Growth Factor Regulation of Cyclin D1 mRNA Expression Through Protein Synthesis-Dependent and -Independent Mechanisms

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Overexpression of the cyclin D1/*PRAD1* oncogene has been observed in a number of tumorigenic cell lines, suggesting that regulation of D1 expression may represent an important step in the control of cellular proliferation. We have examined the mRNA expression of cyclin D1, as well as two related D-type cyclins, D2 and D3, in response to defined growth factors that control the growth of Balb/c-3T3 fibroblasts. Transcripts for all three D-type cyclins were expressed during the G1 phase of the Balb cell cycle, however only D1 and D3 exhibited periodic induction. Although redundantly expressed, message levels of cyclin D1 and D3 were differentially regulated in regard to kinetics of induction; a modest increase in D3 mRNA was detected near the G1/S boundary, 12 h after serum stimulation of quiescent cells, while abundance of D1 transcript increased 20 to 30-fold, peaking 6 h after addition of serum. Factors such as platelet-derived growth factor (PDGF) that induce competence formation in Balb cells, increased D1 message and protein levels to the same extent as serum but did not affect expression of cyclin D3 and did not stimulate entry into S phase. Progression factors contained within platelet-poor plasma stimulated D1 expression only weakly but acted synergistically with low concentrations of PDGF to increase D1 mRNA to maximum levels. Depletion of protein kinase C severely reduced the ability of PDGF and serum to induce D1 mRNA. PDGF- and serum-mediated elevation of steady-state D1 message levels was in part because of a transcriptional activation of the D1 gene that was independent of protein synthesis. However, protein synthesis was required 3–4 h after serum stimulation for the shut down of D1 transcription leading to the normal decline in message levels after peak induction. Our results indicate that overexpression of cyclin D1 message may result from a disruption of negative regulatory events that repress D1 transcription.

INTRODUCTION

The growth of nontransformed eukaryotic cells is regulated by signals in the extracellular environment during the first gap phase (G1) of the cell cycle (Pardee, 1989). Quiescent density-arrested Balb/c-3T3 fibroblasts require the coordinate and sequential action of multiple serum-derived growth factors to progress through G1 and initiate DNA synthesis. Transient exposure to platelet-derived growth factor (PDGF) renders Balb cells competent to respond to progression factors found in platelet-poor plasma (PPP) and traverse G1 (Pledger *et al.*, 1977). The cooperative action of epidermal growth factor (EGF) and insulin-like growth factor I (IGF-1) can replace PPP in the stimulation of competent fibro-

blasts to a restriction point termed commitment just before entry into S phase (Leof *et al.*, 1982). Cells reaching commitment can begin DNA replication and complete the cell cycle in the absence of exogenous mitogens (Wharton, 1983). Although the growth factors necessary for the proliferation of Balb/c-3T3 cells are well defined, intracellular effectors that mediate the mitogenic response to these signals are, at present, incompletely understood.

Budding yeast exhibit a point of cell cycle regulation before the G1/S phase transition called START that is analogous to the mammalian commitment point (Hartwell, 1971). Genetic analysis has demonstrated that passage through START is governed by a highly con-

served serine/threonine kinase, *cdc2*/CDC28 (Reid and Hartwell, 1977; Nurse and Bissett, 1981). Mutational inactivation of *cdc2* results in the block of the yeast cell cycle during G1, as well as at the G2/M phase boundary. Kinase activity of *p34^{cdc2}* is regulated in part through association with the cyclin family of proteins, including the G1-specific cyclins CLN1, CLN2, and CLN3 (Nash *et al.*, 1988; Hadwiger *et al.*, 1989; Richardson *et al.*, 1989). Message levels of CLN1 and CLN2 fluctuate during the cell cycle, peaking during G1 and declining as cells begin DNA synthesis (Wittenberg *et al.*, 1990). Accumulation of the CLN gene products is rate limiting for the execution of START because overexpression of either CLN2 or CLN3 has been shown to reduce the length of G1 (Cross, 1988; Nash *et al.*, 1988; Hadwiger *et al.*, 1989). Functional inactivation of all three CLN genes results in the arrest of cells in G1 and thus prevents transition into S phase (Richardson *et al.*, 1989).

Several human cDNAs have been isolated that rescue the mutant phenotype of yeast deficient in CLN activity (Koff *et al.*, 1991; Lew *et al.*, 1991; Xiong *et al.*, 1991). These putative G1 cyclins display limited homology to the yeast CLN genes and have been grouped into novel categories of C-, D-, and E-type cyclins. Cyclin D1 is the human homologue of CYL1, a murine D-type cyclin identified in mouse macrophages stimulated with colony-stimulating factor 1 (Matsushime *et al.*, 1991). Several lines of evidence have implicated a role for cyclin D in the growth control of mammalian cells. *PRAD1*, a transforming gene that is overexpressed in parathyroid tumors as a result of genomic rearrangement, is identical to the human cyclin D1 gene (Motokura *et al.*, 1991). Furthermore, cyclin D1/*PRAD1* appears to be the *bcl-1* oncogene that is overexpressed in several B-cell leukemias after chromosomal translocation (Rosenberg *et al.*, 1991). Overexpression and amplification of the cyclin D1 locus has also been detected in several breast and squamous cell carcinoma lines (Lammie *et al.*, 1991), suggesting that deregulated expression of cyclin D1 may play a role in the process of cellular transformation.

Induction of cyclin D1 mRNA has also been observed in a number of nontransformed cell lines in response to a variety of mitogenic stimuli (Matsushime *et al.*, 1991; Won *et al.*, 1992). Recently it has been shown that microinjection of antisense plasmids to cyclin D1 in human lung fibroblasts prevents entry into S phase, suggesting that activation of D1 expression is prerequisite for G1 traverse and the initiation of DNA synthesis (Baldin *et al.*, 1993). Upregulation of cyclin D1 message levels may therefore play a critical role in the growth of nontransformed as well as tumorigenic cell lines. We have investigated the regulation of murine cyclin D mRNA expression by those growth factors promoting G1 traverse in Balb/c-3T3 fibroblasts. Although transcripts for all three D-type cyclins were expressed during G1 in Balb cells, only D1 exhibited a dramatic fluctuation in mRNA levels during the cell cycle. Here, we show

that growth factors controlling progression from a quiescent state to mid-G1 synergistically induced expression of cyclin D1 transcript through a protein kinase C(PKC)-dependent mechanism. Growth factor stimulation of Balb fibroblasts ultimately led to a transient increase in D1 gene transcription. Our results suggest that both a positive, protein synthesis-independent pathway and a negative, protein synthesis-dependent pathway influence growth factor-stimulated transcription of cyclin D1.

MATERIALS AND METHODS

Cell Culture

Balb/c-3T3 mouse embryo fibroblasts (clone A31) were cultured in a water-jacketed incubator with a humidified atmosphere (5% CO₂, 95% air) maintained at 37°C. Experimental cultures were grown to confluency in 100-mm Petri dishes using Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated bovine calf serum (Colorado Serum Company, Denver, CO), 4 mM L-glutamine, 50 units/ml penicillin, and 50 µg/ml streptomycin. Cells were used 3–4 d after density arrest.

V Point Arrest

Quiescent cultures were incubated for 50–60 h in amino acid-deficient DMEM containing 1.5% dialyzed fetal bovine serum (dFBS). Amino acid-deficient DMEM was identical to normal DMEM except that it lacked seven essential amino acids: cystine, isoleucine, leucine, lysine, methionine, phenylalanine, and tyrosine. Prestarved cultures stimulated in deficient medium supplemented with 10% dFBS and 10 ng/ml PDGF-BB arrested at the V point as previously described (Olson *et al.*, 1993).

DNA Synthesis Assays

Density-arrested cultures were refed with medium containing 10% serum, 10 ng/ml PDGF-BB, and 5 µCi/ml [³H]thymidine. After the specified time, cells were washed twice with phosphate-buffered saline (PBS), fixed in methanol 10 min, and processed for autoradiography as previously described (Antoniades *et al.*, 1975).

RNA Isolation and Northern Analysis

Total cellular RNA was isolated for Northern analysis by acid guanidinium thiocyanate-phenol-chloroform extraction as previously described (Drozdzoff and Pledger, 1991). Twenty micrograms of RNA/sample was heat denatured for 7 min at 65°C in 2.2 M formaldehyde buffer and separated by electrophoresis in a 1.2% denaturing agarose gel as described (Maniatis *et al.*, 1982). Gels were transferred to nylon membrane (Schleicher and Schuell, Keene, NH) by capillary blotting, and RNA was UV-crosslinked to membrane before prehybridizing in 50% formamide, 5× SSPE, 0.05% bovine serum albumin, 0.1% sodium dodecyl sulphate (SDS), and 400 µg/ml yeast tRNA for 3 h at 42°C. Membranes were hybridized overnight at 42°C in an identical solution containing the denatured DNA probe. Probes were labeled with a random primer kit (Boehringer Mannheim, Indianapolis, IN) to a specific activity of 1–2 × 10⁹ dpm/µg. Membranes were washed in 0.2× SSC, 0.1% SDS at 60°C and autoradiographed at –70°C with intensifying screens.

The following probes were used for Northern analysis: cyclin D1, the 0.9-kilobase (kb) *Eco*RI fragment of pCBZ04.1; cyclin D2, the 0.7-kb *Eco*RI fragment of pCN3-CYL2; cyclin D3, the 1.6-kb *Eco*RI fragment of pCT2.2-CYL3 (plasmids containing partial mouse cDNA's for the cyclin genes were generously provided by Dr. Charles Sherr);

and 1B15, the 0.7-kb *Bam*HI/*Pst* I fragment of pSP65 (Danielson *et al.*, 1988).

Immunoblotting

Whole cell extracts were prepared by sonication in buffer containing 50 mM tris(hydroxymethyl)aminomethane (Tris) (pH 7.4), 250 mM NaCl, 0.1% Triton X-100, 5 mM EDTA, 50 mM NaF, 0.1 mM sodium orthovanadate, 50 μ g/ml phenylmethylsulfonyl fluoride, and 1 μ g/ml leupeptin. Fifty micrograms of protein/sample was boiled 3 min in Laemmli buffer (20% glycerol, 3% SDS, 4% B-mercaptoethanol, 10 mM EDTA, and 0.05% bromophenol blue) and separated on a 10% SDS-polyacrylamide gel. Gels were electrophoretically transferred to nitrocellulose (Bio-Rad, Richmond, CA), and blots were probed with a primary anti-cyclin D1 polyclonal antibody (generously provided by Dr. Gordon Peters, Imperial Cancer Research Fund, U.K.). Proteins were detected using the electrochemiluminescence (ECL) system as per instructions of the manufacturer (Amersham, Arlington Heights, IL).

Nuclear Run-on Analysis

Nuclei were isolated from cells as previously described (Harrington *et al.*, 1990). Briefly, cells from six 100-mm dishes were scraped into PBS and pelleted at 750 $\times$ g. Pelleted cells were resuspended in 4 ml ice-cold nuclear extraction buffer (10 mM Tris [pH 7.5], 10 mM NaCl, 3 mM MgCl₂, 0.5% [vol/vol] Nonidet P-40) and incubated on ice for 5 min. Nuclei were pelleted, washed in extraction buffer, and resuspended in a 100- μ l solution of 50 mM Tris (pH 8.0), 5 mM MgCl₂, 0.1 mM EDTA, and 40% glycerol. Nuclei were flash frozen in a dry ice/methanol bath and stored at -70°C until use. For transcription reactions, nuclei were thawed on ice and added to 100 μ l of reaction buffer (10 mM Tris [pH 8.0], 5 mM MgCl₂, 300 mM KCl, 5 mM dithiothreitol, 1 mM each of ATP, CTP, and GTP, 10 μ M cold UTP, and 100 μ Ci [α -³²P] UTP [3000 Ci/mmol]). Reactions were incubated at 30 $^{\circ}\text{C}$ for 30 min, and labeled transcripts were isolated as described above.

Equal cpm of labeled RNA were used for hybridization to Nytran membranes (Schleicher and Schuell, Keene, NH) containing immobilized sense or antisense strands of cyclin D1 DNA. Briefly, a 4-kb *Bam*HI fragment containing the 3' coding region of cyclin D1 was isolated from a C3H mouse genomic library and subcloned into pBluescript (Stratagene, La Jolla, CA). XL1-Blue *Escherichia coli* were transformed with pBluescript phagemids supplied with genomic cyclin D1 inserts, and single-stranded DNA was rescued after infection with M13K07 helper phage (Stratagene). Three micrograms of DNA were transferred to membranes using a slot blot apparatus. Membranes were UV crosslinked and hybridized as described above.

RESULTS

Cell Cycle Expression of the Cyclin D Genes

To determine the expression pattern of the D-type cyclin genes as cells progressed through the cell cycle, quiescent density-arrested Balb/c-3T3 fibroblasts were stimulated by the addition of fresh medium containing 10% calf serum and 10 ng/ml PDGF. Under these conditions, $\sim 95\%$ of the cells entered the cell cycle and began DNA synthesis after a minimum G1 interval of 12 h (Figure 1A). At various times after stimulation, total RNA was isolated and analyzed by Northern blotting with partial cDNA probes for cyclin D1, D2, D3, and the constitutive 1B15. As seen in Figure 1B, quiescent cells expressed a low basal level of cyclin D1 mRNA. However, message levels of D1 increased dramatically within 4 h after

Figure 1. Expression of cyclin D genes during the cell cycle. (A) Density-arrested Balb cells were stimulated with 10% serum, 10 ng/ml PDGF-BB, and 5 μ Ci/ml [³H] thymidine. At the times indicated, cells were fixed and processed for autoradiography (MATERIALS AND METHODS), and the percentage of labeled nuclei was determined. (B) Density-arrested Balb/c-3T3 cells were stimulated by the addition of fresh medium containing 10% serum and 10 ng/ml PDGF-BB. The last three lanes (+CHX) represent cells stimulated with serum and PDGF in the presence of 1 μ g/ml cycloheximide. Cells were harvested at the times indicated, and total mRNA was isolated as described under MATERIALS AND METHODS. RNA was examined by Northern analysis using the probes indicated. Blots hybridized with cyclin D1 probe were autoradiographed for 24 h, whereas blots hybridized with cyclin D2 or D3 probes were autoradiographed for 48 h.

stimulation. Abundance of cyclin D1 transcript peaked at the midpoint of G1, 6 h before the onset of S phase, and decreased before cells began DNA replication. Thus, the kinetics of cyclin D1 induction in Balb fibroblasts are consistent with an early to mid-G1 regulation of D1 mRNA expression.

Previously it has been reported that induction of cyclin D1 in several cell lines is at least partially dependent on protein synthesis (Matsushima *et al.*, 1991; Won *et al.*, 1992). To test whether the serum/PDGF-mediated

Figure 2. Expression of cyclin D1 and D3 in V point-arrested cells. Density-arrested Balb/c-3T3 cells were incubated for 50 h in amino acid-deficient DMEM containing 1.5% dFBS as described in MATERIALS AND METHODS. Prestarved cells were then stimulated in amino acid-deficient medium (Deficient) or standard DMEM (Complete) supplemented with 10% dFBS and 10 ng/ml PDGF-BB. Total mRNA was isolated at various times after stimulation and analyzed by Northern blotting using the probes indicated. Parallel cultures were stimulated in the presence of 5 μ Ci/ml [3 H] thymidine and processed after 30 h for autoradiography.

increase of D1 mRNA required de novo protein synthesis in Balb cells, fibroblasts were stimulated in the presence of cycloheximide. Treatment of cells with 1 μ g/ml cycloheximide blocked 100% of serum-stimulated protein synthesis as measured by incorporation of [35 S] methionine into acid precipitable counts. In addition, this concentration of cycloheximide completely abolished the late G1 induction of cdc2 mRNA normally seen at 12 h after serum stimulation. However, in contrast to results observed in the mouse macrophage cell line BAC1.2F5A (Matsushima *et al.*, 1991), the presence of cycloheximide did not reduce the serum-stimulated increase of D1 message in Balb fibroblasts (Figure 1B). In fact, cells stimulated in the presence of cycloheximide exhibited a greater induction of D1 transcript than cells treated with serum and PDGF alone, particularly at the later time points of 10 and 12 h when message levels are normally in decline. Treatment of cells with cycloheximide alone had no effect on D1 expression. Thus, induction of cyclin D1 mRNA can occur independently of new protein synthesis. These results indicate that translation of the immediate early gene class, including *c-fos* and *c-myc*, is not required for the expression of D1. Furthermore, as the addition of cycloheximide prevents entry into the cell cycle (Stiles *et al.*, 1979), these data suggest that induction of D1 is not dependent on traverse through G1.

To compare the expression of cyclin D1 to that of other D-type cyclin genes, the Northern blot was stripped and reprobed for cyclin D2 and D3 (Figure 1B). Unlike D1, transcripts for both cyclin D2 and D3 were expressed at relatively high levels in quiescent unstimulated cells. Cyclin D2 displayed a constitutive mRNA expression pattern that was unchanged by the addition of serum and PDGF in the presence or absence of cy-

cloheximide. Thus, cyclin D2 activity is apparently not regulated at the transcriptional level in Balb fibroblasts. In contrast to D2, cyclin D3 mRNA was weakly induced after stimulation, peaking near the G1/S phase boundary. Unlike cyclin D1, increase in D3 message was ablated by cycloheximide treatment. These results demonstrate expression of all three cyclin D genes during G1 in Balb/c-3T3 fibroblasts. However, transcripts for cyclin D1, D2, and D3 are expressed differentially in regard to cycloheximide sensitivity and the time course of induction.

To further dissect the difference between the kinetics of D1 and D3 message induction, the following experiment was performed. Quiescent Balb cells were starved in amino acid-deficient medium for 50 h before stimulation with 10% dFBS and 10 ng/ml PDGF. Cells that are stimulated in amino acid-deficient medium under these conditions arrest at the midway position of G1 (V point), 6 h before the onset of DNA synthesis, whereas cells stimulated in complete medium after starvation progress through G1 and enter S phase with normal kinetics (Olson *et al.*, 1993). Using this system, ~95% of the cells stimulated in deficient medium arrested during G1 and failed to initiate DNA replication (Figure 2). Cyclin D1 mRNA was expressed at comparable levels and with similar kinetics in both cells that blocked at the V point and cells that continued to cycle. Thus, induction of cyclin D1 transcript occurs primarily during the first half of G1. In contrast, cyclin D3 message was not induced in cells that arrested at mid-G1. A small increase in D3 transcript was observed several hours after cells were released from the V point. These data suggest that induction of cyclin D1 mRNA is an early or mid-G1 event, whereas induction of D3 is dependent upon progression into late G1.

Growth Factor Regulation of D1 Expression

The RNA analyzed by Northern blotting in Figures 1 and 2 was isolated from cells stimulated with both serum and PDGF to ensure that a maximal number of fibroblasts entered the cell cycle. However, as shown in Figure 3A, a strong induction of D1 message was observed in cells treated with either 10% serum alone or 25 ng/ml PDGF in medium containing 0.05% serum. No increase in D1 transcript was seen in cells placed in low serum in the absence of PDGF. Kinetics of D1 induction were similar in cells treated with PDGF, serum, or a combination of both. However, the rate of decline in D1 mRNA was much slower in cells receiving only PDGF; elevated levels of D1 transcript were observed ≥ 15 h after PDGF stimulation, whereas D1 message decreased to near basal levels between 9 and 12 h after exposure to serum. In contrast to D1 expression, message levels of cyclin D2 and D3 were not affected by PDGF stimulation (unpublished data). Regulation of cyclin D1 expression by PDGF was not isoform specific as PDGF-AA increased D1 message to comparable levels and with similar kinetics as seen in those cells treated with the BB isoform (unpublished data). In contrast to PDGF, PPP, which does not contain any PDGF isoforms, elicited a very weak induction response when used at concentrations $\leq 10\%$ (Figure 3A). These data suggest that PDGF is one of the primary components of serum responsible for the serum-mediated regulation of D1 mRNA abundance in Balb fibroblasts.

Because Balb cells stimulated with PDGF in the absence of plasma-derived progression factors do not begin DNA synthesis, it is apparent that upregulation of cyclin D1 message is not sufficient for entry into S phase. Furthermore, considerable amounts of cyclin D2 and D3 mRNA are present in quiescent as well as PDGF-stimulated cells (Figure 1B and unpublished data), indicating that simultaneous expression of all three cyclin D genes is not sufficient to stimulate cell growth. One possible explanation is that additional regulatory steps occur at the translational and/or posttranslational levels that are controlled by mitogens other than PDGF. To determine whether PDGF increased the abundance of cyclin D1 protein as well as D1 mRNA, cells were stimulated for various times with 25 ng/ml PDGF or 10% calf serum, and whole cell lysates were analyzed by Western blotting using a polyclonal anti-D1 antibody. As shown in Figure 3B, quiescent Balb cells expressed little, if any, D1 protein. However, highly elevated D1 protein levels were detected within 6 h after treatment of cells with either serum or PDGF. Induction of D1 protein was temporally similar to increase in transcript; however, the peak in D1 protein occurred from 9 to 12 h, somewhat later than the 6- to 9-h peak in D1 mRNA. Protein levels remained elevated up to 15 h after PDGF stimulation but decreased slightly earlier in cells treated with 10% serum. These results suggest that although

Figure 3. Induction of cyclin D1 mRNA and protein by serum and PDGF. (A) Density-arrested Balb/c-3T3 cells were stimulated with fresh medium containing either 10% calf serum (CS) + 10 ng/ml PDGF-BB, 10% CS, 25 ng/ml PDGF-BB + 0.05% CS, or 10% PPP. Total mRNA was isolated at the times indicated and analyzed by Northern blotting with probes for cyclin D1 and cyclophilin (1B15). (B) Density-arrested cultures were incubated with medium containing either 25 ng/ml PDGF-BB + 0.05% CS or 10% serum. At the times indicated, whole cell lysates were prepared and analyzed by Western blotting as described in MATERIALS AND METHODS.

induction of D1 mRNA occurs primarily before the V point, cyclin D1 protein could function at a later point in G1.

Stimulation of fibroblasts with serum consistently activated D1 message expression to levels only slightly less than those observed with 25 ng/ml PDGF (Figure 3A). However, the concentration of PDGF in medium containing 10% calf serum is <1 ng/ml (Bowen-Pope *et al.*, 1989). Dose response studies demonstrated that induction of D1 transcript was minimal in cells treated with 5 ng/ml or less PDGF (unpublished data). These results suggest that PDGF may act synergistically with other serum-derived factors to increase cyclin D1 mRNA expression to maximum levels. In support of this, cells

treated with both PDGF and serum exhibited a reproducibly higher induction of D1 mRNA than cells stimulated with either PDGF or serum alone. Furthermore, PPP did increase D1 expression, albeit weakly, suggesting that factors other than PDGF are capable of regulating D1 message levels.

Because the mitogenic activity of PPP on competent Balb cells can be replaced by the concerted action of EGF and IGF-1, we examined the expression of D1 mRNA in response to these factors, both alone and in combination with PDGF. Similar to the effect seen with plasma treatment, progression factors EGF and IGF-1 stimulated D1 message expression weakly when added to culture medium in the absence of PDGF (Figure 4A). Abundance of the D1 transcript increased faintly at 3 h after exposure to IGF-1 before returning to basal levels at 6 h and later time points (Figure 4A). EGF treatment increased D1 message in a manner that differed from PDGF in both kinetics of induction and intensity of response. As shown in Figure 4A, activation of D1 gene expression was considerably less after EGF stimulation than with PDGF. In addition, the EGF-mediated increase in D1 mRNA peaked at 12 h whereas the PDGF-stimulated peak occurred earlier at 6 h. These findings suggest that different growth factors may act through distinct pathways in the regulation of cyclin D1 message levels.

To assess the ability of progression factors to synergize with PDGF in the stimulation of D1 mRNA expression, cells were treated with 10% PPP in the presence and absence of 5 ng/ml PDGF. Cultures were harvested 6 h after stimulation when PDGF- and serum-treated cells normally exhibit maximum induction of D1 message. As shown in Figure 4B, D1 mRNA was barely detectable by Northern analysis when cells were treated with low doses of PDGF. An equally weak response was observed in cells stimulated with high concentrations of PPP (10%). In contrast, high levels of D1 transcript were seen in cells treated with 10% PPP in the presence of PDGF. Induction of D1 message after treatment with a combination of both PPP and PDGF was greater than that predicted for an additive response. Thus, plasma-derived factors can synergize with PDGF to upregulate the expression of cyclin D1 transcript. To further characterize the cooperative induction of cyclin D1 mRNA, cells were treated with defined progression factors EGF or IGF-1 or both in the presence of 5 ng/ml PDGF. As seen in Figure 4B, both EGF and IGF-1 acted synergistically with PDGF to stimulate expression of D1 message. Furthermore, a combination of both EGF and IGF-1 was as effective as PPP in coordinately upregulating D1 message expression with PDGF. Thus, the three principle growth factors that regulate progression of Balb/c-3T3 cells from a quiescent state to mid-G1 also act in a cooperative fashion to stimulate cyclin D1 mRNA expression.

Figure 4. Regulation of D1 mRNA expression by EGF and IGF-1. (A) Density-arrested Balb/c-3T3 cells were refed with medium containing 0.05% CS and either 25 ng/ml PDGF-BB, 25 ng/ml EGF, or 25 ng/ml IGF-1. Cultures were harvested for Northern analysis at the times indicated and probed for cyclin D1 and 1B15. (B) Density-arrested Balb cells were incubated in medium containing 0.05% CS and various combinations of PDGF-BB (5 ng/ml), EGF (25 ng/ml), IGF-1 (25 ng/ml), and PPP (10%). Cultures were harvested 6 h after stimulation, and total mRNA was probed for cyclin D1 and 1B15 after Northern blotting.

Effect of PKC Depletion on PDGF Induction of Cyclin D1 mRNA

12-O-tetradecanoylphorbol 13-acetate (TPA) is a phorbol ester that has been shown to modulate the growth of Balb fibroblasts by inducing a state of competence (Frantz *et al.*, 1979). As shown in Figure 5A, TPA dramatically increased D1 mRNA expression 6–9 h after stimulation to levels comparable to that observed with PDGF or serum treatment. These results are consistent with a previous study describing TPA induction of D1 expression in diploid human fibroblasts (Won *et al.*, 1992). Mitogenic response to TPA is mediated by the calcium/phospholipid-dependent PKC (Castagna *et al.*, 1982), an enzyme that is also activated by PDGF (Kazlauskas *et al.*, 1988). We therefore sought to determine whether PKC was a necessary component of the PDGF-activated signaling pathway that upregulates expression of cyclin D1 mRNA.

Previously it has been shown that PKC activity is ablated by chronic exposure of cells to high concentrations of TPA (Rodriguez-Pena and Rozengurt, 1985). In our experiments, cells were pretreated for 36 h with 400 nM TPA. No PKC isozymes were detectable by

Figure 5. Effect of PKC depletion on induction of cyclin D1 mRNA by PDGF and serum. (A) Density-arrested Balb/c-3T3 cells were refed with medium containing 200 nM TPA + 0.05% CS. Cultures were harvested at various times after stimulation, and total mRNA was analyzed by Northern blotting with probes for cyclin D1 and cyclophilin (1B15). (B) Density-arrested Balb cells were incubated for 36 h in medium containing either 0.05% serum (Pretreat -; lanes 1, 3, 5, and 7) or 0.05% serum + 400 nM TPA (Pretreat +; lanes 2, 4, 6, and 8). Pretreated cells were harvested at time 0 (lanes 1 and 2) or stimulated for 6 h in medium containing 0.05% CS and either 25 ng/ml PDGF-BB, 10% CS, or 200 nM TPA. Total mRNA was analyzed by Northern blotting using probes for cyclin D1 and cyclophilin (1B15).

Western analysis after TPA pretreatment (Coats *et al.*, unpublished data). Although D1 expression was transiently increased during pretreatment with TPA, abundance of D1 transcript had returned to basal levels by 36 h after stimulation (Figure 5B). As expected, restimulation of cells with TPA after pretreatment did not increase D1 message levels. Similarly, induction of D1 mRNA expression by PDGF or serum was severely diminished in cells lacking PKC. The PDGF-mediated increase in D1 mRNA was reduced by an average of 75% in PKC-depleted cells, whereas the D1 response to serum stimulation was completely abrogated. These results are consistent with a role for PKC in growth factor-mediated expression of cyclin D1 transcript.

Regulation of D1 Transcription by Serum and Cycloheximide

Treatment of Balb cells with cycloheximide alone did not affect D1 expression. However, as seen in Figure 1A, inhibition of protein synthesis resulted in a super-induction of D1 message after stimulation of cells with serum and PDGF. Thus, a protein synthesis inhibitor can act in a cooperative manner with serum-derived factors to increase cyclin D1 transcript levels. The response to cycloheximide after serum stimulation was

most dramatic at later time points when D1 message is normally in decline. To examine the effect of cycloheximide on the temporal regulation of cyclin D1 expression, a more detailed time course was performed. Quiescent fibroblasts were stimulated by the addition of fresh medium containing 10% serum in the presence and absence of 1 μ g/ml cycloheximide. As shown in Figure 6A, cycloheximide did not alter the early kinetics of serum-mediated D1 mRNA induction. At 1 and 2 h after serum stimulation, no differences were observed in D1 expression between cells that received cycloheximide and those that did not. In contrast, cells treated with both serum and cycloheximide maintained highly elevated levels of D1 message for ≥ 15 h, whereas D1 expression decreased to near basal levels after the same time period in cells stimulated with serum alone. These data indicate that protein synthesis is required for the downregulation of cyclin D1 transcript after induction by serum-derived growth factors.

Cycloheximide and growth factors regulating the expression of cyclin D1 could promote accumulation of the D1 message by influencing transcription, stability, and/or mRNA processing. To determine whether transcriptional activation contributed to increased steady-state levels of D1 mRNA, cells were stimulated with 10% serum for 0, 5, or 15 h. At the appropriate time, cells were harvested, and nuclei were isolated for analysis in transcriptional run-on assays. [32 P]-labeled tran-

Figure 6. Effect of cycloheximide on induction of cyclin D1 mRNA by serum. (A) Density-arrested Balb/c-3T3 cells were stimulated with 10% CS or 10% serum + 1 μ g/ml cycloheximide (CS + CHX). Cultures were harvested at the indicated times, and total mRNA was analyzed by Northern blotting using probes for cyclin D1 and 1B15. (B) Density-arrested Balb cells were treated with 10% serum or 10% serum and 1 μ g/ml cycloheximide (+CHX). Cultures were harvested at the indicated times, and nuclear run-on assays were performed as described under MATERIALS AND METHODS. Labeled transcripts were hybridized to single-stranded DNA templates of cyclin D1 anti-sense (as), cyclin D1 sense (s), or Bluescript vector control (vector).

scripts synthesized in the run-on reactions were hybridized to membranes containing single-stranded DNA fragments derived from a genomic clone of cyclin D1. As shown in Figure 6B, D1 transcription was barely detectable above background hybridization in quiescent cells. However, an approximate sixfold transcriptional increase was observed in cells stimulated for 5 h with serum. We have detected a similar transcriptional increase in cells treated with 25 ng/ml PDGF and 0.05% serum. In contrast, nonspecific hybridization to sense strand and vector DNA controls was not affected by serum addition. At 15 h after stimulation, D1 transcription decreased near basal levels observed in untreated cells. Thus, the pattern of serum-stimulated transcriptional activity closely paralleled the accumulation of D1 message detected on Northern blots. These results demonstrate that at least one component of the serum-mediated increase in D1 message is the upregulation of cyclin D1 transcription.

A cycloheximide-mediated stabilization of D1 mRNA could maintain elevated levels of the D1 message after the transcriptional increase subsided. Alternatively, protein synthesis inhibition might prevent repression of D1 transcription and allow accumulation of the message through continued synthesis of the D1 transcript at later time points. As shown in Figure 6B, treatment of Balb cells with cycloheximide did alter the transient induction of D1 transcription by serum. Cells exposed to fresh serum in the presence of cycloheximide for 5 h displayed greater transcription of D1 than cells stimulated with serum alone. Furthermore, transcriptional activity remained elevated for ≥ 15 h in cells receiving cycloheximide, whereas minimal transcription of D1 was observed after the same time period in cells stimulated with serum in the absence of protein synthesis inhibitors. These results suggest that serum-inducible protein(s) are required for the direct or indirect repression of D1 transcription after growth factor-mediated activation.

In the above experiments, cells were exposed to cycloheximide at the time of serum stimulation. However, to ensure that protein synthesis was not induced by serum before the translational machinery of the cell was inactivated, we also examined the mRNA expression of D1 in cells pretreated with cycloheximide for ≤ 6 h before the addition of serum. Identical results were obtained from cells pretreated with cycloheximide and those that received cycloheximide simultaneous with the addition of fresh serum. In contrast, a large difference in cyclin D1 message expression was observed when cells were exposed to cycloheximide at various times after serum stimulation (Figure 7). High levels of D1 transcript were detected 15 h after serum treatment when cells received cycloheximide at any time during the first three h of stimulation. However by 4 h after serum induction, cells were completely refractory to the effect of cycloheximide on D1 expression. Since mRNA

Figure 7. Time course of cycloheximide addition. Density-arrested Balb/c-3T3 fibroblasts were stimulated with 10% calf serum. Cycloheximide (1 μ g/ml) was added to culture medium at indicated times after serum stimulation. The last lane (–) was stimulated with 10% serum but did not receive cycloheximide. The first lane control (Con) did not receive serum or cycloheximide. All cultures were harvested after 15 h. Total RNA was isolated and analyzed by Northern blotting using probes for cyclin D1 and 1B15.

levels of D1 do not peak until 6 h after treatment with serum, it is unlikely that the loss of the superinduction response occurred because transcription of D1 was already shut down at the time of cycloheximide addition. In fact, elevated levels of D1 transcription were detectable by nuclear run-on assay ≥ 5 h after serum stimulation (Figure 6B). These data suggest that negative regulatory proteins influencing transcription of the cyclin D1 gene are synthesized 3–4 h after serum stimulation of quiescent Balb fibroblasts.

DISCUSSION

Cyclin D1 is overexpressed in a variety of tumors including parathyroid adenomas (Motokura *et al.*, 1991), breast and squamous cell carcinomas (Lammie *et al.*, 1991), and certain B-cell malignancies (Withers *et al.*, 1991), suggesting that deregulated expression of D1 may contribute to abnormal cell proliferation. In cases thus far documented, activation of the D1 oncogene appears to result from an increase in cyclin D1 mRNA. Overexpression of D1 message in breast and esophageal cancers involves genomic amplification of the D1 locus (Lammie *et al.*, 1991), whereas in parathyroid adenomas and B-cell leukemias a DNA fragment containing the cyclin D1 gene is juxtaposed against potent enhancer elements after chromosomal inversion or translocation (Tsujiimoto *et al.*, 1986; Rosenberg *et al.*, 1991). Interaction of the D1 promoter with these enhancer elements may induce D1 expression through a direct transcriptional activation. Alternatively, deletion of the normal 5' regulatory region of the D1 gene may allow the accumulation of D1 transcript through the elimination of negative control elements.

Recently it has been shown that expression of cyclin D1 is required for the normal cell cycle progression of nontransformed fibroblasts (Baldin *et al.*, 1993). However, the factors that control D1 expression and the level at which they act is not known. In the present study we have shown that the abundance of cyclin D1 mRNA

is regulated, at least in part, at the level of transcription in the nontransformed Balb/c-3T3 fibroblast cell line. Transcription of the D1 gene increased markedly in response to stimulation of quiescent cells with calf serum or PDGF. Transient induction of D1 transcription after growth factor treatment was reflected by a 30-fold increase in steady-state abundance of D1 message, whereas the subsequent decrease in transcription at later time points correlated with a return of steady-state mRNA levels to basal amounts. Interestingly, inhibition of protein synthesis 3–4 h after serum stimulation blocked the production of factors required for the downregulation of D1 transcription after peak induction. Thus, the overall rate of cyclin D1 transcription is governed by negative as well as positive regulatory signals. Our data indicate that a superinduction of D1 mRNA can result from a disruption of those negative regulatory events that decrease D1 transcription. Loss of potential *cis*-acting response elements that mediate the activity of factors repressing D1 transcription may contribute to the overexpression of D1 message observed in neoplastic cell lines after genomic rearrangement.

Previously a set of PDGF-inducible genes termed immediate-early or competence genes has been described (Lau and Nathans, 1987). This set of genes includes the proto-oncogene transcription factors *c-fos*, *c-jun*, and *c-myc*. As the expression of these growth regulatory proteins temporally precedes that of cyclin D1 and transcription of the D1 gene is increased in response to PDGF treatment, it might be expected that one or more of the immediate-early gene products regulates the activation of the cyclin D1 promoter. However, the growth factor-stimulated increase of cyclin D1 transcription in Balb cells was not reduced in the presence of protein synthesis inhibitors. Therefore translation of the immediate-early genes is not required for increased expression of D1. This conclusion is supported by the observation that introduction of a plasmid encoding an activated *c-myc* protein into Balb/c-3T3 fibroblasts increases the levels of cyclin E but does not induce the expression of cyclin D1 (Jansen-Durr *et al.*, 1993).

As induction of the competence gene class itself is independent of protein synthesis, activation of D1 transcription may be regulated by those factors that govern the expression of certain immediate-early genes. However, the accumulation of cyclin D1 mRNA occurs much later than is typical for competence-associated genes, suggesting that additional regulatory steps may act after transcriptional induction to control the steady-state abundance of cyclin D1 message. We have attempted to determine the half-life of the cyclin D1 transcript using Northern analysis to follow the decay of D1 message in the presence of the RNA synthesis inhibitor actinomycin D. No difference in the half-life of cyclin D1 mRNA was observed in the presence or absence of growth factor, in the presence or absence of cyclohex-

imide, or at different points in the cell cycle. However, cyclin D1 message levels did not decrease appreciably ≤ 8 h after addition of the transcriptional inhibitor, suggesting that actinomycin D itself stabilized the message and prevented an accurate determination of D1 transcript half-life. A similar effect was observed using the RNA synthesis inhibitor 5,6-dichlorobenzimidazole riboside. We therefore are unable to conclude whether an altered mRNA stability may contribute to increased D1 message levels after growth factor stimulation of Balb cells. However, the observation that D1 gene transcription increased four- to sixfold in response to serum or PDGF whereas total mRNA levels increased >30 -fold supports the existence of multiple levels of regulation for D1 expression.

The finding that growth factor-mediated induction of cyclin D1 transcription is not inhibited by cycloheximide indicates that upregulation of the D1 message is signaled through proteins present in quiescent cells. The ability of the phorbol ester TPA to increase D1 mRNA levels suggests that the cellular receptor for TPA, PKC (Castagna *et al.*, 1982), may play a role in signaling D1 induction. PKC, a serine/threonine protein kinase, is also activated by PDGF (Kazlauskas *et al.*, 1988), and desensitization of the PKC pathway has previously been shown to attenuate the PDGF-mediated induction of *c-myc* expression (Coughlin *et al.*, 1985). Several isoforms of PKC are present in unstimulated Balb cells. Depletion of all detectable PKC isozymes considerably reduced induction of cyclin D1 mRNA by PDGF, serum, and TPA, suggesting that a PKC-dependent signaling pathway mediates growth factor-stimulated expression of cyclin D1. It should be noted, however, that failure to activate D1 expression in PKC-depleted cells might result from prior activation of the signaling pathway during TPA pretreatment and may not reflect a requirement for PKC activity. We have demonstrated that D1 transcription is negatively regulated by serum inducible-proteins after activation. If such proteins are also induced by TPA during pretreatment, it is possible they could prevent an increase in D1 message levels after restimulation with serum or PDGF. However, such a negative regulatory activity would have to be extremely stable, because PDGF- and serum-inducible expression of D1 was inhibited for ≥ 48 h after initial TPA treatment. In argument against this theory, we have observed a serum-stimulated increase in D1 mRNA in cells that were released from an early S phase arrest after pretreatment for 24 h with serum, PDGF, and methotrexate (unpublished data). Thus it appears that a PKC-dependent signaling pathway is required for maximal D1 message expression in response to PDGF.

Both cyclin D1 and D3 exhibited a periodic induction of mRNA expression during the G1 period in Balb fibroblasts. Although it has been reported that cyclins D1 and D3 are similarly regulated during late G1 in human diploid fibroblasts (Won *et al.*, 1992), we have found

that D1 and D3 are independently regulated during the G1 interval of Balb/c-3T3 cells. PDGF selectively increased cyclin D1 message levels in Balb fibroblasts but did not affect the expression of cyclin D3. Furthermore, induction of D1 mRNA temporally preceded that of cyclin D3 by several hours. Analysis of cyclin D expression in V point-arrested cells confirmed that D1 message expression was regulated primarily during the first half of G1, whereas induction of the D3 transcript was a later event. The independent regulation of cyclins D1 and D3 through pathways activated several hours apart suggests that D1 and D3 may function to control cell growth through distinct mechanisms or at different points in the cell cycle. Recently it has been shown that cyclins D2 and D3 can associate with the growth regulatory retinoblastoma protein (Rb), and that both D2 and D3 activate the Rb kinase cdk2 (Ewen *et al.*, 1993; Kato *et al.*, 1993). In contrast, cyclin D1 only weakly binds Rb and does not activate cdk2 (Ewen *et al.*, 1993; Kato *et al.*, 1993). Thus, cyclins D2 and D3 could function to remove an Rb-dependent block to S phase entry, whereas D1 may act through a different mechanism. Our results demonstrate distinct cell cycle regulation of the D-type cyclins and are consistent with separate functions for cyclins D1 and D3 during G1 in Balb fibroblasts.

In SAOS-2 cells transfected with a plasmid encoding wild-type Rb, overexpression of cyclin D2, as well as cyclins E and A induces a hyperphosphorylation of Rb and reverses an Rb-dependent growth arrest (Hinds *et al.*, 1992; Ewen *et al.*, 1993). In contrast, cyclin D1 effects a morphological change in SAOS-2 cells correlated with the initiation of cell growth but does not induce phosphorylation of Rb or release of the Rb protein from nuclear tethering, both markers of progression through late G1 (Hinds *et al.*, 1992; Ewen *et al.*, 1993). Cyclin D1 may functionally bypass the requirement for Rb inactivation by activating a growth regulatory pathway directly downstream of Rb. However, D1 is considerably less efficient than cyclins D2, E, and A in overcoming an Rb-dependent block to S phase entry (Hinds *et al.*, 1992; Ewen *et al.*, 1993). It is possible therefore that SAOS-2 cells expressing cyclin D1 initiate growth stimulatory processes and thus reverse phenotypic changes associated with an Rb-induced quiescent state but lack the ability to complete G1 and begin DNA replication in the absence of additional cyclins. The data presented in the current study support such a model. Balb/c-3T3 fibroblasts expressing cyclin D1 protein in response to PDGF treatment activate the growth process but fail to transit the G1/S phase boundary in the absence of plasma-derived progression factors. Hence expression of the cyclin D1 protein, at least at levels induced by PDGF, is not sufficient to stimulate G1 traverse and entry into S phase.

It is not known whether the D1 protein produced in response to PDGF associates with its catalytic subunit

cdk4 (Matsushime *et al.*, 1992) or whether such a cyclin/kinase complex would be active. Although we have found the cdk4 protein to be constitutively expressed during the Balb fibroblast cell cycle (unpublished data) and therefore present at the time of D1 induction by PDGF, progression factors may be required to promote complex formation between cyclin D1 and cdk4 or to activate this complex through specific posttranslation modifications. Thus, it remains formally possible that expression of a functional D1 activity is sufficient to stimulate DNA synthesis although expression of the D1 protein is not. In this regard it is interesting to note that progression factors EGF, IGF-1, and PPP acted synergistically with low concentrations of PDGF to upregulate cyclin D1 mRNA expression. D1 levels may therefore be higher under conditions that allow cell cycle traverse. Such a superinduction might be biologically relevant if a threshold level of cyclin D1 must be attained before an active cyclin/kinase complex is produced.

Paradoxically, progression factors may also be required for the efficient downregulation of cyclin D1. Abundance of the D1 transcript remained elevated above basal levels much longer after initial stimulation in those cells treated with PDGF alone when compared to cells treated with serum or a combination of serum and PDGF. This delayed kinetics of cyclin D1 mRNA decline in PDGF-treated cells was paralleled at the level of D1 protein. It has been hypothesized that D1 must be effectively downregulated to allow progression into S phase (Baldin *et al.*, 1993). Thus, it may be the function of progression factors that promote G1 traverse in PDGF-stimulated cells to decrease D1 levels after peak induction. Although it remains unclear how these growth factors might perform the dual function of increasing and decreasing cyclin D1 levels, our data invoke a model of negative feedback regulation whereby the cooperative action of PDGF and plasma factors may be required to produce an active cyclin/kinase complex that could then "loop back" to terminate D1 expression. As deregulated expression of cyclin D1 mRNA may contribute to the uncontrolled growth of several malignant cell types, it will be of interest to elucidate both the positive and negative mechanisms that control normal expression of the cyclin D1 transcript in a non-transformed cell line such as the Balb/c-3T3 fibroblasts.

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