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Function of a human cyclin gene as an oncogene

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ABSTRACT **The cyclin D1 (*PRAD1*, CCND1) gene is affected by translocations and amplification in the genomes of a number of human tumors, suggesting that these changes confer growth advantage on developing tumor cell clones. We show here that in cultured cells, a cDNA clone of the cyclin D1 gene can contribute to cell transformation by complementing a defective adenovirus *E1A* oncogene. In such cells, this candidate oncogene indeed functions like an oncogene, suggesting a similar role in tumor progression *in vivo*.**

The regulated synthesis and destruction of cyclin proteins appears to be critical for proper cell-cycle control in eukaryotes. Association of these regulatory subunits with cyclin-dependent kinases (cdks) allows the subsequent activation of the complex and may direct the enzymatic activity to specific targets whose modification by phosphorylation is critical to cell-cycle transition (1–6). Five distinct classes of mammalian cyclins (termed A–E) have been described in recent years. The synthesis and function of these cyclins display various patterns of cell-cycle specificity. For example, cyclin A is produced at the G_1 to S phase transition and is required for the onset of DNA synthesis (7, 8), whereas B-type cyclins control passage through M phase (2, 3).

The G_1 to S phase transition may similarly require the prior synthesis and activation of cyclins and cyclin-dependent kinases. The identification of such activities has been an area of much recent interest (5). The D-type cyclins are synthesized in G_1 phase and are induced in response to agents that promote re-entry into the cell cycle (9–12). Further, microinjection of anticyclin D1 antibodies into human fibroblasts suggests that cyclin D1 is required for cell-cycle progression to S phase (13). D-type cyclins are thus good candidates for regulators of the G_1 to S phase transition, despite their rather constitutive synthesis in asynchronous cell populations (14, 15). Indeed, recent work has shown that constitutive overexpression of murine D-type cyclins in rodent cells can shorten the G_1 phase (16), suggesting that the level of these proteins is critical to proper cell-cycle control.

Because loss of cell-cycle control may contribute to tumor formation, it is intriguing that cyclin D1 has been found to be overexpressed in a variety of human tumors. These include parathyroid adenomas, breast carcinomas, B-cell lymphomas, squamous cell carcinomas, and esophageal carcinomas (9, 17–25). Indeed, it was the search for the putative *PRAD1* oncogene, a target of rearrangements with the parathyroid hormone locus in parathyroid tumors, which first led to the cloning of cyclin D1 (9). The proximity of the overexpressed *PRAD1*/cyclin D1 to 11q13 translocation breakpoints in B-cell tumors also strongly suggests its identity as the putative "*bcl-1*" oncogene (9, 21, 22, 24, 26).

If cyclin D1 were to function as an oncoprotein, its overexpression in primary rodent cells might lead to their immortalization and full transformation in cooperation with other oncogenes. We have used a primary rodent cell-transformation assay to test this possibility and have found that cyclin D1 genes can, indeed, participate in cellular transformation. Further, this activity seems to be mediated through an ability of cyclin D1 to circumvent the function of the retinoblastoma protein (RB), a suppressor of cellular proliferation that forms physical complexes with D-type cyclins (27–29).

MATERIALS AND METHODS

Preparation and Transfection of Primary Baby Rat Kidney (BRK) Cells. Primary BRK cells were prepared from 5- to 6-day-old Sprague–Dawley rats as described (30). For calcium phosphate-mediated transfection (31), cells were plated at 1.5–2.0 × 10^6 per 10-cm dish. A total of 6 μg of plasmid DNA consisting of 2 μg of each test plasmid and the vector Rc/CMV (Invitrogen) was mixed with 6 μg of herring sperm DNA and transfected in triplicate in each experiment. Plasmids used included 13S-SVE, containing the adenovirus type 5 13S cDNA under the control of the simian virus 40 early promoter and the identical construct containing the pm928 point mutation (32). Plasmid pucEJ directed the synthesis of an oncogenic allele of Ha-*ras*; all cyclin expression was driven by the construct Rc/CMV as described (33). Foci appeared on transfected cell monolayers within 7 days, and plates were stained (33) 2 to 3 weeks after transfection.

Establishment of Cell Lines and Protein Analysis. Cell lines were established from foci by trypsinization. Cells were initially plated in 24-well dishes and expanded to larger plates as the cultures reached confluence. For protein analysis, cells in 10-cm dishes were lysed and immunoprecipitated as described (33). Immunoprecipitates or whole-cell lysates were separated on polyacrylamide gels and subjected to immunoblot analysis (27). In some cases, cells were labeled with [^{35}S]methionine, and immunoprecipitates were visualized by autoradiography (33). Anti-RB monoclonal antibody 21C9 (33) was used for immunoprecipitation, and monoclonal antibody G3-245 (PharMingen) was used to visualize RB in immunoblots. E1A was analyzed with monoclonal antibody M73 (34). Immunoprecipitation and immunoblotting of cyclin D1 were performed with a rabbit anti-cyclin D1 antiserum (33).

RESULTS

Focus Formation by Cyclin D1 Genes. A sensitive assay of the functional properties of candidate oncogenes derives from the use of embryo cell cultures that can be transfected with these genes singly or in combination. When introduced into rat embryo fibroblasts or BRK cells, oncogenes such as *myc* or *E1A* can immortalize such cells in culture but are unable to fully transform them. Only in the presence of a cointroduced, collaborating oncogene like *ras* can full trans-

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Abbreviations: BRK, baby rat kidney; RB, retinoblastoma protein.

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formation be observed (35–37). This oncogene collaboration test can be used to assay the activity of a putative oncogene by ascertaining whether a gene can substitute for either the *ras* or *myc/E1A* gene in transforming these embryo cells. Activity as a collaborating oncogene is manifested by the appearance of foci and the subsequent outgrowth of cloned, transformed cell lines.

We exploited this assay to gauge the possible function of the cyclin D1 gene as an oncogene. As discussed above, this gene has been strongly implicated in a variety of human tumors, although its ability to function as an oncogene has never been demonstrated directly. For this reason, we transfected a cyclin D1 cDNA clone under the control of a cytomegalovirus (CMV) transcriptional promoter into BRK cells, alone or in combination with other oncogenes, and assayed for foci of transformants.

When transfected alone or in combination with an activated *ras*, *myc*, adenovirus 5 *E1A*, or mutant p53 gene, the cyclin D1 cDNA clone was unable to induce foci. In confirmation of earlier results, we found that none of these potential partner genes could induce fully transformed foci when introduced into BRK cells on its own (data not shown). We concluded that the cyclin D1 gene cannot replace either a *ras*-like or *myc/E1A*-like oncogene in this oncogene collaboration assay. Nonetheless, we considered it possible that the cyclin D1 gene might exert a subset of the functions of one or another of these oncogenes.

One clue to a possible function of the role of the cyclin D1 protein in transformation came from work focused on the retinoblastoma gene (*RB1*), in which we found that the *RB1* gene is cytostatic when introduced into *RB1*$^-$ osteosarcoma cells. This cytostatic effect could be reversed by cointroduction of the cyclin D1 gene into these osteosarcoma cells (33). This observation was consistent with a growth-promoting function of cyclin D1. Further, this protein binds to RB in a manner reminiscent of viral oncoproteins like that encoded by the adenovirus *E1A* oncogene (27). Both proteins carry a Leu-Xaa-Cys-Xaa-Glu (LXCXE) sequence critical for RB association and both appear to target a common domain of RB. According to a widely held model, this association of the E1A protein with RB prevents RB from binding and sequestering a series of cellular growth-promoting proteins including the E2F transcription factor (38, 39) and, as we have proposed elsewhere, the cyclin D1 protein (27). Following this logic, in the presence of E1A protein, these cellular partners of RB are liberated from RB-imposed control and are, thus, free to promote cell growth in a fashion that contributes to the transformation phenotype.

Mutant E1A oncoproteins that fail to bind RB also fail to immortalize and transform primary cells (40–42). This fact demonstrates the essential role of this binding in E1A-mediated transformation and might be explained by the resulting inability of the mutant viral oncoprotein to liberate cellular proteins such as cyclin D1 from RB control. This hypothesis suggested a specific assay for cyclin D1 function, in which we test whether cyclin D1 could complement a transformation-defective E1A oncoprotein that has lost the ability to bind RB.

For such an experiment, we used the pm928 mutant allele of the human adenovirus type 5 *E1A* oncogene (32). This mutation causes the E1A oncoprotein to lose its affinity for RB but does not affect its ability to bind other host-cell proteins including p60, p107, and p300; E1A binding to cellular p130 is reduced somewhat (40–43).

To determine whether constitutive, high-level cyclin D1 expression could compensate for the functional defect in the

Table 1. Focus formation in BRK cells

Plasmids†	*ras*	Foci induced,* no.									Induced,‡ mean % ± SD
		Exp. 1	Exp. 2	Exp. 3	Exp. 4	Exp. 5	Exp. 6	Exp. 7	Exp. 8	Exp. 9	
Rc + Rc	−	1	1	0	0	0	0	1	0	1	1.2 ± 1.6
13S + Rc	−	27	46	66	26	61	30	24	54	69	100.0
D1 + Rc	−	1	0	0	0	0	0	1	1	0	1.1 ± 1.7
GH + Rc	−	3	1			0			0	1	2.8 ± 4.2
KE + Rc	−			0	0		0	2	0	1	1.5 ± 2.9
13S + D1	−	46	34	65	18	30	39	27	87	100	112.4 ± 38.9
13S + GH	−	19	26			22			78	79	84.2 ± 39.3
13S + KE	−			65	16		47	28	35	95	106.5 ± 36.1
928 + Rc	−	3	3	6	2	2	6	3	7	3	9.8 ± 4.9
928 + D1	−	9	9	19	6	6	10	8	18	33	28.2 ± 11.5
928 + GH	−	9	9			4			18	42	30.8 ± 17.9
928 + KE	−			7	1		1	1	3	3	5.2 ± 2.7
Rc + Rc	+	1	0	0	0	0	0	1	1	0	0.4 ± 0.1
13S + Rc	+	54	74	84	45	54	81	82	125	155	100.0
D1 + Rc	+	1	0	0	0	0	0	0	0	0	0.2 ± 0.6
GH + Rc	+	1	0			0			1	0	0.6 ± 0.8
KE + Rc	+			0	0		0	2	0	0	0.3 ± 0.7
13S + D1	+	82	89	90	35	51	98	67	105	116	101.4 ± 24.3
13S + GH	+	81	73			45			109	99	96.6 ± 29.0
13S + KE	+			87	49		96	51	76	90	85.5 ± 25.6
928 + Rc	+	3	6	10	1	3	8	4	5	1	6.0 ± 3.4
928 + D1	+	18	16	26	6	8	19	8	17	24	19.7 ± 7.6
928 + GH	+	22	16			9			17	29	22.6 ± 9.6
928 + KE	+			11	0		3	1	2	1	4.0 ± 4.3

Three 10-cm dishes of BRK cells were transfected with each set of plasmids listed. Visible foci appeared in 5–6 days. Plates were stained after 3 weeks in culture, and the foci were counted.

*Number of foci induced was determined as the average number of foci on three 10-cm dishes independently transfected with the given plasmids.

†Plasmids transfected included 13S-SVE (13S) and the pm928 mutant of 13S-SVE (928); Rc, vector plasmid Rc/CMV; D1, Rc/cycD1; GH, GH mutant of D1; and KE, cyclin box mutant KE, as described in text.

‡Mean percentage of foci induced was calculated by dividing focus numbers for each plasmid combination by the value obtained for 13S alone or 13S plus *ras*. These relative values were determined for each experiment, averaged by the total number of experiments done for the particular plasmid combination, and then multiplied by 100 to obtain the percentage value.

pm928 mutant, a plasmid expressing this mutant viral protein was introduced into BRK cells, either alone or together with a cyclin D1 expression plasmid. As reported (36, 44), we found that wild-type E1A produced immortalized but nontransformed colonies on BRK cell monolayers after transfection. In contrast, the pm928 E1A mutant produced far fewer colonies, averaging only 10% of the control (Table 1 and Fig. 1). The cyclin D1 plasmid was then cointroduced into these cells with either the wild-type or the mutant *E1A* gene. It had no effect on the number of colonies induced by the wild-type gene but increased by a factor of three the number of nontransformed colonies induced by the mutant *E1A* oncogene alone.

When a *ras* oncogene was cointroduced with a wild-type *E1A* gene into these BRK cells, many foci of highly transformed cells appeared rapidly (Table 1 and Fig. 1). As expected (32), the pm928 mutant demonstrated a serious deficiency in the ability to produce such foci in collaboration with the *ras* oncogene. Inclusion of cyclin D1 plasmid had little effect when cointroduced together with wild-type E1A and a *ras* oncogene, recalling its previously observed inability to augment the function of wild-type E1A. However, inclusion of cyclin D1 caused a 3-fold increase in the number of foci produced by pm928 and *ras*. The foci induced by the mutant E1A plus *ras* were small, appeared only with delay, and often showed a nontransformed morphology; many of those induced by pm928 plus *ras* plus cyclin D1 appeared quickly, grew to a large size, and contained cells that appeared highly transformed (Fig. 1). We note that such a 2.5- to 3.5-fold increase in focus number was observed reproducibly in nine independent experiments.

Generation of Cell Lines from Cyclin D1-Immortalized Cells. To demonstrate that transfection with the oncogenes used in these experiments could produce immortal cells, we established cell lines by treating the colonies and foci with trypsin and expanding them in culture. Colonies and foci produced by the wild-type E1A were easily established, as expected (27/28 gave rise to cell lines in culture). In contrast, a small fraction (8/25) of those produced by transfection of the pm928 mutant allele gave rise to established cell lines. Colonies and foci derived from cotransfection of pm928 with cyclin D1 could be established with intermediate efficiency (28/44). The increase in readily clonable BRK colonies produced by pm928 when cyclin D1 plasmid was cotransfected led us to conclude that cyclin D1 can collaborate with the mutant pm928 *E1A* oncogene to produce immortal cell lines and, by extension, can lead to the production of fully transformed cells in collaboration with pm928 and a *ras* oncogene.

Transformation by a Non-RB-Binding Allele of Cyclin D1. We explored three mechanistic explanations that could be invoked to explain these powers of cyclin D1. According to the first of these models, when cyclin D1 is expressed at high levels, it might bind and thus sequester the full cellular complement of RB, thereby mimicking the function of the wild-type E1A protein. To test this model, we studied the activity of a cDNA encoding a mutant cyclin D1 that lacks the ability to bind RB. The mutant protein, termed GH, contains the sequence Leu-Leu-Gly-His-Glu (LLGHE) substituted for the normally present Leu-Leu-Cys-Cys-Glu (LLCCE) sequence in cyclin D1 (27). The amino acid substitution in this GH mutant of cyclin D1 mimics the mutation preventing the pm928 mutant of E1A from binding RB.

Significantly, as can be seen in Table 1 and Fig. 1, this mutant D1 cyclin can still complement the pm928 mutation in focus formation. Indeed, the number of readily established foci produced by this GH mutant in combination with pm928 is reproducibly indistinguishable from that produced by wild-type cyclin D1, indicating that RB binding is dispensable for the oncogenic function of cyclin D1. For this reason, we concluded that cyclin D1 does not act in these cells by binding and thus sequestering their complement of RB.

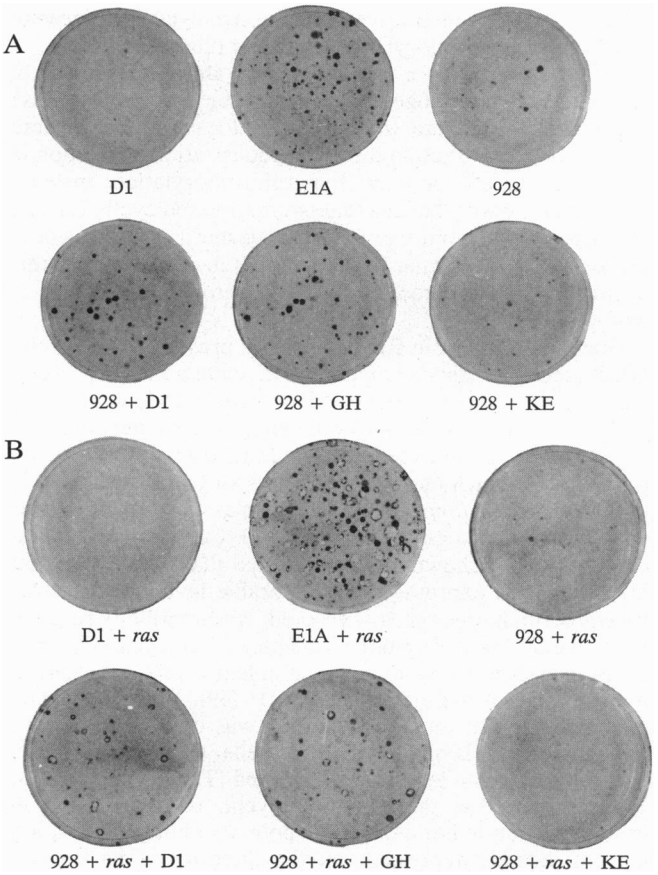

FIG. 1. Focus formation on BRK cell monolayers. (*A*) BRK cells were transfected with the cyclin D1 expression construct Rc/cycD1 (D1), the wild-type 13S E1A cDNA (E1A), the pm928 mutant *E1A* allele (928), or a combination of the pm928 cDNA and cyclin D1. Also included were transfections with pm928 and the GH or KE mutant cyclin D1 cDNAs. (*B*) Transfection of the BRK cells with the same DNAs as above plus activated *ras*. The monolayers were stained 3 weeks after transfection.

Cyclin D1 and RB Expression in Transformed Cells. A second mechanism explaining cyclin D1 action was suggested by earlier observations in human osteosarcoma cells, in which cyclin D1 expression resulted in a profound depression in RB levels (33). Accordingly, we monitored the levels of RB and cyclin D1 in cell lines derived from colonies and foci produced in the various transfections. These data demonstrate that each cell line derived from a cyclin D1 transfection expresses high levels of cyclin D1 (Fig. 2*c*), consistent with a role for this protein in the immortalization of these cells. However, in contrast to the earlier results with osteosarcoma cells, in these BRK cells cyclin D1 has no effect on RB levels (Fig. 2*d*, pRb). Thus, cyclin D1 does not act in these cells by suppressing RB expression.

A third mechanism explaining transformation by cyclin D1 might derive from an ability to induce hyperphosphorylation and attendant functional inactivation of RB, as has been seen in baculovirus-infected insect cells (28, 29). The immunoblots of RB from each cell line shown in Fig. 2 show little difference in the apparent phosphorylation of RB, however. This result was supported by immunoprecipitates of radiolabeled cell lysates, which showed that most newly synthesized RB in these transformed cells migrated at the position of hypophosphorylated RB, regardless of the presence of high cyclin D1 levels (Fig. 3). Thus, while direct action of a cyclin D-dependent kinase on a subset of RB residues cannot be rigor-

ously ruled out, constitutive expression of cyclin D1 does not lead to hyperphosphorylation of RB in these BRK cells.

Transformation by a Kinase-Binding Defective Mutant of Cyclin D1. Taken together with earlier work (27), these various results indicate that cyclin D1 does not function here by affecting RB function through sequestration, reduction in expression levels, or overt hyperphosphorylation; instead, these data support the idea that overexpressed cyclin D1 acts by outpacing the ability of RB to sequester it, yielding a pool of free cyclin D1 similar to that present in cells in which this cyclin is liberated from RB control through the action of wild-type E1A.

Once liberated from RB control, we presumed that cyclin D1 proceeds to associate with and activate a kinase partner, such as the recently identified cyclin-dependent kinase 4 (cdk4) (45, 46). This model was suggested by our observations with a second cyclin D1 mutant used in this assay, termed KE, which has suffered a Lys → Glu substitution in the cyclin box, a domain that mediates association of cyclins with cyclin-dependent kinases (47, 48). Control immunoprecipitations, not shown here, confirmed that this KE mutant D1 cyclin was expressed at comparable levels to the wild-type protein; moreover, as expected, while antibody reactive with cyclin D1 precipitated complexes of cyclin D1 and cyclin-dependent kinases, such complexes were not found in the presence of the mutant cyclin D1 (49). When a plasmid encoding the KE mutant cyclin D1 was cointroduced with pm928 and *ras* plasmids into BRK cells, no increase in foci above background levels was observed (Table 1 and Fig. 1). We conclude that the ability of cyclin D1 to function in transformation is not dependent upon its ability to bind RB and is, indeed, dependent on the integrity of its domain normally used for associating with cyclin-dependent kinases.

DISCUSSION

Our data indicate that overexpressed cyclin D1 protein can participate in cellular transformation, ostensibly through its ability to associate with and activate a kinase partner. This activity can partially overcome the defect in the pm928 adenovirus mutant, which fails to bind to RB, consistent with a proposed role for E1A in freeing sequestered cyclin D1 from RB control.

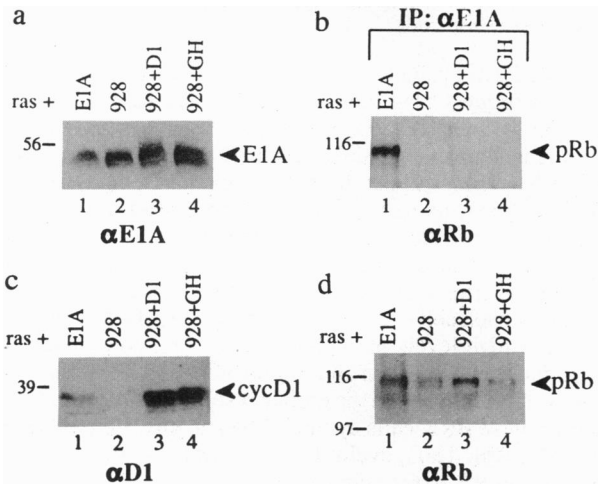

FIG. 2. Protein expression in transformed BRK cells. Representative cell lines established from BRK foci transformed by *E1A* and *ras*, pm928 and *ras*, pm928 plus cyclin D1 and *ras*, or pm928 plus cyclin D1 mutant GH and *ras* were lysed and subjected to immunoblot analysis for E1A (*a*) or were first immunoprecipitated (IP) with anti-E1A antibodies and subsequently immunoblotted for RB (*b*), demonstrating complex formation between RB and wild-type, but not mutant, E1A. (*c*) Immunoblot analysis of protein lysates from transformed BRK cells with anti-cyclin D1 antiserum (33). Lane 2 contained approximately one-half the amount of protein in the other lanes. (*d*) Immunoblot analysis of transformed cell lysates using anti-RB monoclonal antibody G3-245 (PharMingen). Molecular mass markers are indicated at left in kDa. pRb, RB.

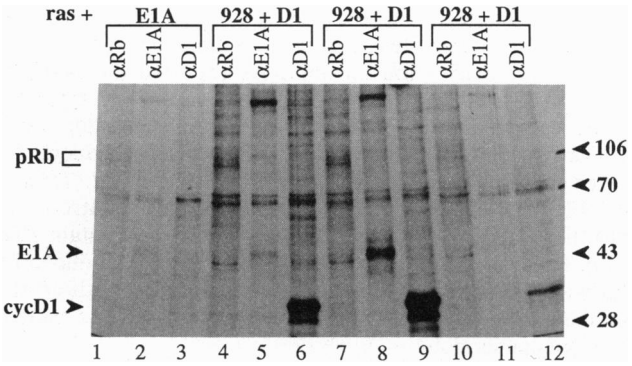

FIG. 3. Immunoprecipitation of metabolically labeled proteins from transformed BRK cells. Continuous cell lines derived from foci produced after transfection of BRK cells with E1A plus *ras* or the pm928 mutant allele of *E1A* plus cyclin D1 plus *ras* were metabolically labeled with [^{35}S]methionine, lysed, and immunoprecipitated with the anti-RB monoclonal antibody 21C9 (αRb), the anti-E1A monoclonal antibody M73 (αE1A), or the anticyclin D1 antiserum (αD1). Immunoprecipitates were separated on a 10% polyacrylamide gel and treated for fluorography. Positions of the specifically immunoprecipitated proteins are indicated at left. Note that the majority of the RB (pRb) signal appears at the fast-migrating, hypophosphorylated position. Molecular mass markers are shown at right in kDa.

If RB, indeed, controls the function of cyclin D1, mutations in cyclin D1 that prevent its association with RB may be expected to increase the activity of cyclin D1 in this assay. Although the GH mutant does not provide such an increase in focus formation here, the extremely high levels of cyclin D1 expression achieved in all of these transfections may mask any additional benefit deriving from this mutation. Indeed, if cyclin D1 transformed cells via a non-RB-dependent mechanism, cooperating with some unidentified function of E1A, cyclin D1 should enhance focus formation by wild-type E1A, an effect not observed here.

Because overexpressed cyclin D1 specifically cooperates with the pm928 allele of E1A, this cyclin in some way probably abrogates a growth-suppressive function of RB. The introduction of murine D-type cyclins into insect cells or of murine cyclin D2 into human cells together with RB can lead to the hyperphosphorylation of RB (28, 29). However, our inability to induce hyperphosphorylation of RB in human cells using any human D-type cyclin (data not shown) and the lack of RB hyperphosphorylation observed here suggests that the oncogenic function of cyclin D1 in these experiments is not due to direct hyperphosphorylation of RB.

The present data do not address the possibility that some of the observed effects of E1A (wild type and mutant) and cyclin D1 are attributable to their ability to interact with other cellular RB-related proteins. Indeed, the cellular protein p130 is a particularly attractive candidate for cyclin D1 interaction because pm928 has a reduced affinity for this protein. The effects of cyclin D1 expression on E1A mutants that bind RB but fail to bind to p130 and fail to transform are being tested and should shed more light on the functional interaction of cyclin D1 and E1A-binding proteins. We note that the present conclusions concerning cyclin D1 function do not depend on whether cyclin D1 associates exclusively with RB or additionally with a set of other RB-related cellular proteins like p130.

We suggest that the genetic alterations causing cyclin D1 overexpression seen in various human tumors overcome negative regulation imposed by RB and analogous proteins

and that the resulting deregulation of cyclin D1 is mimicked by the transfected cyclin D1 plasmids used here. Such deregulation of cyclin D1 may then lead to unchecked cellular proliferation, perhaps by alleviating a rate-limiting step in the late G_1 phase of the cell cycle (13, 16).

The conservation of the Leu-Xaa-Cys-Xaa-Glu domain in cyclins D2 and D3 suggests that they, too, are potential targets of RB (27) and are, thus, encoded by candidate oncogenes. Indeed, both of these cyclins cooperate with pm928 at least as efficiently as cyclin D1 (S.F.D., P.W.H., E.N.G., and R.A.W., unpublished data). In all these cases, we suggest that overproduction of a D cyclin outpaces the ability of RB (and related proteins) to sequester it and, in this way, liberates such a cyclin from its RB-like controllers. This, in turn, enables such a cyclin to activate kinases responsible for triggering late G_1 events required for entrance into subsequent phases of the cell-growth cycle.

The candidacy of cyclin D1 as a human oncogene has been based upon its overexpression associated with chromosomal rearrangement and amplification in primary tumors. However, a functional demonstration of the ability of cyclin D1 to contribute directly to tumorigenesis has been lacking. For this reason, the behavior of cyclin D1 as a cooperating oncogene in the present study provides strong indication that cyclin D1 is indeed a key actor in human tumor development and links disturbances in the cell-cycle machinery to the process of cell transformation.

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