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Cyclin D1 Triggers Autonomous Growth of Breast Cancer Cells by Governing Cell Cycle Exit

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Cyclin D1 controls G_1-associated processes, including G_0-to-G_1 and G_1-to-S transitions. This study demonstrates a novel aspect of cyclin D1 as a regulator of the transition between G_1 and G_0. Overexpression of cyclin D1 in MCF7 breast tumor cells resulted in a continued proliferation under low-serum conditions, whereas nonoverexpressing cells ceased to grow. This difference in growth was due to a reduced exit from G_1 to G_0 in cyclin D1-overexpressing cells. Our data therefore suggest a model in which cyclin D1 overexpression in tumor cells is responsible for hyperproliferation under growth factor-deprived conditions.

The proliferation of mammalian cells is primarily regulated by a decision that occurs at a unique point in the first gap phase (G_1) of the cell cycle: to remain in the cell cycle and proliferate or to leave the cell cycle to become quiescent. Mitogenic signals regulate cell division by triggering a cascade of events and ultimately induce the expression of G_1 cyclins, including the D-type cyclins and cyclin E (33). These cyclins are presumed to control progression through the cell cycle by governing the activity of cyclin-dependent kinases (CDKs). Passage through the unique control point at late G_1 defines the stage at which cells no longer require growth factors. Once a cell passes this checkpoint, also called the restriction point, it will complete the cycle, even in the absence of mitogens (23). Because the G_1 cyclins are critical to the decision to pass the restriction point, it is not surprising that a deregulated expression of G_1 cyclins and/or their CDK partners is often found in malignant cells. For instance, many aberrations in G_1-associated processes are observed in cancer cells, in which cyclin D1 abnormalities are the most prominent. Deregulation of cyclin D1 was found in many different tumor types, such as breast and squamous cell carcinomas (2, 15, 20, 30), parathyroid adenomas (21), and in esophageal carcinomas (11).

Several lines of evidence support the hypothesis that cyclin D1 is rate limiting for the transition of G_0 to S phase: (i) cyclin D1 expression levels peak in the G_1 phase and are associated with either CDK4 or CDK6, which are active during G_1 phase (1, 2, 3, 19, 32); (ii) microinjection of either antisense oligonucleotides or antibodies against cyclin D1 into various cell types arrests the cells in the G_1 phase (1, 2, 25); and (iii) in synchronized (M-phase or G_0-phase) cells, cyclin D1 overexpression causes an increase in the percentage of S-phase cells (12, 22, 25, 27). To further explore the role of cyclin D1 overexpression in G_1-associated processes, we investigated the role of cyclin D1 overexpression in cell cycle exit. For that purpose, we have generated clones from the human epithelial breast cancer cell line MCF7, which can be induced to overexpress cyclin D1. This cell line is normally composed of a high percentage of noncycling cells and is therefore very useful for this study. MCF7 cells do not contain an amplification of the cyclin D1 gene (although cyclin D1 amplification is quite common in this cell type), they are well differentiated, and their cyclin D1 levels are similar to those in normal mammary epithelial cells (2, 4, 17, 32). As in normal cells, cyclin D1 levels in MCF7 cells oscillate during the cell cycle, with peak levels in G_1, and the protein is nuclear (17).

As mentioned above, it was clearly demonstrated that cyclin D1 affects the duration of the several phases of the cell cycle. However, limited and nonuniform data are available concerning the overall effect of cyclin D1 overexpression on population doubling times (11, 25, 27). Because these data are based on normal cells, we investigated the effect of cyclin D1 overexpression on growth in tumor cells of epithelial origin. Our study reveals that overexpression of cyclin D1 accelerates growth under conditions in which growth factors are limiting. We provide evidence that in epithelial tumor cells, overexpression of cyclin D1 controls the transition between proliferation and quiescence by stimulating cells to remain in the cell cycle in the absence of growth factors.

MATERIALS AND METHODS

Cell culture and synchronization. Human epithelial MCF7 breast cancer cells and their transfected derivatives were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), 2 mM glutamine, 100 U of penicillin per ml, and 100 μg of streptomycin per ml. The transfectants were cultured in the presence of 10 μg of tetracycline per ml.

Cells were synchronized in G_0 by serum starvation for 48 h in phenol red-free DMEM with 0.1% serum, and growth was stimulated by supplementation of the medium with 10% serum in the presence (noninduced) or absence (induced) of tetracycline. Progression through the cell cycle was monitored by [^{3}H]thymidine or bromodeoxyuridine (BrdU) incorporation, immunostaining with an antibody against Ki-67, and counting of the number of mitotic cells after synchronization with nocodazole (150 ng/ml).

Expression vectors and transfection methods. Plasmid pUHD15-1 contains the transcriptional transactivator gene; plasmid pUHD10-3 contains seven repeats of the *tet* operator linked to a cytomegalovirus minimal promoter, upstream from a polylinker convenient for cloning and a simian virus 40 polyadenylation signal. pUHC13-3 contains a reporter luciferase gene in expression vector pUHD10-3 (10). These vectors were kindly provided by M. Gossen and H. Bujard (University of Heidelberg, Heidelberg, Germany). To construct the tet-cyclin D1 plasmid, we cloned the *Eco*RI-*Bam*HI 1.3-kb cDNA fragment of cyclin D1 containing the full open reading frame (21) into the *Eco*RI-*Bam*HI site of pUHD10-3.

MCF7 cells were first cotransfected with pUHD15-1 and pSV2neo, using the calcium phosphate precipitation technique. Clones were selected in the presence of 800 μg of G418 (Geneticin; Gibco) per ml and tested for the ability to induce tetracycline-sensitive expression from the tetracycline promoter, using the pUHC13-3 luciferase reporter construct in transient-transfection assays. One positive clone, M6, that lacked luciferase activity in the presence of 10 μg of

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tetracycline per ml was retransfected with the tet-cyclin D1 construct. Stable transfectants were obtained by cotransfection of 0.5 μg of a thymidine kinase-hygromycin plasmid. Cells were selected against 100 μg of hygromycin (Calbiochem) per ml in the presence of 10 μg of tetracycline per ml in the medium. Clones were screened for tetracycline-sensitive cyclin D1 expression by immunoprecipitation of [^{35}S]methionine-cysteine-labeled cells as described below. Starting with one 10-cm-diameter plate, we found three clones showing tetracycline-sensitive cyclin D1 expression.

Southern blot analysis. Genomic DNA was isolated from cells by using standard procedures (29). Ten micrograms of DNA was digested with *Bam*HI and *Eco*RI. DNA was separated by electrophoresis in a 0.6% agarose gel and transferred to a Nytran membrane (Schleicher & Schuell). Hybridization to the ^{32}P-labeled cyclin D1 DNA probe was carried out as previously described (15).

Western blot (immunoblot) analysis. MCF7 and the derived cloned cells were cultured in medium with 0.1, 1, 3, or 10% FCS for 48 h in the presence or absence of tetracycline. Total cell extracts were prepared by lysis of cell monolayers with Laemmli sample buffer without bromophenol blue. Protein concentration was measured by the method of Lowry et al. (16). Equal amounts of total cellular protein extracts (50 μg) were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and blotted to a cellulose nitrate membrane (Schleicher & Schuell).

Immunoblot analysis was performed on different strips of the membrane with an anti-pRb (IF-8; Santa Cruz Biotechnology) or anti-cyclin D1 (DCS-6; Progen) monoclonal antibody, and immunodetection was performed with an enhanced chemiluminescence system (Amersham).

Metabolic labeling and immunoprecipitation. MCF7 transfectants were cultured in methionine-cysteine-free medium for 60 min, and then 250 μCi of [^{35}S]methionine-cysteine (1,000 Ci/mmol; Amersham) per ml was added for 30 min. Metabolically labeled cells were lysed for 1 h at 4°C in 1 ml of radioimmunoprecipitation assay buffer (50 mM Tris [pH 7.8], 150 mM NaCl, 10 mM EDTA, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride, 25 μg of aprotinin per ml, 1 mM sodium orthovanadate, 1 mM sodium fluoride). Centrifuged lysates were incubated with protein A-Sepharose beads precoated with a monoclonal antibody against cyclin D1 for 3 h at 4°C. Pelleted beads were washed once in radioimmunoprecipitation assay buffer, twice in buffer A (10 mM Tris [pH 7.4], 150 mM NaCl, 2 mM EDTA, 0.2% Nonidet P-40), and twice in a high-salt buffer (10 mM Tris [pH 7.4], 500 mM NaCl, 2 mM EDTA, 0.2% Nonidet P-40). The proteins were separated on SDS–12.5% polyacrylamide gels and detected by autoradiography.

Monitoring of DNA synthesis. MCF7 clone 3 cells were seeded at a density of 2.5×10^4 cells per cm^2 in phenol red-free DMEM containing 10% FCS with or without tetracycline. The next day, the medium was replaced by phenol red-free DMEM containing dextran-coated charcoal-treated FCS to remove mitogens with or without tetracycline. After another 48 h, the medium was replaced by medium containing the growth factor insulin (10 μg/ml), 0.1% FCS, or 10% FCS in the presence or absence of tetracycline. To monitor the time course of induction of DNA synthesis by mitogens [*methyl*-^{3}H]thymidine (2 μCi/ml; Amersham) was added in pulses of 2 h. At the indicated time points, the cells were incubated for 15 min with 10% trichloroacetic acid at 4°C and then washed three times with 5% trichloroacetic acid. The cells were then lysed in 0.5 N NaOH and quantified by liquid scintillation counting.

For the BrdU-labeling experiment, MCF7 clone 3 cells were maintained in phenol red-free DMEM containing 0.1 or 10% FCS in the presence or absence of tetracycline. After 48 h, cells were pulse-labeled with BrdU (10 μM) for 10 min. Subsequently, cells were maintained in medium containing the same substances without BrdU for the indicated time periods. Cells were fixed in 70% ethanol and subjected to flow cytometric analysis. The analysis consisted of an acid denaturation step (2 N HCl, 30 min at 37°C), incubation with BrdU-DNA-specific antibody (dilution of 1/1,000; 60 min at room temperature), incubation with a fluorescein isothiocyanate-labeled goat anti-mouse antibody (dilution of 1/50, 30 min at 4°C), and staining of total DNA with a 5-μg/ml propidium iodide solution.

Growth rate analysis. MCF7 cells and their cyclin D1 transfectants were seeded at 10^5 cells per 35-mm-diameter dish in phenol red-free DMEM containing 10% FCS with or without tetracycline. The medium was replaced 24 h later with medium containing 0.1, 1, 3, or 10% FCS in the presence or absence of tetracycline. Every 48 h, the medium was replaced with fresh medium containing the indicated concentrations of FCS with or without tetracycline. At daily intervals, cells were collected by trypsinization, stained with trypan blue, and counted with a hemacytometer. This experiment was done in triplicate.

Growth stimulation experiment. Triplicate cultures of MCF7 clone 3 cells were plated at 10^5 cells on coverslips (18 by 18 mm) in six-well plates in phenol red-free DMEM containing 10% FCS with or without 10 μg of tetracycline per ml. The next day, the medium was replaced with medium supplemented with 0.1% FCS with or without tetracycline. After 48 h of starvation, the medium was replaced by the same medium supplemented with 10% FCS and 150 ng of nocodazole per ml, and at the indicated times, MCF7 cells were scored for rounded mitotic cells or fixed in acetone and immunostained with an anti-Ki-67 monoclonal antibody. At least 4,000 cells were counted at each time point.

Starvation experiment and flow cytometric analysis. MCF7 clone cells were plated at 10^5 cells on coverslips in six-well plates in phenol red-free medium containing 10% FCS with or without tetracycline. The medium was replaced 24 h later with medium containing 0.1% serum in the presence or absence of tetracycline. At the indicated times, cells were fixed and immunostained with an antibody against Ki-67 or stained with propidium iodide (Sigma), 0.6% Nonidet P-40, and 2 mg of RNase (Sigma) per ml. A total of 10,000 stained cells were analyzed in a fluorescence-activated cell sorter (FACS) to determine the percentages of cells in different phases of the cell cycle.

Immunohistochemistry. Cell cultures grown on glass slides were washed twice with phosphate-buffered saline (PBS) and fixed in acetone for 5 min. Immunostaining was then performed with the anti-human Ki-67 monoclonal antibody (dilution of 1:10; Dako) for 1 h at room temperature. The reaction was visualized with fluorescein isothiocyanate-conjugated goat anti-mouse secondary antibody at a 1:100 dilution in PBS–3% bovine serum albumin buffer, and cells were incubated with this mixture for another hour at room temperature. After three additional washes with PBS, the nuclei were counterstained with 50 μg of propidium iodide (Sigma) per ml in the presence of 10 μg of DNase-free RNase (Boehringer Mannheim) and mounted with Vector Shield mounting medium. Cells were stained without the primary antibody to monitor background staining. The data are expressed as the mean value of the number of Ki-67-positive cells compared with the total number of cells scored at a specific time point. Each experiment was performed in triplicate, and two independent experiments were done, resulting in monitoring of at least 4,000 cells per time point.

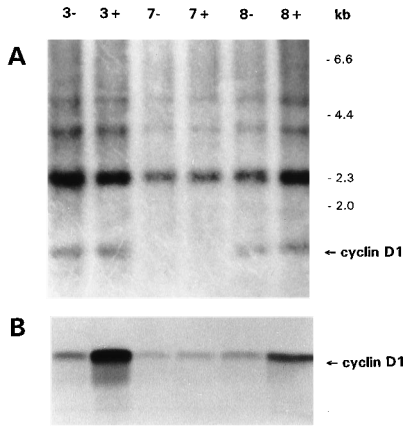

FIG. 1. Transfectants with tetracycline-responsive cyclin D1 expression. (A) Southern blot analysis of cyclin D1 in MCF7 clones in induced (+) or noninduced (−) cells. Genomic DNA was isolated from indicated MCF7 clones, and 10 μg of DNA was digested with *Bam*HI and *Eco*RI. Digested samples were electrophoresed, transferred to a membrane, and hybridized with a ^{32}P-labeled cyclin D1 probe. (B) Expression of cyclin D1 in MCF7 clones in induced (+) or noninduced (−) cells. The G418- and hygromycin-resistant MCF7 clones were labeled for 30 min with [^{35}S]methionine-cysteine, and equal amounts of cell lysate were subjected to immunoprecipitation as described in Materials and Methods.

RESULTS

Transfectants with tetracycline-responsive cyclin D1 expression. Inducible expression of cyclin D1 was obtained in MCF7 cells by using the tetracycline-regulated transcriptional activator as described by Gossen and Bujard (10). Using this method (see Materials and Methods), we obtained three clones of MCF7 cells which showed tetracycline-sensitive expression of cyclin D1. Southern blot analysis of *Eco*RI-*Bam*HI-digested genomic DNA with ^{32}P-labeled cDNA of cyclin D1 revealed that the 1.3-kb DNA fragment containing cyclin D1 was present in clones 3 and 8 but absent in clone 7, containing the empty vector (Fig. 1A). The other hybridizing bands represent endogenous cyclin D1 and cross-reacting DNA fragments, which are likely due to homology in the cyclin box between cyclin genes. The hybridizing 1.3-kb *Eco*RI-*Bam*HI DNA fragment corresponds with the cyclin D1 cDNA fragment inserted in the tet-cyclin D1 construct. Immunoprecipitation of a [^{35}S]methionine-cysteine-labeled lysate of MCF7 clones with a cyclin D1-specific monoclonal antiserum showed tetracycline-sensitive cyclin D1 expression in clones 3 and 8, whereas no

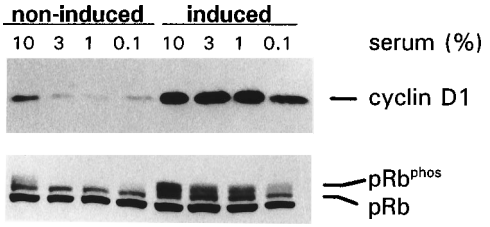

FIG. 2. Expression and activity of cyclin D1 in response to various serum levels in culture medium. Clone 3 cells were cultured for 48 h in DMEM containing the indicated concentrations of serum. Equal amounts of cell lysate were separated on denaturing gels and subjected to Western blotting with an antibody against pRb or cyclin D1 as described in Materials and Methods. pRb^{phos}, hyperphosphorylated pRb.

induction of cyclin D1 was observed in clone 7 (Fig. 1B). Addition of 10 μg of tetracycline per ml to the medium reduced cyclin D1 expression in the positive clones 3 and 8 practically to the level of endogenous cyclin D1. The extent of exogenous cyclin D1 expression varies among the different clones, in clone 3 being fivefold higher than the level of endogenous cyclin D1 expression.

The advantage of using the tetracycline-responsive expression system is that cellular events can be studied as a result of different expression levels of the gene of interest and are not influenced by variations between different clones. We describe in this study predominantly the results of the inducible cell line, clone 3, although clone 8 gave similar results in all experiments.

Cyclin D1 expression and pRb phosphorylation in transfectants in response to serum. Cyclin D1 expression in response to serum was monitored in transfectants and nontransfectants (Fig. 2). In MCF7 clone 3 cells, cyclin D1 expression was dependent on serum levels in the medium under noninduced conditions. Expression was nearly absent under serum-starved conditions (0.1% serum) and clearly present in asynchronous cells maintained in medium with 10% serum. In contrast, in cyclin D1-induced cells, cyclin D1 expression was about five times higher than in asynchronous noninduced cells and was independent of serum concentration. Thus, in MCF7 clone 3 transfectants, cyclin D1 was constitutively expressed both in serum-treated cells and in serum-starved cells.

In parallel with the immunoblot analysis of cyclin D1, we examined the phosphorylation state of endogenous pRb. Hypophosphorylated pRb is a substrate for cyclin D1-associated kinases and becomes hyperphosphorylated in the G_1 phase of the cell cycle. The hypophosphorylated form of pRb shows a slightly increased electrophoretic mobility on SDS-PAGE and is the characteristic form in the G_0 and early to mid-G_1 phases of the cell cycle. In contrast, the appearance of the more slowly migrating hyperphosphorylated form is associated with proliferating cells, especially in the late G_1, S, and G_2/M phases (5, 6).

Figure 2 shows that in the noninduced MCF7 cells, serum deprivation resulted in a decrease in pRb phosphorylation. However, ectopic cyclin D1 expression caused phosphorylation of pRb both in mitogen-stimulated cells and in serum-starved cells. Thus, cyclin D1 overexpression appears to enhance the activity of cyclin D1-associated kinases directly, even when growth factors are absent. Since pRb undergoes phosphorylation in late G_1, the presence of hyperphosphorylated pRb in cyclin D1 overexpressors could indicate that a higher percentage of these cells is cycling and/or there is an alteration(s) in the duration of the several phases of the cell cycle. To investigate the effect of cyclin D1 expression in MCF7 cells in more detail, we performed growth rate analysis and monitored cell cycle kinetics of MCF7 cells in the presence or absence of exogenous cyclin D1.

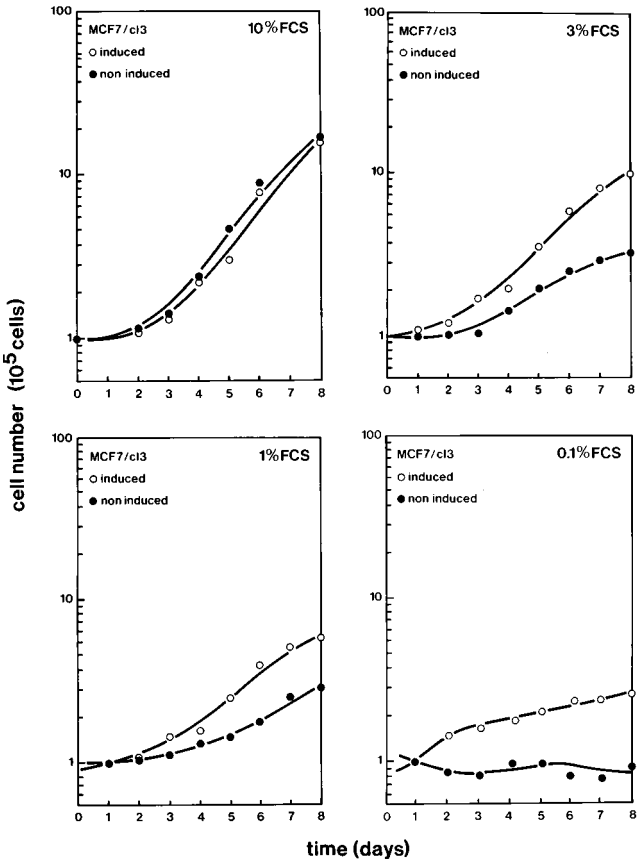

FIG. 3. Effect of overexpression of cyclin D1 on cell proliferation. Triplicate cultures of MCF7 clone 3 (MCF7/cl3) cells were cultured with (noninduced) or without (induced) tetracycline in the presence of various concentrations of serum. The average numbers of cells are plotted versus time.

Cyclin D1 overexpression affects proliferation under low-serum conditions. To investigate how cyclin D1 overexpression affects the response of proliferating breast tumor cells to mitogens, we determined the growth of transfectants and nontransfectants under various conditions. Cell populations expressing exogenous cyclin D1 showed growth similar to that of noninduced cell populations when cultured in medium containing 10% serum (Fig. 3 and Table 1). Analogous results were obtained with parental cells and clone 7 cells maintained in the presence or absence of tetracycline (Table 1). Thus, treatment with tetracycline did not influence the growth of the epithelial cells, and overexpression of cyclin D1 did not affect growth under normal serum conditions. In contrast, cyclin D1-overexpressing cells showed a noticeably higher proliferation level compared with noninduced cells under low-serum (0.1, 1, and 3%) conditions, supporting the idea that cyclin D1 overexpression renders cells less dependent on growth factors. This effect was highly reproducible and statistically significant. However, the growth rate of the induced cells under these low-serum conditions was less than under 10% serum conditions, indicating that the increased amount of cyclin D1 in transfectants was not sufficient to render the cells completely growth factor independent.

Effect of cyclin D1 overexpression on the G_0-to-S transition. To study the effect of constitutive overexpression of cyclin D1

TABLE 1. Doubling times in cells overexpressing cyclin D1 in response to various serum levels[a]

Clone	Cyclin D1 induction	Doubling time (h) at serum concn of:			
		10%	3%	1%	0.1%
3	+	30.3	43.3	47.5	142.8
	−	32.7	67.1	80.0	—[b]
7	+	33.9	71.7	85.6	—
	−	34.8	69.4	88.5	—
8	+	30.9	39.5	51.0	144.0
	−	31.6	52.8	76.5	—

[a] MCF7 clone cells were maintained in medium with (noninduced) or without (induced) tetracycline in the presence of various concentrations of serum. In contrast to clone 7, in clones 3 and 8, tetracycline withdrawal resulted in cyclin D1 induction. At daily intervals, the number of cells was scored and the doubling times of exponentially growing cells were calculated.

[b] —, no growth observed.

in the epithelial breast cancer cell line on the G_0-to-S transition, the stable transfectants were serum starved for 48 h, and then growth was stimulated by addition of serum or growth factors to the medium as described in Materials and Methods. Cells passing the S phase were labeled with tritiated thymidine, and levels of thymidine incorporation are shown in Fig. 4.

In the noninduced cell population, serum deprivation resulted in synchronization of noncycling cells, which was demonstrated by the very low levels of S-phase cells in this population compared with asynchronously growing control cells. Supplementation with the mitogen insulin caused the noninduced cells to enter the cell cycle, with a median time of 30 h for progression into the S phase. Surprisingly, serum deprivation of cyclin D1 overexpressors did not result in a withdrawal from the cell cycle. The numbers of cyclin D1-overexpressing cells in the S phase were very similar for the several growth conditions used in this experiment, ranging from serum-depleted medium to medium containing 10% serum or insulin (10 μg/ml). These data demonstrate that overexpression of cyclin D1 results in MCF7 cells in continuous DNA synthesis even in the absence of mitogens. This could be the result of a sustained stay in the S phase or an increase in the number of growth factor-independent cells.

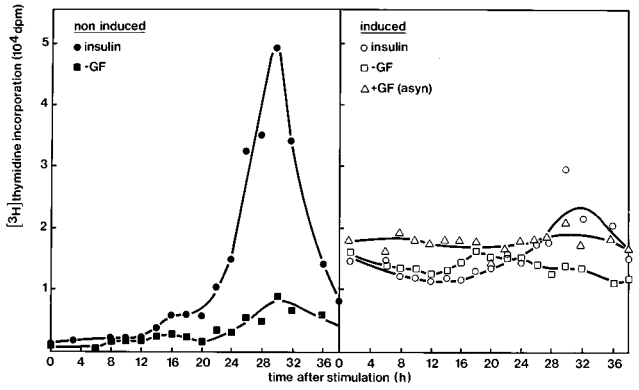

FIG. 4. Effect of ectopic cyclin D1 expression on the G_0-to-S transition. Clone 3 cells were plated, and cyclin D1 was induced by the removal of tetracycline. Cells were starved in 0.1% serum for 48 h and subsequently treated with the growth factor insulin (10 μg/ml) or without growth factor (-GF) as described in Materials and Methods. Asynchronous (asyn) clone 3 cells maintained in 10% serum were used as a control. Cells were treated with tritiated thymidine and lysed at the indicated times after supplementation of growth stimuli. The mean values of [^{3}H]thymidine incorporation are plotted versus time.

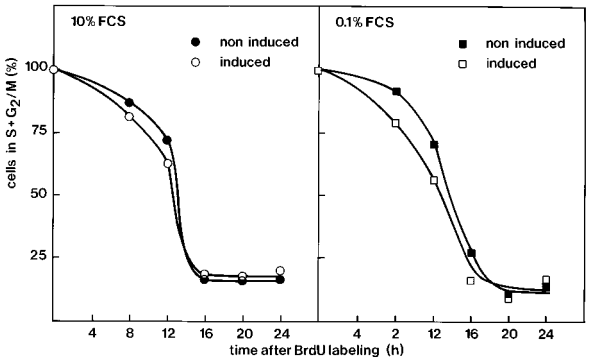

FIG. 5. Effect of cyclin D1 overexpression on progression through the S and G_2/M phases of MCF7 clone 3 cells. Cells were pulse-labeled with BrdU, and then the medium was replaced with medium without BrdU for the indicated time periods. The BrdU-positive cells were monitored by FACS analysis for their presence in the S or G_2/M phase. Each value represents analysis of 10^5 cells and is the mean of two independent experiments.

Cyclin D1 overexpression does not affect the length of the S and G_2/M phase. To elucidate whether cyclin D1 overexpression in mitogen-deprived condition modulates transition through the S phase, MCF7 clone 3 cells were pulse-labeled with BrdU in the presence or absence of tetracycline, and then the medium was replaced with medium without BrdU for the indicated times. The BrdU-labeled cells were screened by FACS analysis for their presence in the different phases of the cell cycle. At $t = 0$, incorporation of BrdU, indicative of transition through the S phase, occurred in 22.7 and 13.5% of the induced and noninduced cells, respectively, in serum-deprived conditions and in 38.5 and 36.1% of the induced and noninduced cells, respectively, in serum-supplemented conditions. Figure 5 shows the fractions of S- and G_2/M-phase cells in the BrdU-labeled cell population at the indicated time periods after pulse-labeling (the percentage of BrdU-positive cells at $t = 0$ was set at 100%). FACS analysis revealed that both cyclin D1-induced and noninduced S-phase cells passed through the S and G_2/M phases to enter the G_1 phase. The lengths of the time periods in which 50% of the initial S-phase cells enter the G_1 phase in serum-deprived conditions were 12.8 and 13.6 h for the induced and noninduced cells, respectively. In the presence of an abundancy of mitogens, the lengths were 12.7 and 12.9 h for the induced and noninduced cells, respectively. This result indicates that the length of the S and G_2/M phase was not substantially modulated by mitogens or cyclin D1 overexpression in breast tumor cells.

Cyclin D1 overexpression affects the quiescent state of MCF7 cells. To clarify whether cyclin D1 overexpression affects the switch between proliferation and quiescence, we performed the following set of experiments. MCF7 transfectants were serum starved under induced and noninduced conditions for 48 h. Subsequently, the cell populations received 10% serum for growth stimulation and were simultaneously treated with nocodazole to prevent progression to the next G_1 phase or quiescent state for different lengths of time. To discriminate between cycling and noncycling cells, we immunostained these cells for the presence of the proliferation marker Ki-67. The monoclonal antibody against Ki-67 that we used detects a nuclear antigen that is present throughout the cell cycle and is absent in resting cells (8, 9, 14). Thus, unlike propidium iodide staining, use of Ki-67 discriminates between resting G_0 cells and cycling G_1 cells. Figure 6B shows that there is a clear difference between the number of Ki-67-positive cells in cyclin

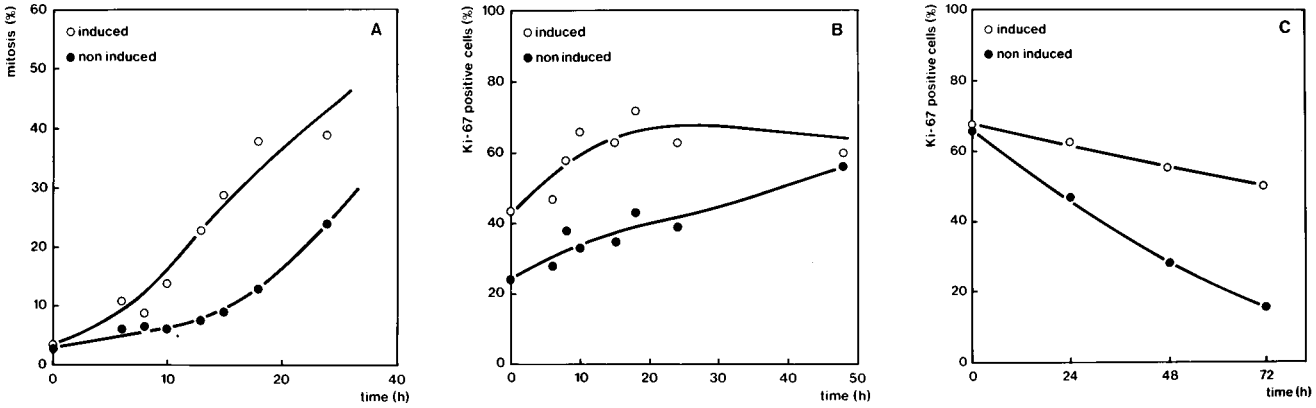

FIG. 6. Effect of cyclin D1 overexpression on the kinetics of the cell cycle. In the growth stimulation studies, clone 3 cells were maintained in medium with or without tetracycline and starved in 0.1% serum for 48 h. Subsequently, cells were maintained in the presence of 10% serum and nocodazole to block the cells in the metaphase. (A) Fraction of mitotic cells in culture. (B) Fraction of cells which were immunostained with a monoclonal antibody against the proliferation marker Ki-67. In the growth starvation assay, asynchronous cells with and without cyclin D1 overexpression were starved in 0.1% serum. Cells were fixed at the indicated times after starvation and immunostained with an antibody against Ki-67. (C) Percentage of Ki-67-positive cells. Each value represents a minimum count of 4,000 cells and is the mean of two independent experiments.

D1-induced and noninduced cells after serum deprivation. The number of cycling cells is twofold higher in the induced cells than in the noninduced cells. This difference was reduced after a subsequent treatment with medium containing 10% serum. Similar data were obtained when the fraction of mitotic cells in this experiment was scored in live cells (Fig. 6A). In the absence of nocodazole (at $t = 0$), no difference was found in the number of mitotic cells between induced cells and noninduced cells, because of the low mitotic index (3%) in proliferating cells. In noninduced cells, it took 24 h before the number of mitotic cells was markedly increased, reflecting the minimal time needed for noninduced cells to switch from the resting phase to enter the cell cycle and subsequently the M phase. Conversely, the number of mitotic cells in cyclin D1 overexpressors gradually increased in response to serum addition. This result strongly suggests that these cells were not growth arrested under the serum-deprived conditions but were still progressing through the cell cycle.

To directly test the hypothesis that cyclin D1 overexpression prevents cell cycle exit in low-serum conditions, cells were maintained in induced and noninduced conditions and subsequently switched from medium containing 10% serum to medium containing 0.1% serum. Before serum withdrawal ($t = 0$), we found that the number of Ki-67-positive cells was not significantly different between the induced and noninduced cells (Fig. 6C). The fraction of cells positive for Ki-67 (which was used as a marker for the number of cycling cells) gradually decreased in response to serum withdrawal in both induced and noninduced cells. However, under these conditions, the decrease in the fraction of Ki-67-positive cells was significantly less pronounced in induced cells ($P \leq 0.005$). Flow cytometric analysis gave similar results (Fig. 7). This finding suggests that synchronization of the cells in G_0 by serum withdrawal had succeeded in noninduced cells but was not effective in cells overexpressing cyclin D1. In summary, our results indicate that the primary effect of cyclin D1 overexpression in tumor cells is to trigger cells to divide in an environment in which proliferation is normally ceased, namely, in growth factor-restricted conditions.

DISCUSSION

In this paper, we report a novel role of cyclin D1 as a regulator of the G_1 phase of the cell cycle. Using various experimental designs, we show that overexpression of cyclin D1 prevents cell cycle exit to quiescent phase (here defined as G_0) in low-serum conditions, indicating that cyclin D1 overexpression renders cells less growth factor dependent by affecting the number of cycling cells.

Our study demonstrates that the length of S and G_2/M phases of the cell cycle was not affected by overexpression of cyclin D1 or serum factors, as expected for cell cycle progression to become insensitive to growth factor requirements after cells have passed the restriction point (Fig. 5). The cell doublings times, however, are dependent on serum concentration as well as cyclin D1 overexpression (Table 1). Our results therefore indicate that transition through the G_1 and/or G_0 phase is affected by either one of these parameters. The increase in cell doubling times as a result of reduced-serum concentrations could have been due to a prolonged stay in G_1 and/or entry into G_0. If serum deprivation had induced a pro-

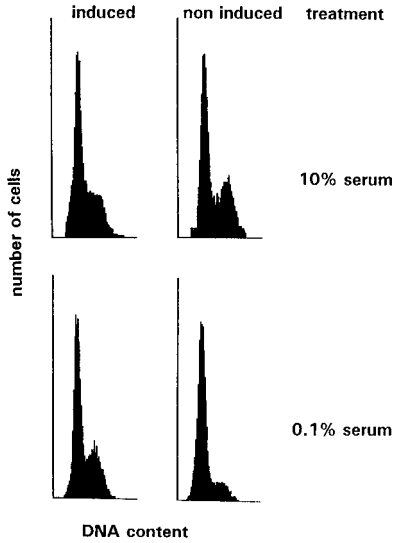

FIG. 7. Effect of overexpression of cyclin D1 on the kinetics of the cell cycle. Clone 3 cells were grown with and without tetracycline in medium containing 10% serum or starved in 0.1% serum for 72 h. Cells were fixed in 70% ethanol, stained with propidium iodide, and subjected to FACS analysis.

longed stay in G_1 without any effect on entry into G_0, this would have resulted in an increase in the fraction of Ki-67-positive cells. However, we observed the opposite (Fig. 6C). This result strongly suggests that reduced growth in low-serum conditions results from increased entry into G_0 and is not due to an enforced stay in G_1. Overexpression of cyclin D1 modulates in minimal growth factor conditions this entry in G_0: in reduced-serum conditions, cyclin D1-overexpressing cells remain more cycling than cells with normal levels of cyclin D1 (Fig. 6C).

Besides this effect on the G_1-to-G_0 transition, overexpression of cyclin D1 also modulates other G_1 processes, including G_0-to-G_1 and G_1-to-S transitions, as has been reported previously (1, 2, 3, 12, 19, 22, 25, 27, 28, 32). Other studies demonstrated an increased entry into the cell cycle, from G_0 to G_1, in cyclin D1-overexpressing cells under low-serum conditions, using either normal cells, with a limited life span (1, 12, 28), or cell lines with almost 100% growth fraction (22). Exit from G_1 to G_0 was not studied in those cells. We used the breast tumor cell line MCF7, which represents breast tumor cells in vivo in that a large proportion (40%) of the cells remain quiescent even in high-serum conditions (Fig. 6C). In these cells, exit from G_1 to G_0 is, under low-serum conditions, affected by overexpression of cyclin D1. Since MCF-7 cells are tumor cells, this effect of cyclin D1 must be viewed in the context of a potentially large number of other oncogenic changes, among which is the absence of the CDK4/CDK6 inhibitor $p16^{INK4}$ in MCF-7 cells (24). A combined effect of overexpression of cyclin D1 on these two G_1 transitions shows a reduced exit from G_1 to G_0 and an increased entry from G_0 to G_1 under low-serum conditions. Together, these two transitions determine the proliferative fraction of tumor cells. Since cyclin D1 is normally rate limiting in these steps, overexpression of cyclin D1 increases the growth fraction and is therefore a marker for proliferation under low-serum conditions. Such an environment is frequently found in vivo, in particular in developing tumors. Cyclin D1 overexpression in breast epithelial tumor cells could in this way result in autonomous growth and increase oncogenic potential of tumor cells.

This model is supported by recent studies (7, 31) describing a lack of massive proliferation of the breast epithelium associated with pregnancy in cyclin D1-deficient mice, suggesting that steroid-induced proliferation of the mammary epithelium may be driven through cyclin D1. In addition, our data indicate that overexpression of cyclin D1 replaces the requirement of growth factors, although cyclin D1 alone was not able to fully compensate for the need of mitogens for growth (Fig. 3).

A reduced entry into G_0 as a result of overexpression of D-type cyclins is also a likely explanation for the inhibition of differentiation by growth factors in normal myoblast cells (26). Furthermore, the inhibition of granulocyte colony-stimulating factor-mediated differentiation in hematopoietic cells is also affected by cyclins D2 and D3 (13). Apparently, overexpression of cyclin D1 in normal cells results in loss of differentiation, whereas in tumor cells it results in proliferation.

This study also shows that a moderate cyclin D1 overexpression enforces the activity of the CDK(s), as demonstrated by phosphorylation of its target, pRb. Rb encodes a nuclear phosphoprotein whose phosphorylation appears essential for traverse through G_1, and it functions as a negative growth regulator (18, 28). pRb is hypophosphorylated during the quiescent state and early G_1 phase, but it undergoes rapid phosphorylation in late G_1 and remains in a hyperproliferated state until cells exit mitosis (5, 6). Surprisingly, under growth factor-limiting conditions, the hyperphosphorylated form of endogenous pRb was clearly visible in cells overexpressing cyclin D1 but was not detectable in nonoverexpressing cells. Under those circumstances, cell growth was increased in cyclin D1-overexpressing cells as well (Fig. 3). A striking result, however, was that the level of pRb phosphorylation did not parallel the extent of cell growth; i.e., the extent of phosphorylation of pRb in cells overexpressing cyclin D1 in 0.1% serum was similar to the extent of pRb phosphorylation in noninduced cells in 10% serum, whereas the growth rate was approximately fivefold less (Table 1). Phosphorylation of pRb is therefore not simply a reflection of the number of cells progressing in late G_1, S, or G_2/M phase but appears to accumulate in cyclin D1-overexpressing cells without achieving the expected progression and cell growth. A likely explanation for this discrepancy might be that phosphorylation of pRb is a requirement for progression and growth but is by itself not sufficient. In that case, other rate-limiting events besides that of pRb are likely involved in regulating the cell cycle. A recent observation that in fibroblasts, cyclins D1 and E have different targets, the one of cyclin D1 being pRb, but that a cooperativity between the two is required for transition through G_1 supports this hypothesis (28).

In this report, we have demonstrated an effect of overexpression of cyclin D1 on exit from the cell cycle. Interestingly, this effect was not observed under normal culture conditions but was measurable only in low-serum conditions. This finding suggests that in cells with a normal cyclin D1 expression pattern (which is triggered by mitogenic signals and peaks in late G_1), this cyclin does not affect entry into the quiescent state. In conclusion, enhancement of proliferative capacities under low-serum conditions could be characteristic for cyclin D1-overexpressing breast tumor cells and is the result of a modulation of the G_1-to-G_0 transition. Therefore, we have proposed a working mechanism of cyclin D1 overexpression in tumor cells which is responsible for hyperproliferation under growth factor-deprived conditions.

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REFERENCES

1. **Baldin, V., J. Lukas, M. J. Marcote, M. Pagano, and G. Draetta.** 1993. Cyclin D1 is a nuclear protein required for cell cycle progression in G_1. Genes Dev. **7:**812–821.
2. **Bartkova, J., J. Lukas, H. Müller, D. Lutzhoft, M. Strauss, and J. Bartek.** 1994. Cyclin D1 protein expression and function in human breast cancer. Int. J. Cancer **57:**353–361.
3. **Bates, S., L. Bonetta, D. MacAllan, D. Parry, A. Holder, C. Dickson, and G. Peters.** 1994. CDK6 (PLSTIRE) and CDK4 (PSK-J3) are a distinct subset of the cyclin-dependent kinases that associate with a cyclin D1. Oncogene **9:**71–79.
4. **Buckley, M. F., K. J. E. Sweeney, J. A. Hamilton, R. L. Sini, D. L. Manning, R. I. Nicholson, A. deFazio, C. K. W. Watts, E. A. Musgrove, and R. L. Sutherland.** 1993. Expression and amplification of cyclin genes in human breast cancer. Oncogene **8:**2127–2133.
5. **Buchkovich, K., L. A. Duffy, and E. Harlow.** 1989. The retinoblastoma protein is phosphorylated during specific phases of the cell cycle. Cell **58:**1097–1105.
6. **Chen, P.-L., P. Scully, J.-Y. Shew, J. Y. Wang, and W.-H. Lee.** 1989. Phosphorylation of the retinoblastoma gene product is modulated during the cell cycle and cellular differentiation. Cell **58:**1193–1198.
7. **Fantl, V., G. Stamp, A. Andrews, I. Rosewell, and C. Dickson.** 1995. Mice lacking cyclin D1 are small and show defects in eye and mammary gland development. Genes Dev. **9:**2364–2372.
8. **Gerdes, J., H. Lemke, H. Baisch, H.-H. Wacker, U. Schwab, and H. Stein.** 1984. Cell cycle analysis of a cell proliferation-associated human nuclear antigen defined by the monoclonal antibody Ki-67. J. Immunol. **133:**1710–1715.

9. **Gerdes, J., U. Schwab, H. Lemke, and H. Stein.** 1983. Production of a mouse monoclonal antibody reactive with a human nuclear antigen associated with cell proliferation. Int. J. Cancer **31:**13–20.
10. **Gossen, M., and H. Bujard.** 1992. Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. Proc. Natl. Acad. Sci. USA **89:**5547–5551.
11. **Jiang, W., S. Kahan, N. Tomita, Y.-J. Zhang, S. Lu, and B. Weinstein.** 1992. Amplification and expression of the human cyclin D1 gene in esophageal cancer. Cancer Res. **52:**2980–2983.
12. **Jiang, W., S. M. Kahn, P. Zhou, Y.-J. Zhang, A. M. Cacace, A. S. Infante, S. Doi, R. M. Santella, and I. B. Weinstein.** 1993. Overexpression of cyclin D1 in rat fibroblasts causes abnormalities in growth control, cell cycle progression and gene expression. Oncogene **8:**3447–3457.
13. **Kato, J. Y., and C. J. Sherr.** 1993. Inhibition of granulocyte differentiation by G_1 cyclins D2 and D3 but not D1. Proc. Natl. Acad. Sci. USA **90:**11513–11517.
14. **Key, G., M. H. Becker, B. Baron, M. Duchrow, C. Schluter, H.-D. Flad, and J. Gerdes.** 1993. New Ki-67-equivalent murine monoclonal antibodies (MIB 1-3) generated against bacterially expressed parts of the Ki-67 cDNA containing three 62 base pair repetitive elements encoding for the Ki-67 epitope. Lab. Invest. **68:**629–636.
15. **Lammie, G. A., V. Fantl, R. Smith, E. Schuuring, S. Brookes, R. Michalides, C. Dickson, A. Arnold, and G. Peters.** 1991. D11S287, a putative oncogene on chromosome 11q13, is amplified and expressed in squamous cell and mammary carcinomas and linked to BCL-1. Oncogene **6:**439–444.
16. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. S. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193:**265–275.
17. **Lukas, J., M. Pagano, Z. Staskova, G. Draetta, and J. Bartek.** 1994. Cyclin D1 protein oscillates and is essential for cell cycle progression in human tumour cell lines. Oncogene **9:**707–718.
18. **Matsushime, H., M. E. Ewen, D. K. Strom, J. Y. Kato, S. K. Hanks, M. F. Roussel, and C. J. Sherr.** 1992. Identification and properties of a atypical catalytic subunit p34PSK-J3 for mammalian D-type G1 cyclins. Cell **71:**323–334.
19. **Matsushime, H., D. E. Quelle, S. A. Shurtleff, M. Shibuya, C. J. Sherr, and J. Kato.** 1994. D-type cyclin dependent kinase activity in mammalian cells. Mol. Cell. Biol. **14:**2066–2076.
20. **Michalides, R., N. van Veelen, A. Hart, B. Loftus, E. Wientjens, and A. Balm.** 1995. Overexpression of cyclin D1 correlates with recurrence in a group of forty-seven operable squamous cell carcinomas of the head and neck. Cancer Res. **55:**975–978.
21. **Motokura, T., T. Bloom, H. G. Kim, H. Juppner, J. V. Ruderman, H. M. Kronenberg, and A. Arnold.** 1991. A novel cyclin encoded by a bcl1-linked candidate oncogene. Nature (London) **350:**512–515.
22. **Musgrove, E. A., C. S. Lee, M. F. Buckley, and R. L. Sutherland.** 1994. Cyclin D1 induction in breast cancer cells shortens G_1 and is sufficient for cells arrested in G_1 to complete the cell cycle. Proc. Natl. Acad. Sci. USA **91:**8022–8026.
23. **Pardee, A. B.** 1989. G_1 events and regulation of cell proliferation. Science **246:**603–608.
24. **Parry, D., S. Bates, D. J. Mann, and G. Peters.** 1995. Lack of cyclin D-CDK complexes in Rb-negative cells correlates with high levels of $p16^{INK4/MTS1}$ tumour suppressor gene product. EMBO J. **14:**503–511.
25. **Quelle, D. E., R. A. Ashmun, S. A. Shurtleff, Y.-Y. Kato, D. Bar-Sagi, M. F. Roussel, and C. J. Sherr.** 1993. Overexpression of mouse D-type cyclins accelerates G_1 phase in rodent fibroblasts. Genes Dev. **7:**1559–1571.
26. **Rao, S. S., and D. S. Kohtz.** 1995. Positive and negative regulation of D-type cyclin expression in skeletal myoblasts by basic fibroblast growth factor and transforming growth factor β. J. Biol. Chem. **270:**4093–4100.
27. **Resnitzky, D., M. Gossen, H. Bujard, and S. I. Reed.** 1994. Acceleration of the G_1/S phase transition by expression of cyclin D1 and E with an inducible system. Mol. Cell. Biol. **14:**1669–1679.
28. **Resnitzky, D., and S. I. Reed.** 1995. Different roles for cyclin D1 and E in regulation of the G_1-to-S transition. Mol. Cell. Biol. **15:**3463–3469.
29. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
30. **Schuuring, E., E. Verhoeven, W. J. Mooi, and R. J. A. M. Michalides.** 1992. Identification and cloning of two overexpressed genes, U21B31/PRAD1 and EMS1, within the amplified chromosome 11q13 region in human carcinomas. Oncogene **7:**355–361.
31. **Sicinski, P., J. L. Donaher, S. B. Parker, T. Li, H. Gardner, S. Z. Haslam, R. T. Bronson, S. J. Elledge, and R. A. Weinberg.** 1995. Cyclin D1 provides a link between development and oncogenesis in the retina and breast. Cell **82:**621–630.
32. **Tam, S. W., A. M. Theodoras, J. W. Shay, G. F. Draetta, and M. Pagano.** 1994. Differential expression and regulation of cyclin D1 protein in normal and tumor human cells: association with cdk4 is required for cyclin D1 function in G_1 progression. Oncogene **9:**2663–2674.
33. **Won, K. A., Y. Xiong, D. Beach, and M. Z. Gilman.** 1992. Growth regulated expression of D-type cyclin genes in human diploid fibroblasts. Proc. Natl. Acad. Sci. USA **89:**9910–9914.