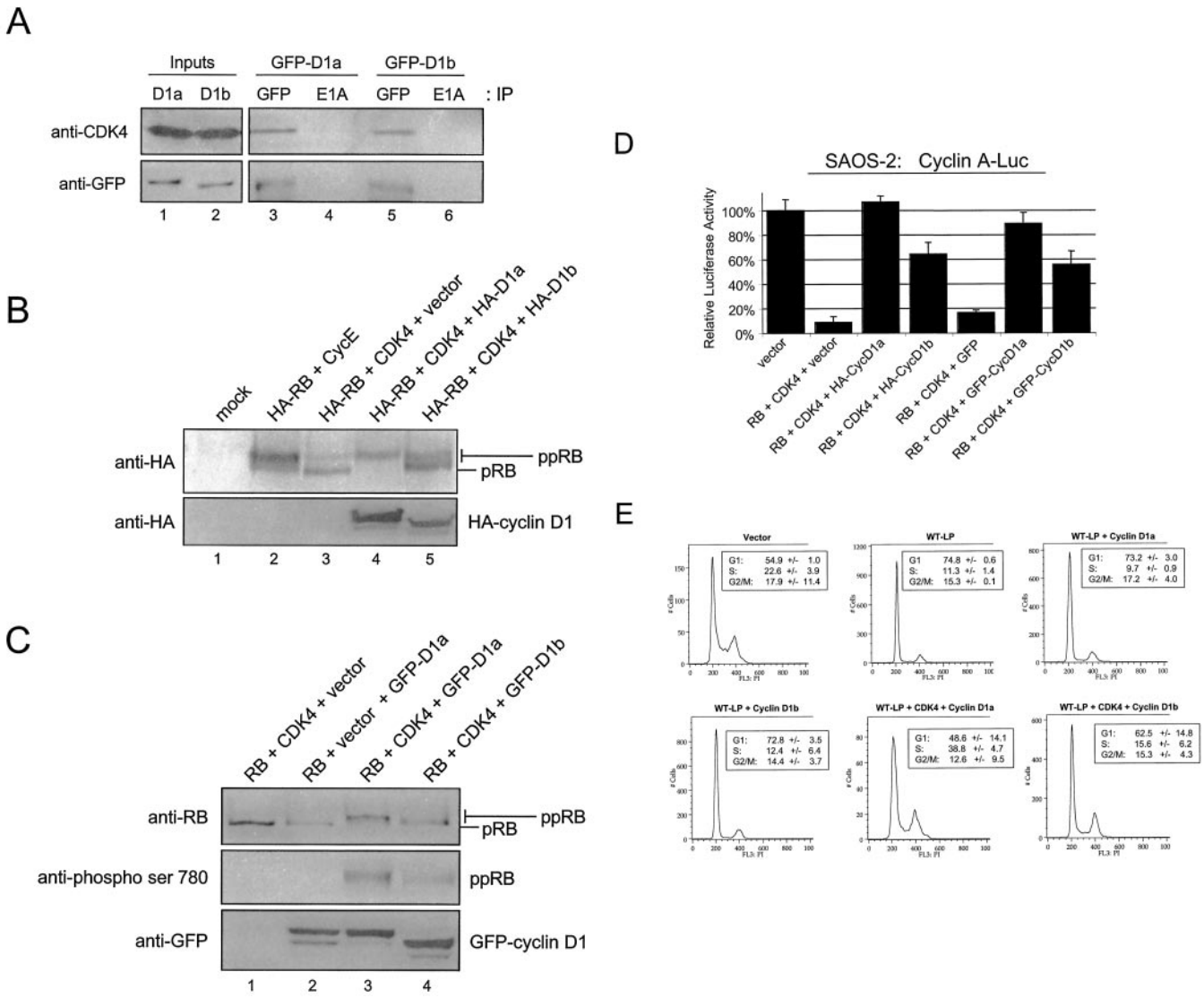


FIG. 5. **Cyclin D1b efficiently associates with Cdk4 but is a weak modulator of the RB pathway.** *A*, C33A cells were co-transfected with expression plasmids encoding CDK4 and GFP-cyclin D1a (*lanes 1*, *3*, and *4*) or D1b (*lanes 2*, *5*, and *6*). Cell lysate was prepared and subjected to immunoprecipitation (*IP*) with GFP (*lanes 3* and *5*) or E1A (*lanes 4* and *6*) antibodies. Input lysate (*lanes 1* and *2*) and the resultant precipitated proteins were resolved by SDS-PAGE. GFP-cyclin D1 and CDK4 proteins were detected by immunoblotting. *B*, SAOS-2 cells were co-transfected with the indicated expression plasmids (*lanes 1–5*). Total cellular protein was resolved by SDS-PAGE. RB and HA-cyclin D1 proteins were detected by immunoblotting. *C*, SAOS-2 cells were co-transfected with the indicated expression plasmids (*lanes 1–4*). Total cellular protein was resolved by SDS-PAGE. RB, phosphoserine 780 RB, and GFP-cyclin D1 proteins were detected by immunoblotting (*D*) SAOS-2 cells were co-transfected as indicated, along with cytomegalovirus β-galactosidase and cyclin A-luciferase reporter plasmids. Forty-eight h post-transfection, cells were harvested and assayed for luciferase activity, which was normalized to β-galactosidase activity. Data are from three independent experiments. *E*, SAOS-2 cells were transfected as indicated. Forty-eight h post-transfection, cells were fixed, stained with propidium iodide, and analyzed by flow cytometry. Representative histograms of 10,000 gated events from two independent experiments are shown. *WT-LP*, wild-type large pocket RB.

that cyclin D1b has enhanced transforming activity relative to cyclin D1a. This finding was surprising given its weakened activity on the RB axis but is consistent with its involvement in tumorigenesis.

DISCUSSION

The association between cyclin D1b expression and tumorigenesis is well described. Given the absence of the C terminus in the transcript b variant, it had been hypothesized that cyclin D1b would function by providing a more abundant/stable cyclin D1 protein that would have enhanced activity. Here, we show that although cyclin D1b is constitutively localized to the nucleus, cyclin D1b protein does not overaccumulate relative to cyclin D1a. Moreover, we show that cyclin D1b is a poor catalyst of RB inactivation, thus drawing an important distinction between cyclin D1a and cyclin D1b function with regard to cell cycle control. However, we show that cyclin D1b is a potent oncogenic agent, capable of transforming NIH-3T3 cells with significantly heightened activity, as compared with cyclin D1a. Together, these data demonstrate the activity of cyclin D1b in cellular transformation and indicate that cyclin D1 variants harbor distinct roles in tumor formation.

The observation that cyclin D1b fails to exhibit enhanced stability is surprising given the lack of a PEST domain and the enhanced nuclear accumulation of cyclin D1b. In general, nuclear cyclin D1a has been associated with enhanced stability (17, 18, 47). Therefore, how cyclin D1b is being turned over in the cell is not explicitly clear. When high levels of cyclin D1b were expressed in cells, it accumulated in discrete peri-nuclear structures that resemble the sites of proteasome accumulation/aggresome localization (data not shown) (52, 53). This structure has been correlated with the degradation of proteins and in principal could be utilized to facilitate the turnover of lower

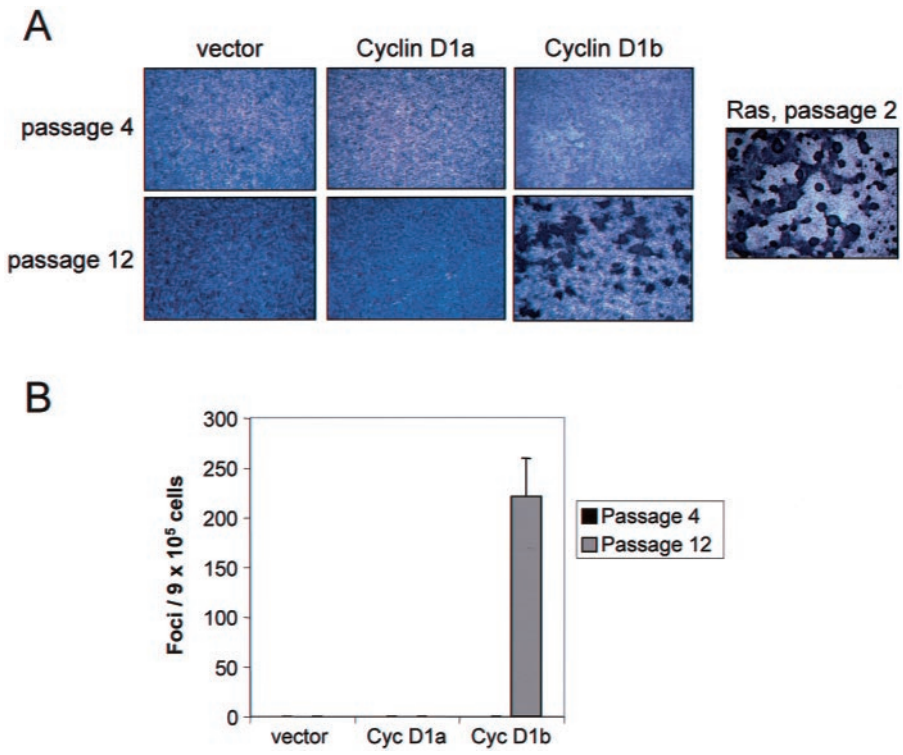


FIG. 6. **Cyclin D1b but not cyclin D1a potently induces transformation in NIH-3T3 cells.** *A*, NIH-3T3 cells were transfected with the indicated expression plasmids and selected with puromycin. Cells were maintained on a 3T9 protocol, and focus forming activity was determined at the indicated passages. Representative photomicrographs of crystal violet-stained cells are shown. *B*, NIH-3T3 cells transfected and passaged were scored for focus formation activity, and data were collected from three independent passaging experiments.

levels of cyclin D1b. Alternatively, N-terminal regions of cyclin D1b (shared with cyclin D1a) can contribute to regulated protein stability (54). The pathways responsible for cyclin D1b turnover will be determined in future studies.

In addition to the disparity between cyclin D1a and D1b with regard to localization, nuclear cyclin D1b exhibited distinct dynamic properties in live cells. Prior studies (18, 47) have indicated that phosphorylation of threonine 286 by glycogen synthase kinase 3β mediates the nuclear export of cyclin D1a. Because threonine 286 is absent in cyclin D1b, the constitutive nuclear localization confirms the importance of exon 5-encoded sequences in nuclear export. The behavior of cyclin D1 in the nucleus of live cells had not been determined. We find that nuclear cyclin D1a in G_1 nuclei is a static protein likely tethered to an immobile complex. This result is consistent with prior studies that have demonstrated that cyclin D1 can interact tightly with other immobile nuclear species, such as the nuclear matrix components or MCMs (55, 56). As cyclin D1a is exported in S-phase, it has been difficult to determine the underlying actions that occur in S-phase nuclei. Because cyclin D1b exhibited two distinct behaviors in nuclei, we immediately postulated that this was related to the cell cycle distribution of the cells expressing GFP-cyclin D1b. By synchronization we were able to show that cyclin D1b is, in fact, disrupted from its nuclear tether in S-phase cells. This suggests that in addition to the conventional phosphothreonine 286-mediated export, there is an additional mechanism for localizing cyclin D1 in the nucleus that involves retention in an immobile phase. At present the nature of this complex is unknown, but because cyclin D1 disruption occurs at the transition to S-phase, it is appealing to speculate that cyclin D1 tethering is associated with the pre-replication complexes as has been reported recently by Gladden and Diehl (55). Despite these alterations in protein mobility, the persistent presence of cyclin D1b in the nucleus is predicted to constitutively drive phosphorylation of nuclear substrates (*e.g.* RB).

Cyclin D1a exerts its biological activity through formation of an active kinase with CDK4/6 that phosphorylates/inactivates the RB tumor suppressor (3, 4, 35, 57). This event is required for the transition into S-phase, and RB is thought to be the principal target of cyclin D1-associated kinase activity (26, 37). In part, this critical action of cyclin D1a is controlled by localization, as cells export cyclin D1a to the cytoplasm where it is degraded during progression into S-phase (18). Because cyclin D1b efficiently interacts with CDK4 and is constitutively nuclear, we reasoned that cyclin D1b would exhibit enhanced activity in phosphorylating/inactivating RB. Surprisingly, we find that cyclin D1b is highly inefficient at mediating both RB phosphorylation and inactivation. These results suggest that critical residues in the C terminus of cyclin D1a may be important for targeting RB as a substrate. This result is surprising, as the known motifs required for cyclins to target specific substrates are retained in cyclin D1b. Specifically, the N-terminal RB binding motif (L*X*C*X*E) is present in both cyclin D1a and D1b (29, 30). Additionally, it has been shown that the presence of an R*X*L substrate-targeting motif in RB or the related protein p107 is an important determinant of the capacity of CDK4/cyclin D1 complexes to phosphorylate these substrates (58, 59). However, the binding site for this motif is present in the hydrophobic patch that is invariant between cyclin D1a and D1b (60). Therefore, cyclin D1b contains those domains previously implicated in efficiently phosphorylating RB, suggesting that additional motifs in cyclin D1a C terminus are required for RB phosphorylation or that the intron 4-encoded sequences compromise substrate recognition. The consequence of poor RB phosphorylation is manifested in both the failure of RB transcriptional repression to be alleviated and in the failure of cyclin D1b to efficiently overcome RB-mediated cell cycle arrest. These results argue against a role for cyclin D1b in overcoming the RB pathway as a means to transform cells. Consistent with this view, the role of cyclin D1b in therapeutic outcome has been observed in non-small cell lung cancers, wherein RB is almost universally inactivated via p16ink4a loss (40). Clearly, studies dissecting the involvement of both cyclin D1b and RB in concert will be required to make this conclusion in primary human cancer specimens.

Despite its inefficient action in overcoming RB-mediated cell cycle arrest, cyclin D1b exhibited markedly enhanced trans-

forming activity relative to cyclin D1a. This ability of cyclin D1b to promote cellular transformation could be attributed to its constitutively nuclear localization. It has been shown previously (47) that cyclin D1 with threonine 286 substituted to alanine harbors the capacity to transform NIH-3T3 cells. This is an attribute that is not present in cyclin D1a. The activity of the threonine 286 alanine allele of cyclin D1a was dependent not only on its expression but on passaging the cells expressing the allele (47). We observe a markedly similar phenotype with cyclin D1b. How constitutively nuclear cyclin D1 proteins promote cellular transformation is unknown. In our studies, the focus forming activity of cyclin D1b was apparent at passage 12. This is earlier than the observations made with the threonine 286 alanine mutant of cyclin D1a (foci detectable after passage 18) (47). This result, combined with the failure to inactivate RB, is consistent with the hypothesis that cyclin D1b may harbor activity not present in cyclin D1a that provides a more potent means of transforming cells. An appealing possibility is that differences in the C terminus enable cyclin D1b to phosphorylate unique substrates. Understanding the nature of these additional targets unique to cyclin D1b are of critical importance to delineate additional risk factors interacting with the cyclin D1 A/A genotype.

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