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Cyclin D2 and Ha-Ras transformed rat embryo fibroblasts exhibit a novel deregulation of cell size control and early S phase arrest in low serum

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The D-type cyclins are growth factor-regulated delayed early functions which peak at the G_1/S transition, are thought to regulate entry into S phase and have been implicated in tumorigenesis. Here, we show that cyclin D2 can co-operate with Ha-Ras to impose a novel transformed state on rat embryo fibroblasts (REF). While clonal cyclin D2/Ha-Ras REF transformants exhibit a characteristic transformed phenotype in high serum, in low serum they arrest cell proliferation and display profound morphological and cytological changes indicating loss of control of cell mass and deregulation of the G_1/S transition. Notably, in low serum, despite re-establishment of actin cables and arrest of proliferation, cell mass continues to increase, creating giant cells up to 10× normal size. Also, during low-serum culture the cells make a very gradual but progressive entry into S phase, reaching a 2.4N DNA content after 6 days. PCNA is expressed and 2N and 4N cells are largely absent, and thus the cells undergo a novel S phase arrest. While transfer to low serum induced the retinoblastoma protein to enter its dephosphorylated state, and cyclin A, cyclin B and cdc2 levels to decrease, all as normal, cyclin E, cdk4, cdk2 and the exogenous cyclin D2 persisted at high levels. These results indicate that cyclin D2 and Ha-Ras can transform cells when mitogenic signals from growth factors are provided. However, in low serum, co-operation of cyclin D2 and Ha-Ras provides only a subset of the progression signals and these are sufficient for G_1-related cell mass increase and S phase entry, but are insufficient for full cell cycling.

Key words: cell cycle functions/cell size control/cyclin D2/oncogenic activation/S phase arrest

Introduction

Cell proliferation is primarily regulated in the G_1 phase of the cell cycle. Deregulation of the transition from G_1 to S phase can cause neoplastic transformation. Cyclins are key regulators of the cell cycle in yeast and higher eukaryotic cells. So far, in mammalian cells three different types of cyclins are expressed in G_1: cyclin C, D and E (Sherr, 1993). Three different D-type cyclins are known and their individual expression is tissue specific (Matsushime *et al.*, 1991). Amongst hematopoietic cells, cyclin D1 was found to be expressed in BAC1.2F5 macrophages, but not in pre-B lymphoid cells, HT-2 T-lymphoid cells or 32D13 myeloid cells. Cyclin D2 was shown to be expressed in each of these cells, whereas cyclin D3 was exclusively expressed in HT-2 T-lymphoid cells.

D-type cyclins are expressed as delayed early genes in response to growth factor stimulation of cell proliferation (Matsushime *et al.*, 1991). Their expression, as well as their kinase activities, peak at the G_1/S transition of the cell cycle (Matsushime *et al.*, 1994). This suggests a possible role for D-type cyclins in the regulation of the G_1/S transition. D-type cyclins were shown to interact directly with the retinoblastoma protein, Rb (Dowdy *et al.*, 1993; Ewen *et al.*, 1993). It has been proposed that D-type cyclins regulate the phosphorylation of the Rb protein, a crucial step in the progression of cells from G_1 to S phase (Dowdy *et al.*, 1993; Ewen *et al.*, 1993).

One finding which led to the discovery of the D-type cyclins was the elevated expression of cyclins D1 and D2 in different tumors, which also suggests that they are oncoproteins (Motokura and Arnold, 1993). Deregulated expression of cyclin D1 has been implicated in the pathogenesis of a subset of benign parathyroid adenomas. DNA from the long arm of chromosome 11 at band 11q13, originally called *PRAD1* and later shown to be the cyclin D1 locus, was clonally rearranged with the parathyroid hormone gene and transcriptionally activated in these tumors (Motokura *et al.*, 1991). It has also been suggested that the oncogene of the *BCL*-1 chromosomal translocation found in B-cell neoplasia is the *cyclin* D1 gene (Tsujimoto *et al.*, 1984; Motokura and Arnold, 1993). Recently, deregulated expression of the *cyclin* D2 gene has also been implicated in neoplastic transformation (Hanna *et al.*, 1993). The *vin*-1 gene, identified by insertional mutagenesis in murine leukemia virus-induced T-cell leukemias, was shown to be the *cyclin* D2 gene (Tremblay *et al.*, 1992; Hanna *et al.*, 1993).

The expression of an activated Ha-*ras* oncogene in primary cells can contribute to cell transformation in cell culture, as found with the co-expression of activated Ha-Ras and the c-Myc or E1A oncoproteins in primary cells (Land *et al.*, 1983; Ruley, 1983). Very recently, it has also been shown that cyclin D1 can co-operate with activated Ha-Ras in the transformation of rat embryo fibroblasts (REF) (Lovec *et al.*, 1994). In our studies, we have investigated the ability of cyclin D2 to transform cells in cell culture. We show that cyclin D2 and Ras-val12 can co-operate in the transformation of primary REF. Results from the analysis of a cell line subcloned from a focus of REF cells transfected with *cyclin* D2 and *ras*-val12 suggest that cyclin D2 plus Ras-val12 confer a novel, partially transformed phenotype in low serum characterized by a proliferative arrest in S phase, but deregulated G_1 activity. Full transformation is serum dependent and potentially requires a growth factor-

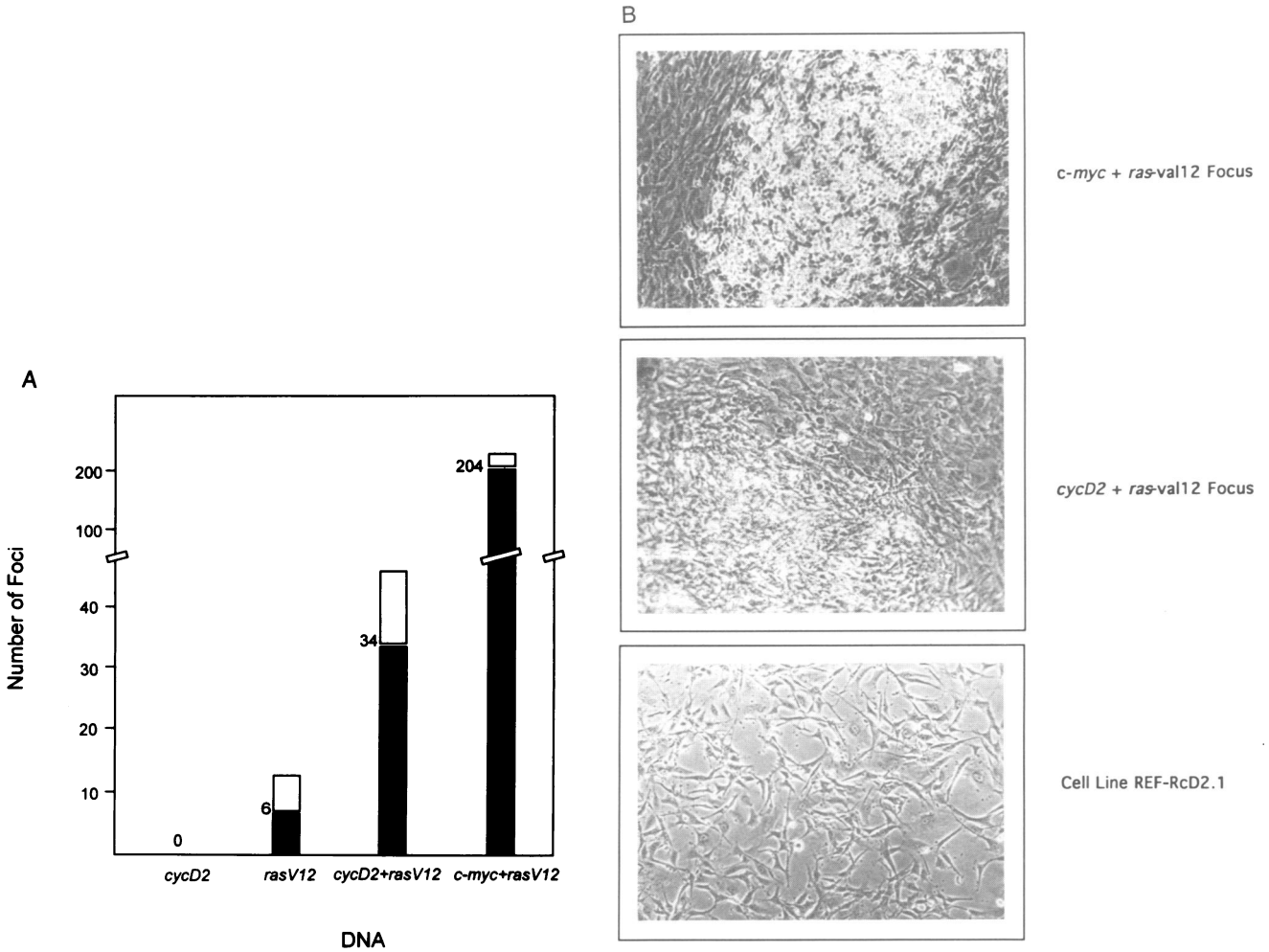

Fig. 1. Rat embryo fibroblast transformation assay. (**A**) Primary REF were transfected with eukaryotic expression vectors expressing *cyclin* D2, *ras*-val12, *cyclin* D2 plus *ras*-val12, and *ras*-val12 plus c-*myc*. The number of foci of transformed cells of six individual transfections on a standard 6 well tissue culture plate was counted and plotted in a bar diagram. The counted foci are shown as black bars, the calculated errors are shown as white bars. (**B**) The morphology of a typical focus resulting from a transfection of expression vectors expressing ras-val12 plus c-myc and ras-val12 plus cyclin D2 is shown. In addition, the morphology of REF-RcD2.1 cells, subcloned from a focus of REF transfected with expression vectors expressing ras-val12 plus cyclin D2, is documented.

induced signal. We provide evidence that cyclin D2 and Ras-val12 provide a subset of the functions necessary for full transformation and that the remaining functions, including expression of cyclin A and phosphorylation of the Rb protein, may be dependent on high serum.

Results

By transfecting primary REF with eukaryotic expression vectors constitutively expressing Ha-*ras*-val12 (pT22) and *cyclin* D2 (pSG5-cycD2), we show that activated Ha-Ras and *cyclin* D2 can co-operate in the transformation of primary REF. Cells transfected with Ha-*ras*-val12 plus *cyclin* D2 exhibited a 5-fold increase in the number of transformed foci compared with transfection with Ha-*ras*-val12 alone (Figure 1A). No transformed cells could be detected following transfection with *cyclin* D2 alone (Figure 1A). However, the oncogenic activity in this assay of Ha-Ras-val12 plus cyclin D2 was much lower than that of Ha-Ras-val12 plus c-Myc since transfection with eukaryotic expression vectors constitutively expressing Ha-*ras*-val12 and c-*myc* (LTR-Hmyc) resulted in a 34-fold increase in the number of foci of transformed cells compared with the transfection with Ha-*ras*-val12 alone (Figure 1A). No transformed foci were observed after transfection with c-*myc* alone (data not shown). Transformed cells in both cyclin D2/Ras-val12 and Myc/Ras-val12 foci were very refractile (Figure 1B). The Myc/Ras-val12 transformed cells are spherical in shape, whereas the cyclin D2/Ras-val12 cells are rod shaped (Figure 1B).

We have subcloned three different cell lines from individual foci of primary REF transfected with Ha-*ras*-val12 plus *cyclin* D2 (REF-RcD2.1-3). These cell lines, grown under conditions of high serum, are morphologically different from wild-type REF cells. REF cells are flat and non-refractile, while REF-RcD2.1-3 cells are refractile and spindle shaped (Figure 1B). REF-RcD2 cells show an altered expression of cell adhesion proteins since the cells attach only very weakly to tissue culture plates, a property which necessitated their passage on collagen-coated plates. While the growth of wild-type REF is contact inhibited, REF-RcD2.1 cells in contrast show no contact inhibition and continue to proliferate even when confluency is reached.

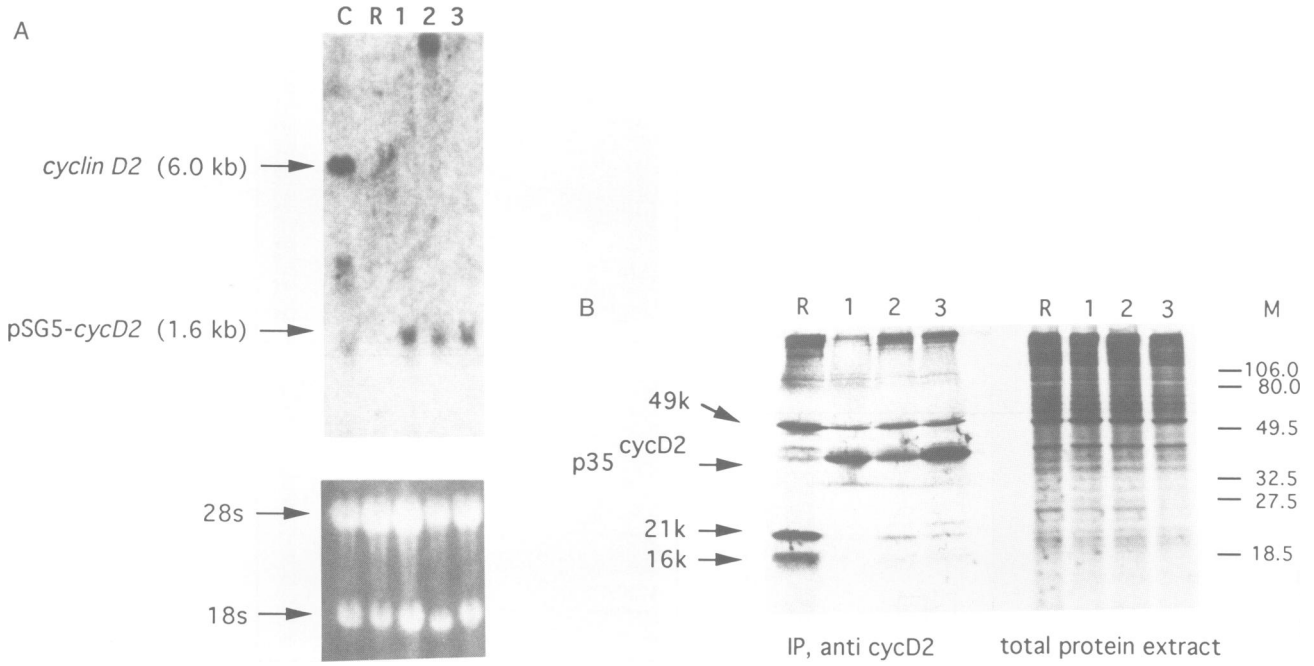

Fig. 2. Gene expression in cell lines subcloned from foci of REF transfected with expression vectors expressing *ras*-val12 plus *cyclin* D2 (REF-RcD2.1,2,3). (**A**) Total RNA from a murine cytotoxic T-cell line CTLL-2 expressing high levels of endogenous *cyclin* D2 (C), subconfluent primary REF (R) and the cell lines REF-RcD2.1 (1), REF-RcD2.2 (2) and REF-RcD2.3 (3) was analyzed by Northern blot analysis. The blotted RNA was hybridized with a [α-^{32}P]dCTP-labeled *cyclin* D2 cDNA probe. The bands were visualized by autoradiography. The migration of the endogenous (6.0 kb) and exogenous (1.6 kb) *cyclin* D2 mRNA is marked. (**B**) Subconfluent primary REF (R) and the cell lines REF-RcD2.1 (1), REF-RcD2.2 (2) and REF-RcD2.3 (3) were metabolically labeled with [^{35}S]methionine. The cells were lysed and the proteins from immunoprecipitations using a polyclonal rabbit serum raised against bacterially expressed recombinant cyclin D2 protein (α-D2), as well as the proteins of the total cellular extracts were separated on a 12% SDS−polyacrylamide gel. The bands were visualized by autoradiography. For size determination, a pre-stained SDS−PAGE standard (Bio-Rad, low range) containing proteins of an apparent mol. wt of 106, 80, 49.5, 32.5, 27.5 and 18.5 kDa was used.

Northern blot analysis confirmed that all three subcloned cell lines express an exogenous cyclin D2 mRNA of ~1.6 kb (Figure 2A). Only an extremely weak or no endogenous expression of cyclin D2 mRNA of the size of 6000 bases could be detected in either the wild-type REF or the REF-RcD2.1-3 cell lines in the Northern blot experiments (Figure 2A), indicating a very low expression of the endogenous cyclin D2 mRNA.

Immunoprecipitation of extracts from cells metabolically labeled with [^{35}S]methionine using a polyclonal antiserum specific for cyclin D2 (alpha-D2) revealed that each clonal cell line expresses high levels of exogenous p35 cyclin D2 protein (Figure 2B). In wild-type REF cells, similar immunoprecipitation resulted only in a very weak band of the size of the cyclin D2 protein, indicating an at least 4- to 10-fold overexpression of cyclin D2 protein in REF-RcD2.1-3 cells (Figure 2B). We also detected low levels of a 21k protein in the cyclin D2 immunoprecipitations (Figure 2B, lanes 2 and 3), which co-migrated with a strong band in the REF cell immune precipitate (Figure 2B, lane R). However the REF cell 21k band and bands of 49k and 16k were also seen in control pre-immune serum immunoprecipitations, and thus further experiments are necessary to resolve the identity of these bands and the significance of their levels.

While the REF-RcD cells were spindle shaped and exhibited a loss of actin stress fiber structure when cultured under conditions of high serum, an unexpected difference was seen under conditions of low-serum growth. Under conditions of high-serum growth, actin immunostaining of REF-RcD2.1 cells revealed a reorganization of their actin cable structure similar to that previously documented in cells expressing an oncogenic Ras protein (Figure 3) (Bar-Sagi and Feramisco, 1986; Prendergast and Gibbs, 1993). Instead of displaying actin stress fibers such as those present in wild-type REF-cells, REF-RcD2.1 cells show a collapsed actin structure (Figure 3). However, when REF-RcD2.1 cells were incubated for 5 days under low-serum conditions, analysis of their actin structure by anti-actin antibody immunofluorescent cell staining revealed that the oncogenic actin rearrangement under high-serum conditions was reversible, and that the actin stress fibers had reappeared. The cells lost their spindle-shaped appearance and assumed a flattened morphology under low-serum conditions. Most notably, the actin staining revealed an increase of up to 30-fold in the cell adhesion area of REF-RcD2.1 cells under low-serum growth conditions compared with high-serum growth conditions (Figure 3), indicating a large increase in cell mass. These changes indicated that the properties of the cells were highly dependent on the level of serum in medium used for their culture. Immunofluorescent cell staining using a monoclonal rat antibody specific for cyclin D2 suggested a perinuclear localization of the cyclin D2 protein in REF and REF-RcD2.1 cells under high- and low-serum conditions, extending in some cells to within the nucleus of REF-RcD2.1 cells in high serum (Figure 3). A perinuclear localization was previously reported for cyclin D1 in a rat embryo fibroblast cell line Rat6 (Jiang *et al.*, 1993). The PCNA protein is a subunit of DNA polymerase delta (Wang, 1991). PCNA was shown to associate with D-type cyclins (Xiong *et al.*,

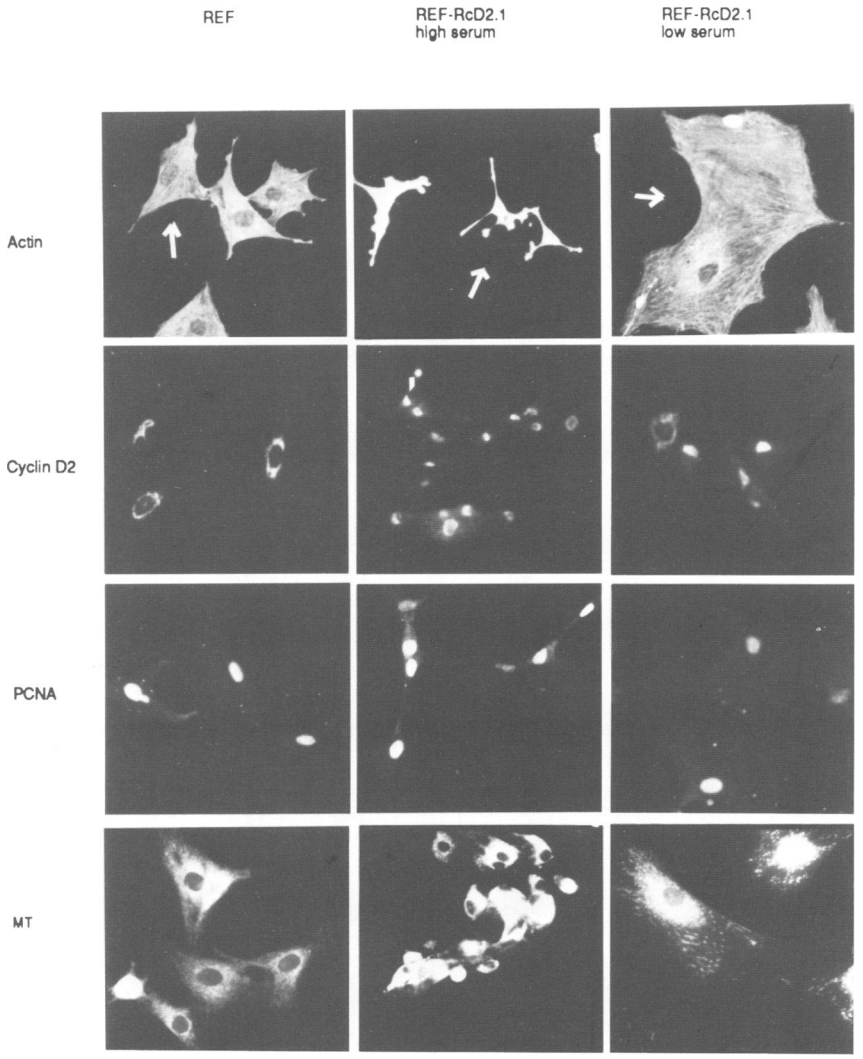

Fig. 3. Immunofluorescence staining of REF and REF-RcD2.1 cells under high- (10%) and low-serum (1%) conditions. REF and REF-RcD2.1 under high-serum (10%) conditions and REF-RcD2.1 cells incubated for 5 days in medium containing 1% serum were fixed with methanol. The fixed cells were incubated with primary antibodies against actin (clone 4, Boehringer), cyclin D2 (34B1-3, Oncogene Science) and PCNA (PC-10, Oncogene Science). Protein–antibody complexes were fluorescently labeled with secondary FITC-labeled anti-IgG antibodies. REF and REF-RcD2.1 cells under high- (10%) and low-serum (1%) conditions (5 days) in cell culture were stained for their mitochondria organelles (MT) with Rhodamine 123. The cells were analyzed with the help of a fluorescence microscope (40× magnification). The arrow-labeled cells stained for actin were analyzed for their cell adhesion areas by measuring the weight of paper they cover in the photograph. The relative adhesion areas are REF/REF-RcD2.1 (high serum)/REF-RcD2.1 (low serum) = 1/0.3/8.9.

1992). PCNA levels normally decline in quiescent, growth-arrested cells (Vriz *et al.*, 1992). Immunofluorescent cell staining disclosed a nuclear localization of the PCNA protein in proliferating REF and REF-RcD2.1 cells under both high- and low-serum conditions (Figure 3).

REF-RcD2.1 cells in low serum undergo a novel arrest in early S phase

Because comparison of the REF-RcD2.1 cells in high and low serum revealed profound morphological changes and persistent expression of PCNA upon serum withdrawal, we further characterized the low-serum state of REF-RcD2.1 cells by flow cytometry. We found that in low serum (1%), although the cells are unable to proliferate, they exhibit a DNA content of early S phase cells. Thus, after 6 days of serum starvation, the peak fraction of the cells exhibited a 2.4N DNA content (Figure 4A). In addition to chromosomal DNA, mammalian cells also contain small amounts of mitochondrial DNA. The observed increase of 20% of total cellular DNA (addition of ~1.2×10^9 nucleotides) would correspond to an addition of ~70 000 new mitochondrial DNA genomes (16 298 nucleotides/mitochondrial genome; Gadaleta *et al.*, 1989). We could not detect any abnormal changes in the distribution of mitochondria organelles in REF-RcD2.1 cells under high- and low-serum growth conditions (Figure 3). We were further unable to detect any DNA outside the nucleus of proliferating and serum-starved REF-RcD2.1 cells by fluorescence microscopy. Therefore, we exclude the possibility that the observed increase in cellular DNA is due to an increase of mitochondrial DNA. In agreement with the presence of a protein necessary for chromosomal DNA replication (PCNA) in the nucleus of serum-starved REF-RcD2.1 cells, it is therefore most likely that the observed increase of DNA is due to an increase in chromosomal DNA. By analyzing the kinetics of appear-

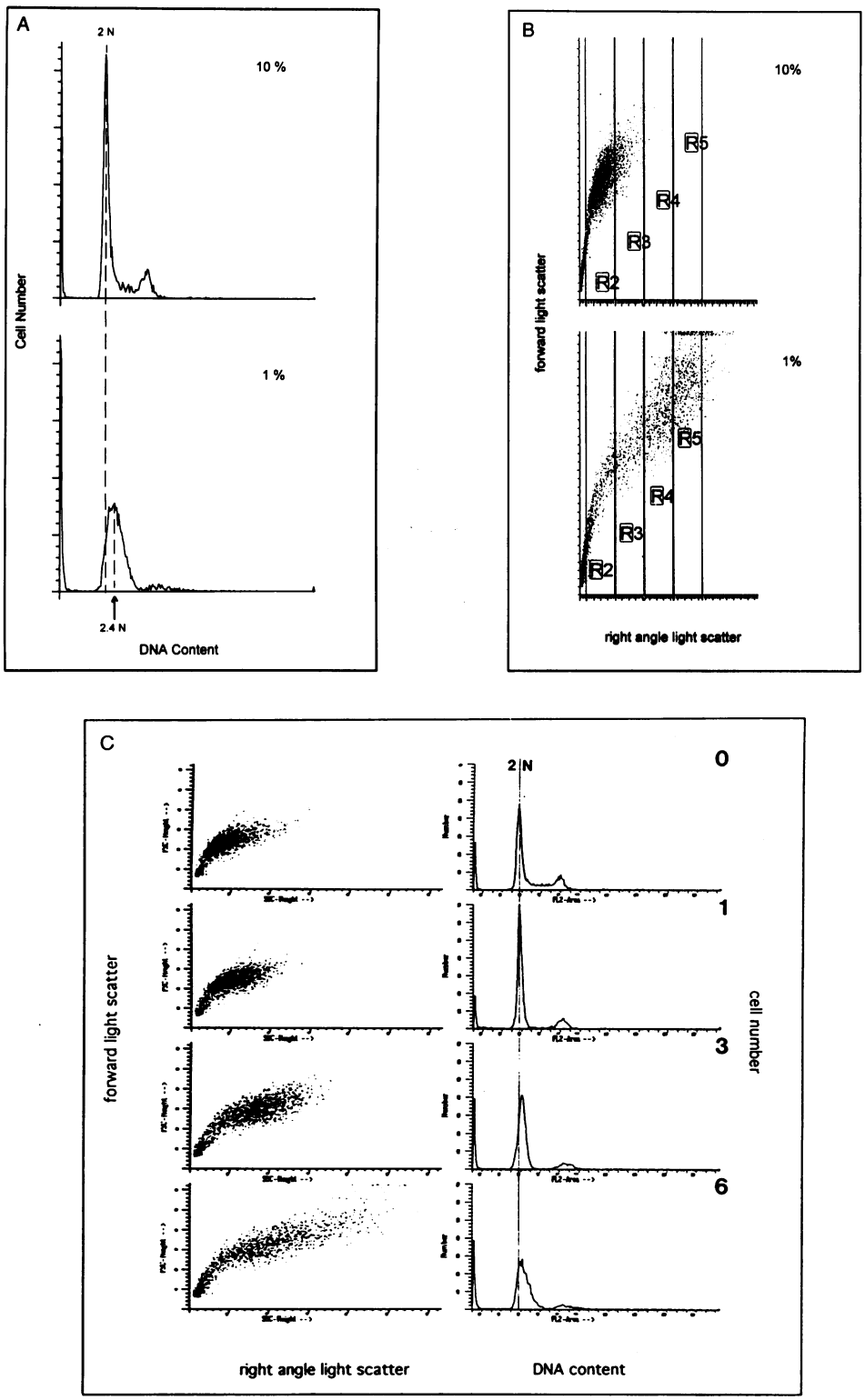

Fig. 4. Cell cycle analysis and light-scattering behavior of REF-RcD2.1 cells under high- (10%) and low-serum conditions (1%). (**A**) REF-RcD2.1 cells incubated in medium containing 10% serum and REF-RcD2.1 cells incubated for 6 days in the presence of medium containing 1% serum were fixed with ethanol, stained with propidium iodide and analyzed for their DNA content by flow cytometry. A plot of the cell number against the DNA content is shown. (**B**) A plot of the forward light scatter values against the right angle light scatter values of the cells described in (A) is shown. The data were obtained by flow cytometry analyses. The percentages of cells in the outlined regions of different light-scattering behavior are: high serum (10%): R2 = 73%, R3 = 20%, R4 = 5%, R5 = 1.4%; low serum (1%): R2 = 23.9%, R3 = 18.9%, R4 = 29%, R5 = 28%. (**C**) REF-RcD2.1 cells incubated in medium containing 10% serum (0) and REF-RcD2.1 cells incubated for 1 (1), 3 (3) and 6 (6) days in the presence of medium containing 1% serum were fixed with ethanol, stained with propidium iodide, and analyzed for their light-scattering behavior and DNA content by flow cytometry. A plot of the forward light scatter values against the right angle light scatter values and a plot of the cell number against the DNA content is shown.

ance of this novel phenotype, we found that the cells initially accumulate in G_1 phase after 1 day of culture in low serum and then shift to a DNA content of early phase (>2N) after 3–6 days of serum deprivation (Figure 4C). Light-scattering properties of cells are dependent on cell size. In a randomly proliferating cell population, the larger cells in G_2/M phase have increased forward and right angle light scatter values compared with their smaller counterparts in G_1 phase. Light-scattering analyses of REF-RcD2.1 cells revealed that the cells also keep growing in size, consistent with the observation by microscopy presented in Figure 3, resulting in giant cells with highly increased forward and right angle light scatter values (Figure 4B and C). Quantification of the light-scattering properties of REF-RcD2.1 under high- and low-serum conditions revealed that >50% of the cells cultured for 6 days under low-serum conditions have higher forward and right angle light scatter values than the largest proliferating cells under high-serum conditions (Figure 4B). This result indicates that after 6 days of serum deprivation most of the starved cells have at least doubled their cell mass. The large increase in the light-scattering properties of these cells further supports estimations, obtained by microscopy of trypsinized cells, of a cell mass increase of up to 10-fold. These data indicate that in low serum, the REF-RcD2.1 cells exhibited a partial capacity to traverse the G_1–S boundary. Although the population could enter S phase and augment their DNA content, they could not complete S phase and undergo mitosis; hence, the absence of cells with 4N content and the failure of cell number to increase. This capacity to enter, but not complete, S phase was accompanied by an abnormal and unregulated increase in cell mass.

To establish the molecular basis of the observed phenotype, we first analyzed a cell function which is characteristic of the progression from G_1 phase through the cell cycle: the phosphorylation of the retinoblastoma protein Rb (Buchkovich *et al.*, 1989; Goodrich *et al.*, 1991; Templeton *et al.*, 1991). Rb becomes phosphorylated when cells progress from G_1 to S phase, suggesting that this phosphorylation is required for cells to progress through the cell cycle (Mittnacht *et al.*, 1991; Templeton *et al.*, 1991). After mitosis, the Rb protein becomes dephosphorylated. To analyze phosphorylation of the Rb protein, REF-RcD2.1 cells were cultured under different serum conditions and then incubated for 2 h in the presence of [^{32}P]orthophosphate in phosphate-free medium. Cell extracts were prepared and immunoprecipitated using a monoclonal mouse antibody recognizing phosphorylated and unphosphorylated forms of the Rb protein (XZ133). Similar extents of phosphorylation of the Rb protein were detected in wild-type REF cells and REF-RcD2.1 cells cultured in high serum (Figure 5). However, no phosphorylation of Rb could be detected in REF-RcD2.1 cells incubated for 1 or 3 days under low-serum conditions (Figure 5). Constitutive expression of the Rb protein under high- and low-serum conditions was confirmed by immunoblotting (Figure 6A). This indicated that REF-RcD2.1 cells retained the ability to regulate the phosphorylation of Rb in response to changes in serum and that incorrect deregulation of Rb phosphorylation was not the basis for the gradual movement of the cells in S phase or the abnormal increase in cell size seen in low serum.

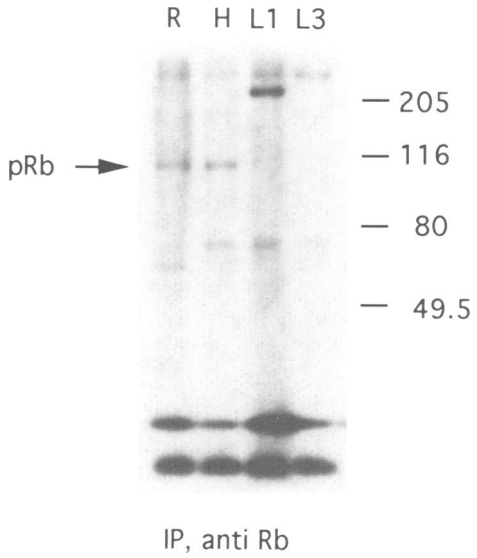

Fig. 5. Phosphorylation of the retinoblastoma protein Rb in REF cells and REF-RcD2.1 cells under high- and low-serum conditions. Subconfluent REF cells (R) and REF-RcD2.1 cells under high-serum conditions (10%) (H) and REF-RcD2.1 cells incubated in medium containing 1% serum for 1 (L1) and 3 (L3) days were incubated in the presence of [^{32}P]orthophosphate for 2 h. The cells were lysed and the Rb proteins were immunoprecipitated using a monoclonal antibody against the Rb protein (XZ133). The precipitated proteins were separated on a 7.5% SDS–polyacrylamide gel. Bands were visualized by autoradiography. For size determination, a pre-stained SDS–PAGE standard (Bio-Rad, high range) containing proteins of an apparent mol. wt of 205, 116, 80 and 49.5 kDa was used.

We next examined the expression of different cell cycle-related proteins in REF-RcD2.1 cells under high- and low-serum conditions by immunoblotting. As expected, we found that the level of the exogenously expressed cyclin D2 protein was not altered under the different serum conditions (Figure 6A). By comparing the cyclin D2 levels in REF and REF-RcD2.1 cells, we confirmed our previous results that cyclin D2 is much more highly expressed in the transformed cells than in the wild-type fibroblasts (Figure 6A). We further analyzed the synthesis of cyclin D2 protein by metabollic labeling of proliferating and serum-starved REF-RcD2.1 cells with [^{35}S]methionine and immunoprecipitation of the cyclin D2 proteins. In REF-RcD2.1 cells cultured for 3 days under low-serum conditions, we found a marked reduction of the rate of cyclin D2 protein synthesis compared with the rate in proliferating cells (Figure 6B). The total amount of cyclin D2 protein measured by Western blot analyses did not show any significant changes (Figure 6B). This result indicates that serum starvation causes a reduction of the rate of protein synthesis as well as of the rate of protein degradation, and suggests a general downregulation of metabolic processes in REF-RcD2.1 cells cultured under low-serum growth conditions.

To further examine cell cycle regulatory protein expression in REF-RcD2.1 cells, we measured the levels of other cyclin proteins in high serum, conditions which support proliferation and the low-serum, growth-arrested state. Surprisingly, the expression of the G_1-related cyclin, cyclin E (Koff *et al.*, 1992; Sherr, 1993), did not change between the different serum conditions (Figure 6A). Cyclin E levels

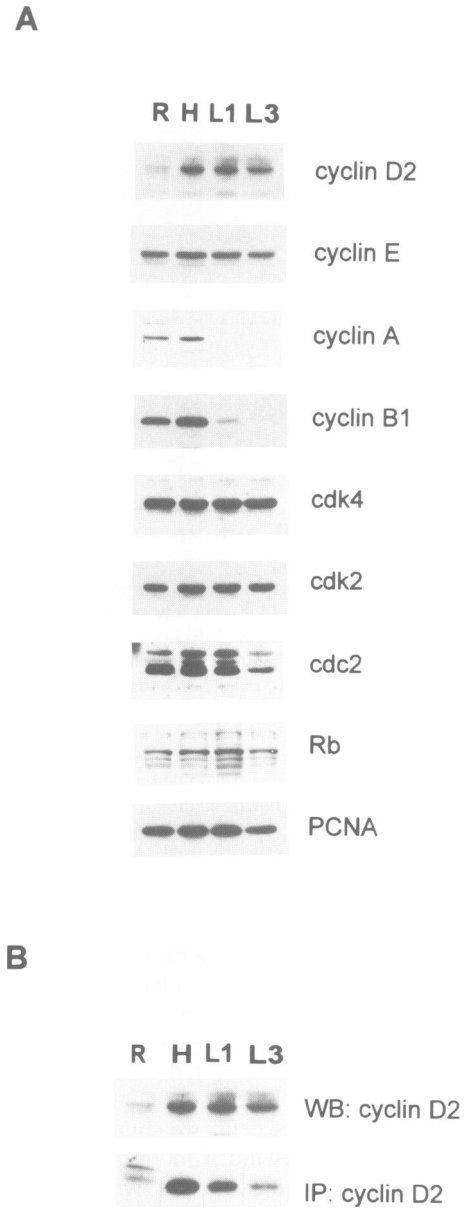

Fig. 6. Protein expression in REF and REF-RcD2.1 cells under high- and low-serum conditions. (**A**) Total protein extracts from subconfluent REF cells (R) and REF-RcD2.1 cells under high-serum conditions (10%) (H) and REF-RcD2.1 cells incubated in medium containing 1% serum for 1 (L1) and 3 days (L3) were separated on a 10% SDS–polyacrylamide gel and the expression of the indicated proteins was analyzed by immunoblotting. (**B**) Subconfluent REF cells (R) and REF-RcD2.1 cells under high-serum conditions (10%) (H) and REF-RcD2.1 cells incubated in medium containing 1% serum for 1 (L1) and 3 days (L3) were metabolically labeled with [^{35}S]methionine. The cells were lysed and the proteins from immunoprecipitations using a polyclonal rabbit serum raised against bacterially expressed recombinant cyclin D2 protein (α-D2) were separated on a 12% SDS–polyacrylamide gel. The 35k fraction of the autoradiogram is shown (IP cyclin D2). The radioimmunoassay is compared with the immunoblot for cyclin D2 described in (A) (WB cyclin D2).

normally decline as cells withdraw from the cell cycle, but cyclin E persisted at high levels in the REF-RcD2.1 cells under both low- and high-serum conditions. The expression of the S and G_2/M phase-related cyclin A and cyclin B1 proteins (Minshull *et al.*, 1990; Pines and Hunter, 1990), however, decreased very rapidly after serum deprivation (Figure 6A). Virtually no protein could be detected after 1 day of serum withdrawal and therefore, unlike cyclin E, these cyclins were still subject to regulation by serum.

The cyclins serve as regulatory subunits of the cdk family of protein kinases. D-type cyclins were shown to interact with the cyclin-dependent kinases cdk4, cdk2 and cdk5 (Matsushime *et al.*, 1992; Xiong *et al.*, 1992). Cyclin E was shown to interact with cdk2 and cdc2 (Koff *et al.*, 1991). No changes were observed in the expression of the cyclin-dependent kinases cdk4 and cdk2 under high- and low-serum conditions (Figure 6A). In contrast to the kinases cdk4 and cdk2, the level of the cdc2 kinase, which becomes active in late S phase (Norbury and Nurse, 1992), was distinctly decreased within 3 days of culture under low-serum conditions (Figure 6A). The immunoblot data revealed no changes in the expression of the PCNA protein (Figure 6A), in agreement with the result of immunostaining in Figure 3.

The expression of cyclin A has been shown to be essential for S phase initiation and progression through the cell cycle. The G_1 cell cycle arrest of SAOS-2 osteosarcoma cells by the introduction of the RB gene could be rescued by the constitutive overexpression of cyclin A (Hinds *et al.*, 1992). We therefore asked whether constitutive overexpression of the cyclin A protein can induce cell proliferation in REF-RcD2.1 cells under low-serum conditions. As a control, we have also asked whether the expression in REF-RcD2.1 of the E1a and c-Myc oncoproteins, which were shown to co-operate with oncogenic activated Ha-Ras in the transformation of REF cells (Land *et al.*, 1983; Ruley, 1983), results in cell lines able to proliferate under low-serum conditions. Subconfluent proliferating REF-RcD2.1 cells were transfected with eukaryotic expression vectors expressing the *cyclin* A (Rc-CMV-cycA) or E1a (pSVE1a) or c-*myc* (LTR-Hmyc) gene. Two days later, the cells were incubated in the presence of low-serum (1%) growth medium for 10 days. The transfected cells were analyzed for proliferation under low-serum conditions. Only the transfection of REF-RcD2.1 cells with the E1a gene resulted in foci of cells capable of proliferating under low-serum conditions (Figure 7). No such foci could be detected in the transfection assays with the *cyclin* A gene (Figure 7). The overexpression of the c-*myc* gene also did not induce cell proliferation under low-serum conditions (Figure 7).

Discussion

Co-operation of cyclin D2 with activated Ras induces a novel serum-dependent transformed phenotype

Our results show that overexpression of cyclin D2 in co-operation with expression of activated Ha-Ras transforms primary REF. Cyclin D2 can therefore be regarded as a proto-oncogene. This conclusion is also supported by previous results reporting elevated expression of cyclin D2 in rodent T-cell leukemia (Tremblay *et al.*, 1992; Hanna *et al.*, 1993).

The cyclin D2-overexpressing line shows a novel partially transformed phenotype. Under high-serum conditions, these cells are transformed in the sense that they

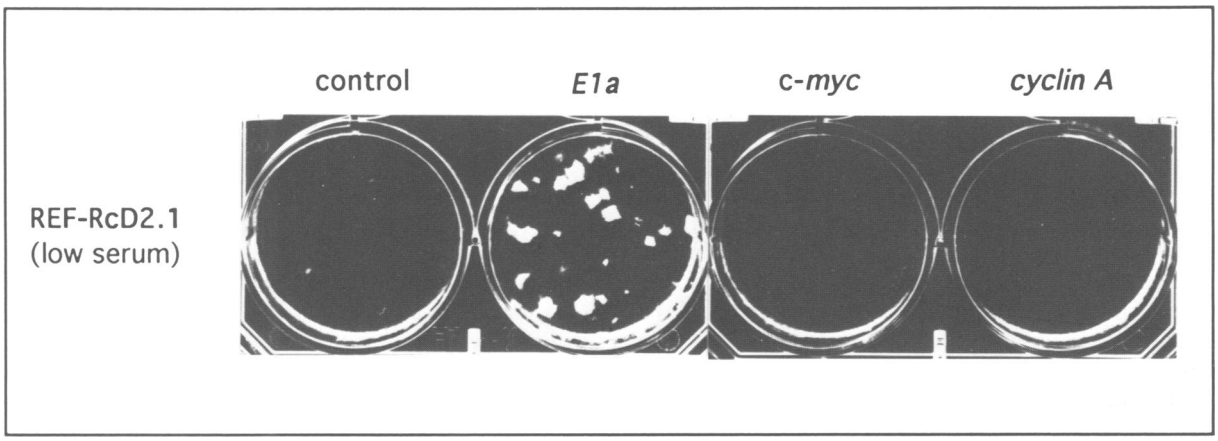

Fig. 7. Proliferation of REF-RcD2.1 cells under low-serum conditions is specifically mediated by E1a. Subconfluent proliferating REF-RcD2.1 cells were transfected by a high-efficiency transfection method with eukaryotic expression vectors expressing the E1a (pSVE1a) or c-*myc* (LTR-Hmyc) or *cyclin* A (Rc-CMV-cycA) gene. As a control, the cells were transfected with plasmid DNA (pSV-K3, Pharmacia). Two days after the transfection, the cell were incubated in the presence of low-serum (1%) growth medium for 10 days. The cells were fixed with methanol. Foci of cells able to proliferate under low-serum conditions appear as white spots.

are immortalized, morphologically different from wild-type REF, show the typical oncogenic actin rearrangement of Ras transformed cells and their growth is no longer contact inhibited. However, the transformed phenotype is reversible by serum deprivation. Under low-serum conditions, REF-RcD2.1 cells are not able to proliferate, but keep growing in size and accumulate a DNA content of early S phase cells. The partial entry of the cells into S phase is in agreement with our finding that the cells express PCNA in the low-serum growth conditions. PCNA is a subunit of DNA polymerase delta (see Wang, 1991) and its expression is an indicator of the presence of the DNA replication machinery in REF-RcD2.1 cells. Expression of components of the replication apparatus is consistent with the ability of the cells to attain a 2.4N DNA content in low serum over a period of several days. The data, however, also reveal that overexpression of cyclin D2 in the presence of an activated Ha-Ras is not sufficient to drive cells through the cell cycle. These data are consistent with a model that cyclin D2 plus Ha-Ras expression induces an incomplete set of factors required for cell proliferation. Other factors stimulated or controlled by serum are required to induce cell proliferation.

Studies in yeast and higher eukaryotic cells have shown that very similar mechanisms control the progression through the cell cycle. In the yeast system, cell size is a critical parameter in the mechanisms which control cellular proliferation (Nasmyth, 1993). In the presence of nutrients, G_1 cells enter the S phase only after a period of growth. A minimum cell size is required for the cells to undergo START, a point in the late phase of G_1 where the cells become committed to completing the mitotic cell cycle. Three different G_1-specific cyclins are expressed in budding yeast, CLN1–3, and there is good evidence that they play a key role in triggering START (Nasmyth, 1993). Nothing is known of how the activity of the G_1 cyclins and cell size are correlated.

In mammalian cells, five different cyclins have been discovered which are expressed and active in G_1: cyclin C, D1, D2, D3 and E (Sherr, 1993). It is not yet known whether the cell cycle of mammalian cells is controlled by cell size and whether they have a point similar to START in yeast. The expression patterns of CLN1 and 2 are reminiscent of mammalian cyclin E, whereas that of CLN3 is similar to mammalian D-type cyclins (Hatakeyama *et al.*, 1994). The functions of these G_1-specific cyclins appear to be very similar. The overexpression of the yeast CLN cyclins and mammalian cyclins D1, D2 and E results in a shortening of the G_1 phase and also leads, with the exception of cyclin D2, to a reduced cell size (Nasmyth, 1993; Ohtsubo and Roberts, 1993; Quelle *et al.*, 1993; Tyers *et al.*, 1993). In yeast, the reduced cell size can be explained by a premature triggering of START.

A triple CLN mutant budding yeast cell shows a phenotype very similar to that observed in REF-RcD2.1 cells under low-serum conditions since these cells continue to grow in the absence of cell division (Richardson *et al.*, 1989). This suggests that there is no need for G_1 cyclin activity for positive cell growth regulation in yeast. Cell growth and proliferation in yeast are mainly determined by nutrients. The regulation of growth and proliferation of mammalian cells, however, is much more complicated since it requires cell type-specific growth factor signals in addition to nutrients. The Ras protein is activated in the signal cascade of several of such growth factors and may therefore play an important role in the growth and cell size regulation of mammalian cells. However, a deregulation of cell size has never been observed in cells expressing activated Ras alone, suggesting a co-operation of activated Ras and other proteins in the observed phenotype, and a possible role of cyclin D2 in positive cell growth regulation. A possible involvement of D-type cyclins in positive cell growth regulation also results from differentiation studies conducted in PC12 cells. NGF-treated PC12 cells differentiate into a neuron-like phenotype by slowly ceasing the cell cycling and developing neurites. During their differentiation program, these cells accumulate high amounts of cyclin D1 (an up to 30-fold amplification) in their nuclei (G.-Z.Yan and E.B.Ziff, submitted). The accumulation of cyclin D1 protein in these cells therefore suggests a possible role of the cyclin in differentiation-specific cell growth regulation.

Although under low-serum conditions REF-RcD2.1

cells continue to grow in size, they undergo an abnormal proliferative arrest in early S phase. Analysis of functions important for cell cycling revealed that the cells continue to express specific G_1 cyclins, including the exogenously introduced cyclin D2 and the endogenous cyclin E. The cells also continue to express the cyclin-dependent kinases cdk4 and cdk2 which interact with cyclin D2 and cyclin E. The overexpression of cyclin D2 or E in fibroblasts was shown to cause a shortening of the G_1 phase (Ohtsubo and Roberts, 1993; Quelle *et al.*, 1993), suggesting a crucial role for these two cyclins in the regulation of the progression from G_1 to S phase. It is uncertain which protein substrates are targeted by the cyclin D2- or E-mediated kinase activities. Both cyclins were shown to mediate the phosphorylation of the retinoblastoma protein Rb in an *in vitro* kinase assay (Koff *et al.*, 1992; Ewen *et al.*, 1993; Harper *et al.*, 1993; Matsushime *et al.*, 1994). However, in REF-RcD2.1 cells under low-serum conditions, the Rb protein was found to be in the unphosphorylated state, indicating that Rb phosphorylation activity is downregulated in these cells despite the presence of cyclin D2 and E, and cdk 2 and cdk4. This observation suggests either (i) that cyclin D2 and cyclin E are unable to form active kinase complexes in the REF-RcD2.1 cells under conditions of low-serum growth, or (ii) that the Rb protein is not a phosphorylation target for kinase activity mediated by these cyclins.

The fact that REF-RcD2.1 cells are able to enter early S phase gradually under low-serum conditions favors the model that the cyclins D2 and E found under these conditions are at least partially active. However, it remains possible that these kinases are present in an inactive form. Recently, two proteins of size 16 kDa (p16) and 21 kDa (p21) were cloned (Gu *et al.*, 1993; Gyuris *et al.*, 1993; Harper *et al.*, 1993; Serrano *et al.*, 1993; Xiong *et al.*, 1993b) which were shown to be regulatory subunits of cdk/cyclin complexes. Furthermore, it was shown that p21 and p16 were lost from cdk/cyclin complexes in multiple transformed cell lines (Xiong *et al.*, 1993a; Kamb *et al.*, 1994; Nobori *et al.*, 1994). A potential function of p16 as a tumor suppressor protein has been discussed. In preliminary studies, we have not yet resolved the status of p21 or p16 in REF and REF-RcD2.1 cells. Further investigations are necessary to analyze the roles of p16 and p21, as well as cyclin D2 and E and cdk2 and 4, in establishing the observed early S phase arrest phenotype.

Rodent fibroblasts were found to have a strong selection against the establishment of cell lines which overexpress D-type cyclins (Quelle *et al.*, 1993). By the use of microinjection, it was shown that overexpression of cyclin D1 in G_1 phase causes a cell cycle arrest in late G_1. The cell cycle block could be overcome by co-expression of PCNA (Pagano *et al.*, 1994). Since it was shown that cyclin D1 and PCNA are able to form complexes (Xiong *et al.*, 1992), the release by PCNA leads to the model that overexpression of cyclin D1 causes a cell cycle arrest in late G_1 by sequestering the PCNA protein from the replication machinery (Pagano *et al.*, 1994). It is not known whether cyclin D2 is also able to form complexes with the PCNA protein. We note that our immunofluorescence data indicate different cellular localizations of the PCNA and cyclin D2 proteins in REF and REF-RcD2.1 cells. In the latter cells, although the patterns are distinct, a portion of cyclin D2 may reside with PCNA in the nucleus. Experiments analyzing the possible interaction of the cyclin D2 and PCNA proteins, and its loss during cell transformation by cyclin D2, will be necessary to test the model for REF-RcD2.1 cells.

In normal cells, cyclin A periodically accumulates during the cell cycle in S phase and G_2 phase (Minshull *et al.*, 1990; Pines and Hunter, 1990). The presence of cyclin A has been shown to be essential for S phase initiation and progression, and for entry into mitosis (Girad *et al.*, 1991; Pagano *et al.*, 1992; Baldin *et al.*, 1993). Microinjection of cyclin A-specific antibodies into human fibroblasts during G_1 or S phase was shown to prevent DNA replication (Baldin *et al.*, 1993). In REF-RcD2.1 cells, cyclin A expression is serum dependent. The protein is undetectable after 1 day of serum deprivation. The lack of the cyclin A protein under low-serum conditions may explain the observed proliferative arrest in early S phase.

Transfection experiments employing overexpression of cyclin A in REF-RcD2.1 cells under low-serum conditions, however, have shown that the presence of cyclin A protein alone is not sufficient to induce cell proliferation under these conditions (Figure 7). The Myc and E1a oncoproteins can induce cell proliferation of immortalized fibroblasts (NIH3T3 cells) under low-serum conditions (Kelekar and Cole, 1987). In REF-RcD2.1, however, only the E1a protein, but not the Myc protein, was able to rescue the cells from the cell cycle block imposed by serum deprivation. This suggests that a specific transformation state of the cell is necessary for the induction of proliferation under low-serum conditions. This is also supported by the observation that Myc/Ras transformed REF cells are dependent on serum for their growth and survival (G.Evan, personal communication). In the presence of Ras, neither Myc nor cyclin D2 can overcome the requirement for serum and neither provides the complementation afforded by E1a. When activated Ras is introduced with a temperature-sensitive T antigen into Schwann cells (Ridley *et al.*, 1988) or established REF (Hirakawa and Ruley, 1988), the cells are transformed at the permissive temperature, but arrest growth at the non-permissive temperature, indicating that Ras alone inhibits growth. This is consistent with the inability of activated Ras alone to transform (Land *et al.*, 1983; Ruley, 1983) and with the inhibition of Schwann cell (Ridley *et al.*, 1988) and PC12 cell (Bar-Sagi and Feramisco, 1985) proliferation by microinjected Ras protein.

It is still unclear which functions are provided by cyclin D2 and Ha-Ras for the regulation of the cell cycle. Here we show that they can co-operate in the transformation of cells. Constitutive expression of cyclin D2 and oncogenic activated Ras in REF-RcD2.1 cells leads to deregulated cell size control and initiation of DNA replication under low-serum conditions. The expression of activated Ras may be essential for the survival of REF-RcD2.1 cells. Long-term 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA) treatment induces massive cell death of REF-RcD2.1 cells under high- and low-serum conditions (E.Kerkhoff, unpublished). Long-term TPA treatment inactivates protein kinase C (Rodriguez and Rozengurt, 1984; Ballester and Rosen, 1985), a kinase which was shown to be an important activator in the Ras/Raf signal transduction cascade (Kolch *et al.*, 1993). Cell survival mediated by activated Ras

in our cells is also supported by the results of other investigators who find a strong selection against the overexpression of D-type cyclins in immortalized cell lines (Quelle *et al.*, 1993). Our data show that in co-operation with activated Ras, it is very easy to establish cell lines overexpressing cyclin D2. The role of an activated Ras/Raf signal transduction cascade in cell survival is also implicated for cells expressing the Myc oncoprotein (Troppmair *et al.*, 1992). The specific roles of cyclin D2 and high serum in our transformants remain to be established. In one model, cyclin D2 may stimulate or may be required for G_1 phase functions leading to S phase, a role consistent with CLN function in yeast. However, an ultimate shut-off of D cyclin function may also be a pre-requisite for actual entry into S phase. This would be consistent with the apparent growth- and DNA synthesis-inhibitory effects of cyclin D1 (Quelle *et al.*, 1993; Pagano *et al.*, 1994). If this were the case, our data suggest that high serum could override the D cyclin block and allow the cells to move from a G_1 phase active state into S phase.

Further studies of REF-RcD2.1 cells under high and low serum may reveal important information about the role of Ha-Ras and cyclin D2 in the control of G_1 specific functions, and the regulation of the full set of functions necessary for the G_1/S transition.

Material and methods

Cell culture

REF and REF-RcD2 cells were grown in Dulbecco's modified Eagle medium (Gibco) supplemented with 10% fetal calf serum (Hyclone), 100 U/ml penicillin and 100 μg/ml streptomycin. The cells were incubated at 37°C, 5% CO_2 and 90% humidity. REF-RcD2.1 cells were passaged on collagen-coated tissue culture plates.

Transformation assay and transfections

Second-passage REF (Whittaker Bioproducts) were transfected by a high-efficiency liposome transfection method. One microgram of plasmid DNA in 0.1 ml OPTI-MEM I medium (Gibco) was mixed with 6 μl of Lipofectamine Reagent (Gibco) dissolved in 0.1 ml OPTI-MEM medium. For complex formation of the liposomes and the nucleic acids, the solution was incubated for 30 min at room temperature. A 0.8 ml volume of OPTI-MEM I medium was added and the solution was applied to ~8×10^4 second-passage REF in a well of a flat-bottom 6 well tissue culture plate (Falcon). The fibroblasts were previously washed twice with OPTI-MEM I medium. The cells were incubated for 5 h in an incubator at 37°C, 90% humidity and 5% CO_2 in the presence of the lipid–nucleic acid complexes. All medium was then removed and substituted by normal growth medium. The following eukaryotic expression vectors were used for the transfection: pT22 (*ras*-val12) (Land *et al.*, 1983); pSG5-cycD2 (*cyclin* D2) (Ewen *et al.*, 1993); LTR-Hmyc (c-*myc*) (Kelekar and Cole, 1987); pSV-K3 (carrier DNA) (Pharmacia). The pSV-K3 vector was used to bring the total DNA in each transfection experiment to 1 μg. Subconfluent REF-RcD2.1 cells were transfected by the same method as described above. The cells were transfected with 1 μg of the following eukaryotic expression vectors: pSVE1a (E1a) (Velcich and Ziff, 1985); Rc-CMV-cycA (*cyclin* A) (Hinds *et al.*, 1992); LTR-Hmyc (*c-myc*) (Kelekar and Cole, 1987); pSV-K3 (control DNA) (Pharmacia).

Northern blot analysis

Total RNA was isolated by the guanidinium–thiocyanate–phenol–choloroform single-step extraction method (Chomcynski and Sacchi, 1987). Fifteen micrograms of total RNA per lane were separated on a formaldehyde–agarose gel and transferred to a Duralon-UV membrane (Stratagene) by capillary elution. c-DNA fragments were purified by gel elution and labeled with [α-^{32}P]dCTP by random-primed DNA labeling (Feinberg and Vogelstein, 1984). The following DNA fragment was used: *cyclin* D2, 1.2 kb *Eco*RI fragment of pBluescript-cyl2 (Matsushime *et al.*, 1991). For hybridization, the labeled DNA probe was heat denatured and transferred to the Duralon membrane, which was pre-hybridized for 30 min at 63.5°C in 15 ml Quikhyb solution (Stratagene). The hybridization was performed for 4 h at 63.5°C. The membrane was washed for 30 min at room temperature using a solution containing 300 mM sodium chloride, 30 mM sodium citrate and 0.1% SDS. A second washing step was performed for 30 min at 59.5°C using a solution containing 15 mM sodium chloride, 1.5 mM sodium citrate and 0.1% SDS. The blots were autoradiographed.

Radioimmunoprecipitation assay

For metabollic labeling of REF or REF-RcD2 cells, the cells of an 80% confluent 10 cm tissue culture dish were washed once with Dulbecco's modified Eagle medium without L-methionine (Gibco) or phosphate (Gibco) supplemented with 10% dialyzed fetal calf serum (Hyclone). Two milliliters of the above medium containing 0.5 mCi [^{35}S]methionine or 1 mCi [^{32}P]orthophosphate were added to the cells, and the cells were incubated for 2 h at 5% CO_2, 37°C and 90% humidity. The medium was removed and the cells were washed once with phosphate-buffered saline (PBS) buffer. One milliliter of lysis buffer [250 mM NaCl, 50 mM Tris–HCl (pH 7.4), 0.1% NP40, 2 μg/ml leupeptin, 2 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml pepstatin, 10 mM NaF, 0.2 mM sodium orthovanadate] was added and the cells were incubated for 20 min at 4°C for lysis. The lysate was spun for 20 min at 4°C and 14 000 r.p.m. in the lab centrifuge. The protein concentration of the supernatant was determined by a Bio-Rad detergent compatible protein assay. The protein concentration was adjusted to 0.4 mg/ml by dilution with lysis buffer. One hundred microliters of 10% protein A–Sepharose (in lysis buffer) and 3 μl of 10× diluted normal rabbit serum were added to 0.9 ml of the cell extract and the suspension was rocked for 1 h at 4°C. After removal of the protein A–Sepharose by centrifugation, 7 μl of a polyclonal rabbit serum against recombinant cyclin D2 (α-D2; Ewen *et al.*, 1993) or 100 μl tissue culture supernatant of hybridoma cells expressing a mouse monoclonal antibody against the Rb protein (XZ133; Hu *et al.*, 1991) were added and the solution was incubated for 1 h on ice. When using a mouse monoclonal antibody, 3 μg of rabbit anti-mouse IgG1 (Pharmingen) were added and the solution was incubated for 20 min on ice. One hundred microliters of 10% protein A–Sepharose solution were added and the suspension was rocked for 1 h at 4°C. Afterwards, the protein A–Sepharose was pelleted and washed four times with lysis buffer; 20 μl SDS protein sample buffer were added and the sample was boiled. The proteins were separated on a 12 or 7.5% SDS–polyacrylamide gel.

Flow cytometry analysis

Light-scattering and cell cycle distribution experiments were performed with a FACScan flow cytometer (Becton & Dickinson). A total of 5×10^5 cells were pelleted by centrifugation and resuspended in 0.5 ml PBS buffer supplemented with 1% (v/v) fetal calf serum. While vortexing, 5 ml of ice cold ethanol were added. The cell suspension was incubated overnight at 4°C. The fixed cells were pelleted by centrifugation and resuspended in 0.75 ml PBS buffer supplemented with 1% (v/v) fetal calf serum. RNase A was added to a final concentration of 0.1 mg/ml. After the addition of propidium iodide to a final concentration of 0.05 mg/ml, the suspension was incubated for 2 h at 37°C. The ethanol-fixed and propidium iodide-stained cells were analyzed by flow cytometry for light-scattering properties and DNA content.

Immunoblot

Cells were lysed in protein sample buffer [60 mM Tris–HCl (pH 6.8), 10% (w/v) glycerine, 3% (w/v) SDS, 5% (w/v) 2-mercaptoethanol, 0.005% (w/v) bromophenol blue]. A total of 100 μg of total protein extract were separated on an SDS–polyacrylamide gel. The proteins were transferred to a nitrocellulose membrane (Bio-Rad, 162-0114) using a tank blot procedure (Bio-Rad Mini-Protean II). A transfer buffer containing 25 mM Tris, 190 mM glycine and 20% (v/v) methanol was used. The blot was performed at 150 mA for 1 h. The membrane was blocked overnight at 4°C in PBS buffer supplemented with 5% (w/v) non-fat dried milk and 0.05% (v/v) Tween 20, and incubated on a platform shaker at room temperature for 3 h with appropriate dilutions of antibodies in blocking buffer. Afterwards, it was washed three times for 10 min with PBS buffer supplemented with 0.05% Tween 20 and incubated on a platform shaker at room temperature for 1 h in PBS buffer supplemented with 1.6% (w/v) non-fat dried milk and a 1:5000 dilution of anti-rabbit or anti-mouse or anti-rat Ig, horseradish peroxidase-linked F(ab′)$_2$ fragment (Amersham). The blot was washed three times for 10 min with PBS buffer supplemented with 0.05% (v/v) Tween 20

and the protein bands were visualized with the help of the ECL detection system (Amersham). For reprobing the membranes, the primary and secondary antibodies were removed by incubating the membranes for 30 min at 50°C in a 2% (w/v) SDS, 62.5 mM Tris−HCl (pH 6.8) and 100 mM 2-mercaptoethanol buffer. Afterwards, the filters were washed twice for 10 min with PBS buffer supplemented with 0.05% (v/v) Tween 20 and blocked overnight at 4°C in blocking buffer. The following primary antibodies were used: cyclin D2, 34B1-3, rat monoclonal, 2 μg/ml (Oncogene Science); cyclin E, α cyclin E, rabbit polyclonal, 1:3000 (J.Roberts); cyclin A, α cyclin A, rabbit polyclonal, 1:800 (T.Hunter); cyclin B1, 05-158 cyclin B1, mouse monoclonal, 1 μg/ml (UBI); cdk4, cdk4 C-22, rabbit polyclonal, affinity purified, 1.5 μg/ml (Santa Cruz Biot.); cdk2, 06-148 cdk2, rabbit polyclonal, affinity purified, 1.5 μg/ml (UBI); cdc 2, α cdc2 7845, rabbit polyclonal, 1:1000 (Ziff Lab); Rb, Rb (C-15), rabbit polyclonal, affinity purified, 1 μg/ml (Santa Cruz Biot.); PCNA, PC-10 PCNA, mouse monoclonal, 1 μg/ml (Oncogene Science).

Immunofluorescence cell staining

The cells were grown on glass cover slides. The medium was removed and the cells were washed twice with PBS. Cold methanol (−20°C) was added and the cells were fixed for 7 min at −20°C. After washing three times for 5 min on a platform shaker with PBS buffer at room temperature, the fixed cells were blocked by incubating them for 1 h in blocking buffer [PBS buffer supplemented with 1% (v/v) fetal calf serum and 0.1% (w/v) bovine serum albumin]. The primary antibody was diluted to the appropriate concentration in blocking buffer and the cells were incubated in this solution for 1 h. After washing the fixed cells three times for 10 min with PBS buffer, the cells were incubated for 1 h in the presence of 5 μg/ml goat anti-mouse IgG fluorescein isothiocyanate (FITC)-labeled polyclonal antibodies (Pharmingen) or 5 μg/ml rabbit anti-rat IgG FITC-labeled polyclonal antibodies (Pharmingen) in blocking buffer. The fixed and stained cells were than washed three times for 10 min with PBS buffer and mounted. The following antibodies were used: actin, anti-actin C4, mouse monoclonal, 10 μg/ml (Boehringer); cyclin D2, 34B1-3, rat monoclonal, 7.5 μg/ml (Oncogene Science); PCNA, PC-10 PCNA, mouse monoclonal, 6 μg/ml (Oncogene Science).

Localization of mitochondria

The mitochondria organelles in REF and REF-RcD2.1 cells were localized by Rhodamine 123 (Sigma) staining as described in Johnson *et al.* (1980).

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