[CANCER RESEARCH 63, 7056–7061, November 1, 2003]

Advances in Brief

An Alternatively Spliced Cyclin D1 Isoform, Cyclin D1b, Is a Nuclear Oncogene[1]

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Abstract

Glycogen synthase kinase-3β-dependent phosphorylation of cyclin D1 at a conserved COOH-terminal residue, Thr-286, promotes CRM1-dependent cyclin D1 nuclear export at the G_1-S boundary. Mutations that perturb the phosphorylation of cyclin D1 at Thr-286 contribute to cell transformation, although to date, no such mutations have been found in human cancers. *Cyclin D1 (CCND1)* undergoes alternative splicing leading to the production of an mRNA predicted to encode a unique cyclin D1 isoform, cyclin D1b, which lacks Thr-286. We have cloned and expressed cyclin D1b, and find that it retains the ability to bind to and activate CDK4. Unlike canonical cyclin D1a, cyclin D1b remains nuclear through the cell cycle where its constitutive expression facilitates cellular transformation. Using antisera specific for cyclin D1b, the protein was detected in a high percentage of esophageal cancer-derived cell lines and in primary esophageal carcinomas. Therefore, alternative splicing leads to expression of a nuclear, oncogenic cyclin D1 isoform that is expressed in human cancer.

Introduction

Overexpression of cyclin D1 occurs at a high frequency in a variety of carcinomas including those of breast, esophageal, and pancreatic origin (1, 4–7, 11). Paradoxically, overexpression of canonical cyclin D1 alone is not sufficient to induce transformation (2, 3). In contrast, expression of the cyclin D1 mutant, cyclin D1-T286A, which cannot be phosphorylated by GSK-3β[3] and is stabilized in the nucleus throughout interphase, transforms murine fibroblasts in the absence of a collaborating oncogene (2) suggesting that deregulation of cyclin D1 nuclear export results in increased cyclin D1 oncogenic capacity. Whereas there is no evidence for mutations that specifically target Thr-286 of cyclin D1, recent evidence suggests the presence of an alternatively spliced cyclin D1 mRNA, cyclin D1b, which is selectively expressed in a number of human cancers and is predicted to encode a cyclin D1 isoform that lacks Thr-286 (reviewed in Ref. 4).

We now demonstrate that the protein encoded by *cyclin D1b* encodes an oncogenic cyclin isoform. Whereas cyclin D1b retains the capacity to bind to and activate CDK4, the cyclin D1b protein is refractory to GSK-3β- and CRM1-dependent nuclear export, and is, thus, constitutively nuclear. Strikingly, in contrast to canonical cyclin D1a, expression of cyclin D1b is sufficient to drive transformation of NIH-3T3 murine fibroblasts. Finally, we demonstrate that cyclin D1b is expressed in tumor-derived cells and primary human esophageal tumors.

Received 7/18/03; accepted 9/4/03.

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[1] Supported by grants from the NIH CA93237 and the American Cancer Society RPG-00-303 (to J. A. D.) and NIH/National Institute of Diabetes and Digestive and Kidney Diseases Center for Molecular Studies in Digestive and Liver Diseases (P30 DK050306) and its Morphology Core Facility.

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[3] The abbreviations used are: GSK-3β, glycogen synthase kinase-3β; CDK, cyclin-dependent kinase; GST, glutathione *S*-transferase; RB, retinoblastoma; HFF, human foreskin fibroblast.

Materials and Methods

Cell Culture Conditions, Transfections, and Generation of Cyclin D1a and D1b cDNAs. All of the cells were maintained in DMEM containing glutamine supplemented with antibiotics (BioWhitaker) and 10% FCS (Hyclone). Insect Sf9 cells were grown in Grace's medium supplemented with 10% heat-inactivated FCS. Transient expression of plasmids encoding Flag-tagged cyclin D1a, D1b, and CDK4 was achieved by using Lipofectamine Plus (Invitrogen) according to the manufacturer instructions. cDNAs encoding cyclin D1a and cyclin D1b were generated for the human non-small cell lung cancer cell line, A549. cDNAs were sequenced in their entirety to ensure their identity and compared with published sequence to ensure their identity (5–7). The respective cDNAs were inserted into the pFlex-1 vector as *Eco*RI fragments as described previously for murine cyclin D1 (8) to generate Flag-tagged molecules.

Immunoblotting, Immunoprecipitation, and Kinase Assays. For direct Western analysis, cells (or tumors) were lysed in Tween 20 buffer [50 mM HEPES (pH 7.5), 150 mM NaCl, 10 mM $MgCl_2$, 1 mM EDTA, 0.1% Tween 20, 1 mM phenylmethylsulfonyl fluoride, 20 units/ml of aprotinin, 5 μg/ml leupeptin, 0.4 mM $NaVO_4$, and 0.4 mM NaF]. Total cellular protein was resolved on denaturing polyacrylamide gels, transferred to membranes (MSI, Westborough, MA), and blotted with an isoform-specific cyclin D1 antibody (Ab3, Oncogene Research Products or the D1b-specific R3 antiserum). The R3 antiserum was generated in a rabbit injected with a peptide synthesized with the sequence "SEGDVPGSLAGAYRGRHLVPRK," underlined in Fig. 1*A*. Protein:antibody complexes were visualized by one of two methods: use of secondary antibodies coupled to horseradish peroxidase followed by enhanced chemiluminescence or use of secondary antibodies conjugated with Cy5.5 (Amersham Pharmacia Biotech) and visualized using the LI-COR Odyssey IR Imaging System (LI-COR Biosciences). Detection of cyclin D1-dependent kinase activity and cyclin/CDK complexes was performed as described previously (9, 10).

Immunofluorescence and Immunohistochemistry. NIH-3T3 cells seeded on glass coverslips were transfected with expression vectors encoding the indicated DNAs. Cells were fixed using either 3% paraformaldehyde or methanol-acetone (1:1). Visualization of cyclin D1a and D1b in transfected mouse fibroblasts was achieved with the Flag-specific M2 monoclonal antibody. In the tumor-derived cell lines, cyclin D1a was visualized with the Ab3 antibody followed by biotin-conjugated antimouse and Texas Red conjugated streptavidin (Vector Laboratories). Cyclin D1b was then visualized in the same cell using the rabbit R3 antibody followed by FITC-conjugated antirabbit (Amersham Pharmacia Biotech).

After antigen unmasking (Vector Laboratories, Burlingame, CA), endogenous peroxidase activity of paraffin-embedded esophageal tumor samples was blocked by incubation of samples in 1% H_2O_2 for 15 min. Samples were then preblocked in 4% BSA, 2% normal goat serum for 20 min, and then incubated with primary antibody R3 (5 μg/ml) or Ab3 (5 μg/ml; Oncogene Research Products, Boston, MA) overnight at 4°C. After extensive washing primary antibodies were visualized by incubation with biotinylated secondary antibodies followed by avidin-biotin complex method-horseradish peroxidase incubation and treatment with 3, 3′-diaminobenzidine (Vector).

Cell Transformation and Tumorigenicity Assays. NIH-3T3 cells were plated at 3×10^5 cells/60-mm dish and the following day were transfected with the indicated plasmids. Transfected cells were cultured in medium containing 5% FCS, and foci were visualized after 21 days with Wright Giemsa Stain (Sigma). Images were captured by phase/contrast microscopy before Giemsa stain. Anchorage-dependent growth of NIH-3T3, D1a-3T3, and D1b-3T3 was determined by analyzing cell growth in semi-solid medium as described

previously (2). For experiments examining tumor formation in SCID mice, 10 million cells were resuspended in 0.2 ml of PBS and injected s.c. into the right and left flanks of 6-week-old male SCID mice (Charles River).

Results

Cyclin D1b Assembles with CDK4 as an Exclusively Nuclear Complex Refractory to Nuclear Export. A variant cyclin D1 transcript is expressed in a number of human cancers (4–7, 11). This transcript is predicted to encode a cyclin D1 protein (D1b) with a unique COOH terminus that is encoded by sequences specified by intron 4 (Fig. 1*A*). The fact that the cyclin D1b transcript has only been detected in cancer-derived cell lines has led to the hypothesis that it encodes an oncogenic variant of cyclin D1. To functionally characterize the cyclin D1b protein, both cyclin D1b and canonical cyclin D1a were engineered to encode an NH_2-terminal Flag-epitope tag (Flag-D1b or Flag-D1a). Placement of this epitope tag at the NH_2 terminus of D-type cyclins does not alter their known biochemical properties (8). We initially tested whether cyclin D1b can bind to and activate CDK4. NIH-3T3 cells were transfected with plasmids encoding CDK4 and either Flag-D1a or Flag-D1b. Cyclin D1/CDK4 complexes were isolated from whole cell lysates using a monoclonal antibody (M2) directed against the Flag-tag, and the presence of D1a, D1b, and CDK4 was determined by immunoblotting with either the M2 antibody or a CDK4-specific antibody, respectively. Flag-D1a and Flag-D1b were expressed at equal levels (Fig. 1*B, top*). CDK4 was detected at equivalent levels in both the D1a and D1b precipitates (Fig. 1*B, bottom,* compare *Lanes 2* and *3*). As shown, Flag-D1b migrates with increased mobility relative to Flag-D1a because of its smaller size (273 *versus* 295 residues). To determine whether cyclin D1b/CDK4 complexes were catalytically active, lysates prepared from NIH-3T3 cells transfected as above were subjected to precipitation with the M2 monoclonal antibody. Precipitates were then mixed with recombinant GST-RB and [^{32}P]γATP. Both D1a/CDK4 and D1b/CDK4 complexes phosphorylated RB (Fig. 1*C, Lanes 2* and *3*).

Cyclin D1a is phosphorylated at threonine 286 by GSK-3β, which in turn targets the cyclin D1a/CDK4 complex to the cytoplasm via the CRM1 nuclear exportin (2). As cyclin D1b lacks the GSK-3β phosphorylation site, which is encoded by exon 5, we reasoned that it might be refractory to nuclear export directed by GSK-3β and CRM1. Therefore, we transfected NIH-3T3 cells with plasmids encoding either CDK4 and Flag-D1a or Flag-D1b with or without either GSK-3β or CRM1. In the absence of either ectopic GSK-3β or CRM1, a significant fraction of Flag-D1a (Fig. 1*D, panel a*) and a greater fraction of Flag-D1b (Fig. 1*D, panel g*) were localized primarily to the nucleus as determined by indirect immunofluorescence. As demonstrated previously with murine cyclin D1, overexpression of either CRM1 (Fig. 1*D, panel b*) or GSK-3β (Fig. 1*D, panel c*) resulted in the redistribution of cyclin D1a to the cytoplasm. Thus, human cyclin D1a like its murine counterpart is subject to phosphorylation-dependent nuclear export. We confirmed that human D1a is phosphorylated on Thr-286 in a GSK-3β-dependent manner (data not shown). In contrast, ectopic expression of either CRM1 (Fig. 1*D, panel h*) or GSK-3β (Fig. 1*D, panel i*) did not promote the redistribution of cyclin D1b to the cytoplasm (Fig. 1*D*). In this respect, cyclin D1b behaves like our previously engineered D1-T286A mutant that remains nuclear throughout interphase.

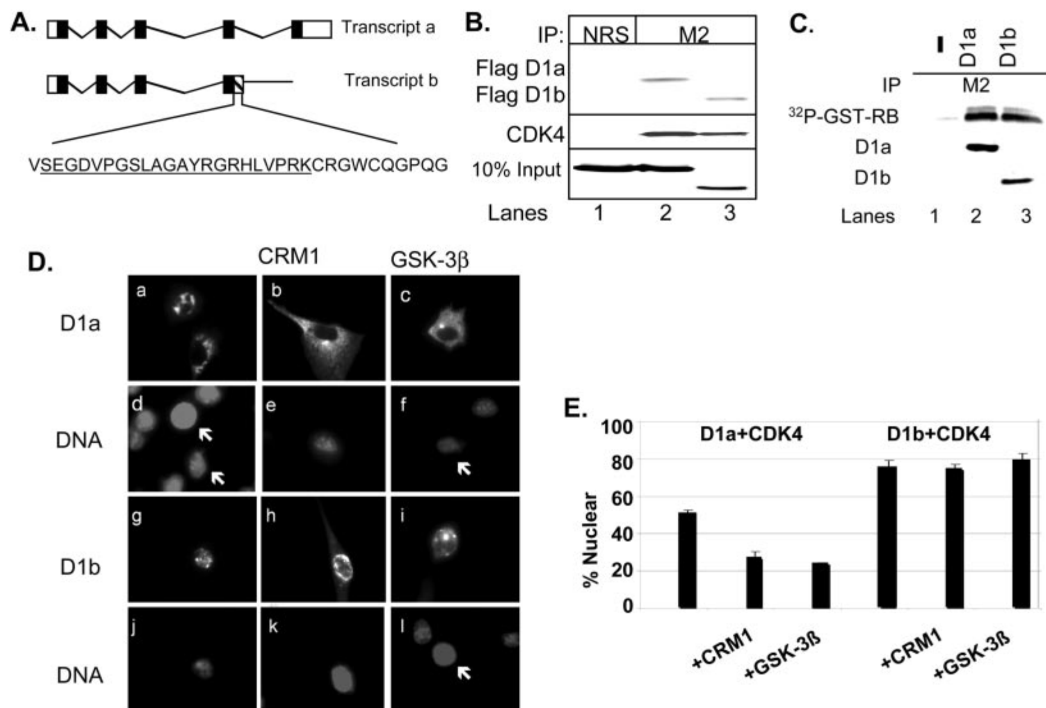


Fig. 1. Activation of CDK4 and nuclear accumulation of cyclin D1b/CDK4 complexes. *A,* genomic structure and splicing of cyclin D1; exons indicated by *shaded areas* and introns by *lines*. Cyclin D1 sequences derived from intron 4 are indicated by ▧: the *underlined portion* indicates the region to which the R3 antibody is derived. *B,* lysates were prepared from NIH-3T3 cells transfected with vectors encoding Flag-tagged cyclin D1a (*Flag-D1a*) or cyclin D1b (*Flag-D1b*) along with CDK4 and were precipitated with antibodies directed toward the Flag epitope (*M2*) or as a control normal rabbit serum (*NRS*). Precipitated proteins were assessed by Western analysis with antibodies directed toward CDK4 (*third panel*) or the Flag epitope (*M2, top panel*). One tenth of the total cell lysates was subjected to direct Western analysis to confirm expression levels of each cyclin D1 isoform. *C,* lysates transfected with plasmids encoding the proteins indicted at the *top of each panel* were precipitated with the M2 monoclonal antibody and mixed with recombinant GST-RB and [$^{32}\gamma$P]ATP. Radiolabeled GST-RB was assessed by SDS-PAGE, transferred to nitrocellulose membrane, and visualized by autoradiography. Levels of precipitated cyclin D1a and D1b were confirmed by immunoblot with the M2 antibody. *D,* NIH-3T3 cells were transfected with plasmids encoding Flag-D1a and CDK4 (*a–f*), or Flag-D1b and CDK4 (*g–l*). Where indicated, cells were also cotransfected with vectors encoding either HA-CRM1 (*b, e, h,* and *k*) or GSK-3β (*c, f, i,* and *l*). Cyclin D1 localization was assessed by immunofluorescent staining with the M2 monoclonal antibody and DNA was visualized with Hoechst Dye. *E,* quantitation of cells expressing exclusively nuclear cyclin D1. *Bars,* ±SD between three independent experiments as performed in *A*.

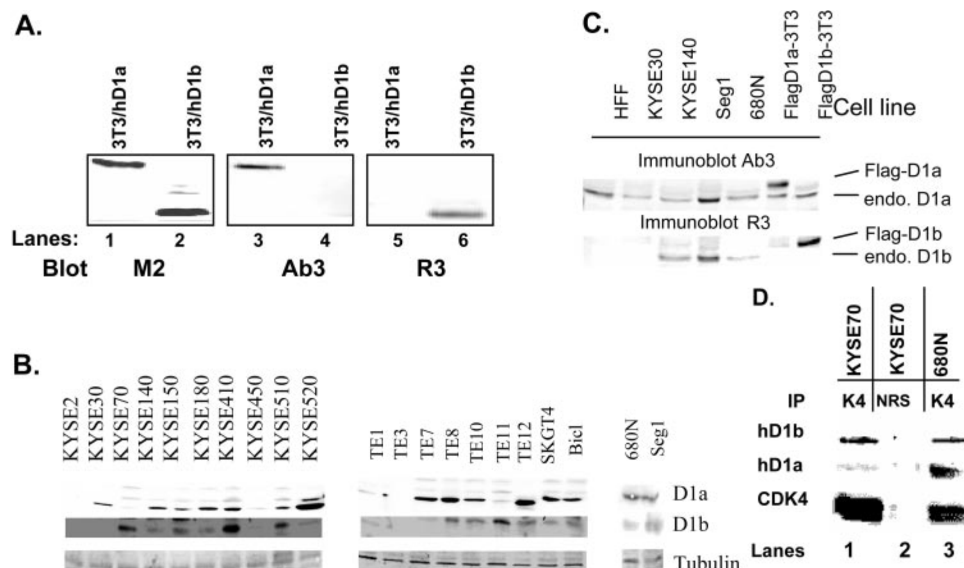


Fig. 2. Expression of cyclin D1a and D1b in cancer-derived cell lines. *A,* NIH-3T3 cell lysates programmed with either Flag-D1a or Flag-D1b were blotted with either the M2 monoclonal antibody *(Lanes 1* and *2),* an antibody derived against D1a *(Lanes 3* and *4, Ab3)* or an antibody raised against a peptide encoding the intron-derived sequences unique to D1b *(Lanes 5* and *6, R3).* *B,* a panel of 21 cell lines independently derived from esophageal tumors were analyzed for expression of cyclin D1a (*top panel*), cyclin D1b (*middle panel*), and tubulin (*bottom panel*). *C,* lysates prepared from HFFs, esophageal tumor cell lines (*KYSE30, KYSE140, Seg1,* and *680N*), or NIH-3T3 cells programmed to overexpress either Flag-D1a or Flag-D1b were subjected to immunoblot analysis with either the Ab3 (*top panel*) or the R3 (*bottom panel*) antibody. *D,* lysates prepared from the cell lines indicated were precipitated with a CDK4 specific antibody followed by immunoblot with antibodies directed toward CDK4, cyclin D1a, and cyclin D1b.

Nuclear Accumulation of Cyclin D1b in Esophageal Tumor-Derived Cell Lines. Cyclin D1b mRNA has been detected in several human cancers including breast (12) and hereditary nonpolyposis colorectal carcinoma (7). However, the lack of antibodies specific for cyclin D1b, has precluded analysis of D1b protein expression. Therefore, we generated an antibody (designated R3) against the unique COOH terminus of cyclin D1b. We confirmed the specificity of our D1b antisera (R3) *versus* a commercially available monoclonal antibody (Ab3) using protein produced in NIH-3T3 cells (Fig. 2*A*). Using antibodies to cyclin D1a, D1b, and tubulin as a control, we screened a panel of esophageal carcinoma cell lines by immunoblot analysis for differential expression of cyclin D1 isoforms. Of 21 cell lines, 16 were found to express D1b (Fig. 2*B, middle*). D1a was detected in 17 of the cell lines (Fig. 2*B, top*). Whereas the ratio of D1a:D1b was variable, generally, cell lines that expressed D1b also expressed D1a. We also determined whether cyclin D1b could be detected in normal cells. Here, we used HFF that have been life-extended with the telomerase catalytic subunit, human telomerase reverse transcriptase (13), along with a subset of the esophageal cancer-derived cell lines used in Fig. 2*B,* and NIH-3T3 cells that express ectopic Flag-D1a or Flag-D1b. Lysates prepared from these cells were analyzed by immunoblot with either the Ab3 antibody (Fig. 2*C, top*) or the R3 antibody (Fig. 2*C, bottom*). Equal protein loading was confirmed by Ponceau S stain (data not shown). Wild-type cyclin D1a was apparent in all of the cell lines at variable levels. In contrast, cyclin D1b was detected in KYSE140, Seg-1, and 680N, but it was not detectable in HFF cells (Fig. 2*C, bottom*).

To ensure that endogenous cyclin D1b was incorporated into CDK4 complexes, we precipitated CDK4 from proliferating KYSE70 or 680N cells, and blotted for the presence of cyclin D1b and cyclin D1a. Cyclin D1b and D1a were readily detected in CDK4 precipitates, but not in control precipitates (Fig. 2*D*). Higher levels of D1b, relative to D1a, were detected in CDK4 complexes isolated from the KYSE70 cells consistent with our detection of higher levels of cyclin D1b in this cell line. Coupled with our demonstration that active cyclin D1b/CDK4 complexes can be reconstituted in NIH-3T3 cells, these data demonstrate that endogenous cyclin D1b/CDK4 complexes are assembled *in vivo*.

On the basis of the results presented in Fig. 1, we reasoned that cyclin D1b should be exclusively nuclear in asynchronously proliferating tumor-derived cell lines, whereas cyclin D1a should be nuclear in the fraction of cells that are in G_1 phase as it is shuttled to the cytoplasm at the G_1-S boundary (2, 14, 15). To test this notion, we examined cyclin D1b and D1a localization by indirect immunofluorescence staining in asynchronously proliferating 680N cells, which express both cyclin D1 isoforms. Staining with the Ab3 antibody revealed that cyclin D1a localized to both the nuclear and cytoplasmic compartments (Fig. 3*A, panel b*). The overlay of cyclin D1a with DNA, revealed readily apparent cytoplasmic localization of cyclin D1a with some nuclear overlap (Fig. 3*A, panel c*). Quantitation revealed that D1a was predominantly nuclear in only 35% of cells (Fig. 3*B*). In contrast, D1b was predominantly nuclear in >95% of these same cells (Fig. 3*A, panel d;* Fig. 3*B* quantitation). An overlay of cyclin D1b staining with DNA revealed that essentially all of cyclin D1b localized to the nucleus (Fig. 3*A, panel e*). Similar results were obtained using the KYSE410 cell line (data not shown). As a control, we also assessed localization of cyclin D1 in KYSE30 cells, which contain no detectable cyclin D1b as determined by Western analysis. Immunofluorescent staining with the Ab3 antibody revealed cyclin D1a expression throughout both nuclear and cytoplasmic compartments (Fig. 3*C, panel a*). In contrast, incubation with the R3 antibody revealed no detectable cyclin D1b (Fig. 3*C, panel b*). Taken together with the results shown in Fig. 1, we conclude that cyclin D1b is an exclusively nuclear protein that is refractory to signals that promote cyclin D1a nuclear export.

Proteolysis of cyclin D1 is dependent on the 26S-proteasome and on phosphorylation-dependent nuclear export (8). Cyclin D1b lacks critical residues that direct its nuclear export and phosphorylation-dependent cytoplasmic degradation. Therefore, we investigated whether cyclin D1b might exhibit an increased half-life indicative of reduced proteolysis. Flag-D1a or Flag-D1b was first expressed in NIH-3T3 cells. To measure their respective rates of turnover, cycloheximide was added to these cell lines to block new protein synthesis, and we followed the rate of cyclin D1 decay by direct Western blot analysis using the D1a-specific Ab-3 antibody (Fig. 3*D, top*) or the D1b specific R3 antibody (Fig. 3*D, bottom*). The cyclin D1a half-life was estimated at 20–30 min, whereas that of cyclin D1b was only marginally longer, 50–60 min. Whereas these results indicate a marginal decrease in cyclin D1b turnover, we note that it remains a relatively unstable protein, in contrast to the D1-T286A mutant published previously (8).

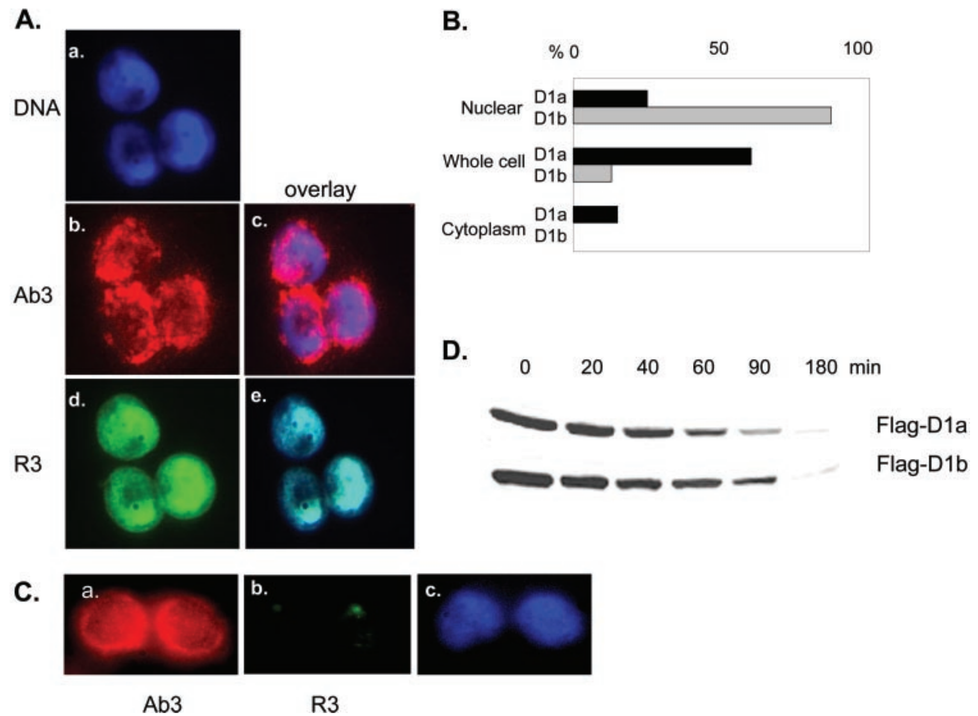


Fig. 3. Nuclear localization of cyclin D1b in tumor-derived cell lines. *A,* immunofluorescence analysis of cyclin D1a or cyclin D1b localization in human esophagus squamous cell line 680N. *a,* Hoechst nuclear staining; *b,* Ab3 mouse monoclonal antibody directed against D1a (Texas Red labeled 2nd antibody, *red*); c, overlay of Ab3 and Hoechst dye (*a* + *b*); *d,* R3 rabbit antibody directed against D1b (FITC-conjugated 2nd antibody, *green*); *e,* overlay of R3 and Hoechst dye (*a* + *d*); *B,* quantitation of the number of cells expressing exclusively nuclear cyclin D1. *C,* immunofluorescence analysis of cyclin D1a and cyclin D1b localization in human esophageal squamous cell line KYSE30. Staining was performed as described in *A. D,* proliferating NIH-3T3 cells stably expressing either Flag-D1a (*top*) or Flag-D1b (*bottom*) were treated with cycloheximide and harvested at the intervals indicated. D1 proteins were visualized using isoform specific antibodies, Cy5.5 conjugated secondary antibodies, and the LI-COR imager.

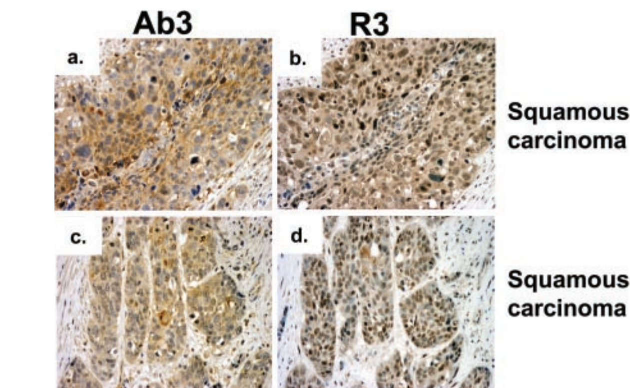


Fig. 4. Cyclin D1b is expressed in primary esophageal carcinoma. Paraffin-embedded tissue was subjected to immunohistochemical analysis with the Ab3 (*a* and *c*) or the R3 (*b* and *d*) antibody. Sections were counterstained with hematoxylin.

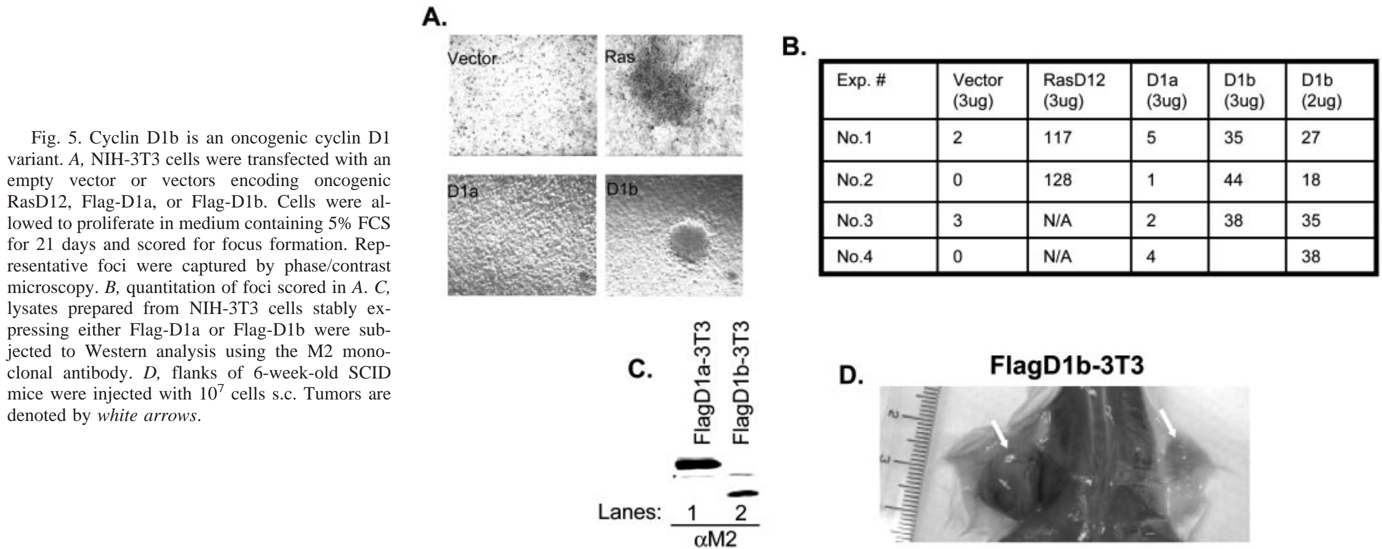
Cyclin D1b Expression in Primary Human Esophageal Cancers. To asses whether cyclin D1b is expressed in primary cancer, we performed immunohistochemical analysis on 12 independent esophageal tumors using both the Ab3 and the R3 antibodies. The intensity of R3 staining ranged from no staining to intense nuclear staining of all of the observed epithelial cells (Fig. 4, *panels b* and *d*). Eight of 12 tumors exhibited readily detectable staining with the R3 antibody (Table 1). Staining with the Ab3 antibody revealed expression of cyclin D1a in only 10 of 12 samples (Fig. 4, *panels a* and *c;* Table 1). Whereas cyclin D1b was generally nuclear, cyclin D1a appeared cytoplasmic or nuclear and cytoplasmic in most samples. These data demonstrate that cyclin D1b is expressed in primary human esophageal carcinoma.

Cyclin D1b Is a Nuclear Oncogene. To evaluate the oncogenicity of cyclin D1b, we transfected NIH-3T3 cells with plasmids encoding cyclin D1a, cyclin D1b, or as a positive control, oncogenic RasD12. After transfection, cells were allowed to grow for 21–28 days and examined for focus formation. As expected, expression of RasD12 resulted in the formation of numerous foci (Fig. 5*A, top right;* Fig. 5*B*). Whereas expression of wild-type cyclin D1a did not promote focus formation above background levels (Fig. 5*A, bottom left;* Fig. 5*B*), expression of D1b resulted in a 10-fold increase in focus formation (Fig. 5*A, bottom right;* Fig. 5*B*). Cyclin D1b was less potent than Ras at conferring this transformed phenotype. Expression of RasD12 resulted in an ∼4-fold increase in colony formation relative to D1b. Expression of cyclin D1b also promoted growth in semi-solid medium, whereas cyclin D1a did not (data not shown). To conclusively establish the oncogenicity of cyclin D1b, we determined whether cells expressing Flag-D1b would form s.c. tumors in immune-compromised mice. We established NIH-3T3 cell lines that stably overexpressed either Flag-D1a or Flag-D1b. In these cell lines, expression of Flag-tagged cyclin D1a exceeded that of Flag-tagged D1b as determined by immunoblot with the M2 monoclonal antibody (Fig. 5*C*). As a positive control we injected late passage NIH-3T3 cells that express a nonphosphorylatable cyclin D1 mutant, D1-T286A. As expected, the D1-T286A-3T3 cells readily produced tumors (Table 2). Injection of early passage, p7, D1b-3T3 cells did not result in tumor formation, whereas injection of later passage D1b-3T3 cells, p12, readily induced

Table 1 *Expression of cyclin D1b in primary esophageal tumors*

Paraffin sections from esophageal tumors were subjected to immunohistochemical analysis using either the Ab3 (D1a) or R3 (D1b) antibodies as indicated in columns 1 and 2. Intensity of antibody staining was classified as no signal (−), light staining in a fraction of epithelial cells (+/−), light staining in a majority of epithelial cells (+), moderate staining in a majority of epithelial cells (++), and intense staining in all epithelial cells (+++).

Ab3	R3	Pathology
+	+++	squamous carcinoma
++	+	squamous carcinoma
+	+	squamous carcinoma
++	−	squamous carcinoma
±	−	squamous carcinoma
+	±	squamous carcinoma
±	++	squamous carcinoma
+	+	squamous carcinoma
+	+++	squamous carcinoma
±	++	squamous carcinoma
−	−	squamous carcinoma
−	−	squamous carcinoma



Exp. #	Vector (3ug)	RasD12 (3ug)	D1a (3ug)	D1b (3ug)	D1b (2ug)
No.1	2	117	5	35	27
No.2	0	128	1	44	18
No.3	3	N/A	2	38	35
No.4	0	N/A	4		38

Fig. 5. Cyclin D1b is an oncogenic cyclin D1 variant. *A,* NIH-3T3 cells were transfected with an empty vector or vectors encoding oncogenic RasD12, Flag-D1a, or Flag-D1b. Cells were allowed to proliferate in medium containing 5% FCS for 21 days and scored for focus formation. Representative foci were captured by phase/contrast microscopy. *B,* quantitation of foci scored in *A. C,* lysates prepared from NIH-3T3 cells stably expressing either Flag-D1a or Flag-D1b were subjected to Western analysis using the M2 monoclonal antibody. *D,* flanks of 6-week-old SCID mice were injected with 10^7 cells s.c. Tumors are denoted by *white arrows*.

tumor formation in SCID mice (Fig. 5*D;* Table 2). This is consistent with previous findings that expression of nuclear cyclin D1 does not immediately result in an overt transformed phenotype (2); rather, nuclear cyclin D1 expression appears to sensitize cells to stochastic second hits that result in neoplasia. Injection of D1a-3T3 cells of equal or later passage failed to promote tumor development. Expression of Flag-D1b in tumors was confirmed by immunoblot analysis (data not shown). Collectively, these data demonstrate that the protein encoded by the cyclin D1 alternative transcript, cyclin D1b, is a more potently transforming cyclin D1 isoform.

Discussion

Cyclin D1 was simultaneously isolated as a growth-responsive gene (16) through a yeast complementation screen (17) and in a parathyroid adenoma containing the inversion (Ref. 8; p15;q13) on human chromosome 11 (18). Whereas the high frequency of cyclin D1 overexpression in human malignancy strongly suggests its involvement in the neoplastic process, *in vitro* studies have failed to reveal strong oncogenic potential (3, 19, 20). One primary mode of maintaining regulated cyclin D1 activity involves its timely nuclear export (2). Phosphorylation of cyclin D1 on a conserved COOH-terminal residue, Thr-286, by GSK-3β triggers association of cyclin D1 with CRM1 and subsequent nuclear export during S phase. Strikingly, expression of a nonphosphorylatable, stable cyclin D1 mutant was sufficient to induce cell transformation (2). This observation suggested that whereas overexpression of cyclin D1 may be observed frequently in cancer, mutations, direct or indirect, that promote constitutive cyclin D1 nuclear localization are likely the key oncogenic events.

Mutations that target threonine 286 of cyclin D1 have not been found. However, human cyclin D1 does undergo alternative splicing, which generates a unique cyclin D1 transcript (5); the resulting protein will lack critical COOH-terminal regulatory sequences necessary for cyclin D1 nuclear export. We anticipated that this novel cyclin D1 protein, D1b, would be a constitutively nuclear, cyclin D1 isoform. Indeed, we found that whereas the cyclin D1b cDNA encodes a translatable protein with many of the same biochemical properties as wild-type cyclin D1a, unlike cyclin D1a, cyclin D1b is refractory to phosphorylation-dependent nuclear export and is, thus, a constitutively nuclear protein. This reflects the fact that cyclin D1b lacks the COOH-terminal sequences targeted by GSK-3β and CRM1 (2).

Rapid proteolysis of wild-type cyclin D1a is also associated with phosphorylation of Thr-286 (8, 15). Mutation of Thr-286 to a nonphosphorylatable residue resulted in a $>$5-fold increase in the measured cyclin D1 half-life (8). Cyclin D1 turnover is reduced when its nuclear export is blocked, either by inhibition of CRM1 (2) or overexpression of Cip/Kip proteins (22). Taken together, these results suggest that cyclin D1 degradation occurs more efficiently in the cytoplasm. Given that cyclin D1b lacks Thr-286 and is refractory to CRM1-dependent nuclear export, we anticipated that it would have an extended half-life relative to cyclin D1a. However, we found that cyclin D1a and cyclin D1b have similar rates of protein turnover when expressed in normal mouse fibroblasts. These data suggest that cyclin D1b must be more susceptible to nuclear degradation than cyclin D1-T286A.

Similar to our conclusions, Solomon *et al.* (23) have reported recently that cyclin D1b is a constitutively nuclear protein when overexpressed in tissue culture cells. Upon characterization of cells overexpressing ectopic D1b, it was noted that D1b-expressing cells lost contact inhibition but did not determine whether expression of D1b conferred a truly transformed phenotype. We find that expression of cyclin D1b promotes not only focus formation, but also growth in soft agar (data not shown) and tumor formation in immune-compromised mice, thereby demonstrating its overt oncogenic potential. The abrogation of cyclin D1b nuclear export correlates with the capacity of cyclin D1b to induce focus formation, anchorage-independent growth, and tumor formation in SCID mice. It is important to note that in the cell lines used for tumor formation studies, cyclin D1a expression exceeded that of cyclin D1b. This is consistent with previous work demonstrating that loss of cyclin D1 nuclear export is a critical factor in cyclin D1-driven cell transformation (2), rather than overt cyclin overexpression. Our detection of cyclin D1b protein in cells derived from esophageal tumors and in primary esophageal tumors also supports the notion that expression of cyclin D1b was an oncogenic event during the neoplastic process. Collectively, these data

Table 2 *In vivo tumor growth of D1b-expressing cells*

The ability of cells from the indicated cell line and passage number (p) to form tumors in SCID mice was determined by injecting 10^7 cells s.c. into the flanks of 6-week-old SCID mice. Mice were monitored daily for tumor formation.

Cell line	Passage no.	No. of injections	No. of tumors
D1a-3T3	p13	4	0
D1b-3T3	p7	2	0
D1b-3T3	p12	4	4
D1-T286A-3T3	p31	2	2

suggest that cyclin D1b expression will be prevalent in many cancers that are associated with "cyclin D1 overexpression."

Elucidation of the mechanisms that determine D1a *versus* D1b expression will contribute to our understanding of the cancer-specific expression of cyclin D1b. Cyclin D1b mRNA has been associated with a G/A polymorphism at codon 870 at the exon 4/intron 4 boundary (5, 6, 11, 24). This polymorphism is predicted to alter the splice-donor, splice-acceptor sequence thereby influencing the frequency of intron excision. Yet, it remains unclear whether its presence fully accounts for allele-specific expression of cyclin D1b in cancer. In principle, the cyclin D1b mRNA may be spliced at low levels in many cell types that normally express cyclin D1 leading to only marginal protein accumulation. However, upon exposure of cells to aberrant proproliferative/oncogenic signals, the balance of cyclin D1 splicing is shifted to favor D1b. Such an event could represent an early event in the neoplastic process.

It is tempting to speculate that constitutively nuclear cyclin D1b might contribute to genomic instability through perturbations in the fidelity of DNA synthesis. Consistent with this idea, we have demonstrated recently that cyclin D1 associates with MCM7 (25). Whereas both the D1a and D1b isoforms retain the ability to associate with MCM7 (data not shown), only cyclin D1b remains in the nucleus, when DNA is undergoing active replication. Alternatively, constitutively nuclear cyclin D1b/CDK4 complexes may induce transformation through perturbation of the normal RB phosphorylation/dephosphorylation cycle. Consistent with this idea D1b retains a similar capacity to activate CDK4 catalytic activity toward RB when compared with cyclin D1a, and assembles with CDK4 in both reconstituted cell lines and in tumor derived cells. Future efforts will certainly focus on the elucidation of the mechanisms whereby cyclin D1b expression contributes to neoplasia.

Acknowledgments

We would like to thank Charles J. Sherr (St. Jude Children's Research Hospital, Memphis, TN) for critical comments regarding this work and for providing NIH-3T3 cells; Anil K. Rustgi (Abramson Family Cancer Institute, University of Pennsylvania, Philadelphia, PA) for assistance with immunohistochemistry and analysis of samples; Michael Olson (Abramson Family Cancer Institute) for providing the RasD12 expression vector; and Mingzhou Guo (John Hopkins Oncology Center, Baltimore, MD) for providing the esophageal cancer cell lines.

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