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Cyclin D1 Messenger RNA Is Inducible by Platelet-derived Growth Factor in Cultured Fibroblasts[1]

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ABSTRACT

In fibroblasts in culture, the levels of cyclin D1 mRNA are growth regulated. In mouse and in human fibroblasts, both serum and platelet-derived growth factor increase cyclin D1 mRNA levels with similar kinetics of induction. Insulin-like growth factor 1 by itself does not induce cyclin D1 expression, and an antisense oligodeoxynucleotide to the insulin-like growth factor 1 receptor RNA does not affect the growth-regulated levels of cyclin D1 mRNA.

INTRODUCTION

Cyclins are proteins originally discovered for their important role in the early cleavage divisions of marine invertebrate eggs (1). Two types of cyclins, A and B, are known to be necessary for the normal regulation of mitosis in yeast as well as in mammalian cells (2, 3). In addition to these M-phase cyclins, another class of related proteins known as G_1 cyclins has been identified. In *Saccharomyces cerevisiae*, at least three G_1 cyclins are known to exist that exhibit limited but significant sequence similarities to M-phase cyclins (4, 5). Recently, G_1 cyclins have also been identified in mammalian cells. A cyclin D1 has been isolated (and sequenced) from a complementary DNA library prepared from a human glioblastoma cell line (6) and three more G_1 cyclins were identified in mouse cells (7). One of these murine cyclins is the mouse counterpart of the human D1 cyclin and is inducible in macrophages by colony-stimulating factor 1 (7).

Mammalian D-type cyclins are thought to be putative oncogenes (5, 8, 9). Indeed, overexpression of a human cyclin D1 (*PRAD1*) has been found in parathyroid tumors (8). This gene is also amplified and overexpressed in primary breast cancer and squamous cell carcinoma lines (9). Cyclin D1 is genetically linked to the *bcl-1* locus, which is rearranged in some B-cell leukemias (10).

Although mammalian D-type cyclins have been cloned through their ability to complement yeast mutants defective in the expression of cyclin genes important in G_1-S transition, the involvement of G_1 cyclins in the progression of mammalian cell cycle is not yet understood. Cyclin D1 (both murine and human) can bind *cdc2* or *cdc2*-related proteins (7, 8), but the functional role of these complexes in cell proliferation has not been elucidated.

The growth of cells in culture is regulated by the growth factors in the environment; fibroblasts and other cells usually require at least two growth factors for optimal growth. PDGF[2] and IGF-1 are the two growth factors necessary for the growth of fibroblast-like cells such as 3T3 cells (11, 12). Other cells may have different requirements, but many types of cells require IGF-1 for growth (for a review, see Ref. 13). In a previous paper (14), we have shown that the levels of *cdc2* mRNA are regulated by IGF-1; in this paper, we show that PDGF increases the levels of cyclin D1 mRNA, whileIGF-1 has no effect.

MATERIALS AND METHODS

Cell Lines and Stimulation of Cell Proliferation by Growth Factors and Inhibition of Growth by Antisense Oligodeoxynucleotides. In this study we used BALB/c 3T3 mouse fibroblasts, human WI-38 fibroblasts, and HL-60 human promyelocytic cell line (15).

BALB/c 3T3 cells were grown as previously described (12) in DMEM containing 5% calf serum and 5% fetal calf serum. BALB/c 3T3 cells were made quiescent by plating at a concentration of 5×10^5/100-mm plate and allowing growth for 24 h before replacing the growth medium with DMEM supplemented with 1% calf serum for 72 h. Quiescent 3T3 cells were stimulated with combined or individual growth factors: PDGF (GIBCO BRL), 1 ng/ml; and IGF-1 (GIBCO BRL), 50 ng/ml.

WI-38 fibroblasts were plated at a concentration of 1×10^6/100-mm plate in minimum essential medium containing 10% fetal bovine serum and grown for 10 days to reach quiescence. Quiescent cells were stimulated with minimum essential medium containing 20% fetal bovine serum or with individual growth factors: PDGF (5 ng/ml) or IGF-1 (50 ng/ml). For antisense experiments cells were treated first with 40 μg/ml of missense or antisense oligonucleotides 24 h before stimulation and then with another 20 μg/ml of oligonucleotides 2 h before stimulation. The sequences of the oligodeoxynucleotides are given below.

HL-60 cells were plated at a concentration of 5×10^5 in 10 ml of DMEM containing 10% heat-inactivated fetal calf serum. Twenty-four h after plating cells were treated with either antisense or sense/missense oligonucleotides for 48 h; 40 μg/ml of oligonucleotides were added for 24 h, and then 20 μg/ml of the same oligonucleotides were added for the next 24 h. In this study we used phosphorothioate oligodeoxynucleotides.

As antisense to the hIGF-1R mRNA we used oligomer 5′ CTG CTC GTC TGC TAT GAA GA 3′; as missense to the hIGF-1R mRNA oligomer 5′ CTC CTC GTC TGC TAT GAA GA 3′ was used. We also used oligomers 5′ GTG CCG GGG TCT TCG GC 3′ and 5′ GCC CGC AGA CCC CGG CAC 3′ (16) as antisense and sense to c-*myb* mRNA, respectively. To evaluate efficacy of antisense oligonucleotides in inhibition of cell growth, we determined the number of cells in populations treated with antisense or sense/missense oligomers. We established that antisense to hIGF-1R mRNA decreased growth of HL-60 by 60% and antisense to c-*myb* mRNA inhibited growth by 85%, both compared with the effects observed after addition of sense or missense.

RT-PCR. RT-PCR was performed as described before (17). Since the sequences of human and mouse cyclin D1 complementary DNA are very similar (6, 7), we were able to design and use the same amplimers and the same probe for detection of either human or mouse cyclin D1 mRNA. Amplimers used were 5′ ACC TGG ATG CTG GAG GTC TG 3′ and 5′ GAA CTT CAC ATC TGT GGC ACA 3′. Oligomer 5′ CTG GCC ATC AAC TAC CTG GAC 3′ was used as the probe in Southern analysis of PCR amplification products. To evaluate the amount of input RNA in RT-PCR analysis, we performed parallel RT-PCR reaction to detect *pHe7* mRNA. *pHe7* is a gene coding for a ribosomal protein, the mRNA level of which is constant during the cell cycle and is not decreased in growth-inhibited cells. Amplimers used for detection of *pHe7* mRNA were 5′ ACC ATT GAT TCT GTT ACT TC 3′ and 5′ ATA CTC TGT GAC ATT CTT AA 3′. Oligomer 5′ CTG CTT CCT

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[2] The abbreviations used are: PDGF, platelet-derived growth factor; IGF-1, insulin-like growth factor 1; IGF-1R, IGF-1 receptor; hIGF-1R, human IGF-1R; DMEM, Dulbecco's modified Eagle's medium; RT, reverse transcriptase; PCR, polymerase chain reaction.

CTC CTA GGA TGA 3′ was utilized as the probe for hybridization. The amounts of amplification products were quantitated by densitometry and normalized to the amount of *pHe7* mRNA. The *pHe7* control is shown only in Fig. 3.

RESULTS

Our first experiments were carried out in BALB/c 3T3 cells, which are exquisitely growth regulated (11, 18) and require both PDGF and IGF-1 for growth (12, 18). Cells were made quiescent and subsequently stimulated with PDGF plus IGF-1 (see "Materials and Methods"). Cyclin D1 mRNA was detected by RT-PCR as described in "Materials and Methods," where the controls used in these experiments are also given. Fig. 1*A* shows a Southern blot of the amplification products hybridized to a specific mouse cyclin D1 probe. Cyclin D1 mRNA is undetectable (under these conditions) in G_0 cells but becomes detectable (faintly) 1 h after stimulation, reaching peak levels at 8 h and decreasing after that time. At 8 h, by densitometry, the increase in cyclin D1 RNA levels over G_0 is 15-fold (after normalization to the levels of *pHe7* RNA).

The experiment was repeated 3 times and the increase in cyclin D1 mRNA levels over G_0 varied from 11- to 15-fold. The kinetics of induction suggests a PDGF-inducible gene. We therefore repeated the experiment shown in Fig. 1*A*, except that the quiescent cells were now stimulated either with PDGF (1 ng/ml) or IGF-1 (50 ng/ml). The results (Fig. 1*B*) indicate that cyclin D1 mRNA levels are increased by stimulation with PDGF, reaching a peak between 4 and 8 h after PDGF. By densitometry, the increase over G_0 cells is 10-fold (ranging, in 4 experiments, from 7- to 10-fold). No such increase was observed when the cells are stimulated with IGF-1 only, although the experiment was repeated 3 times. In Fig. 1*B*, some cyclin mRNA is detectable by RT-PCR in G_0 cells. The difference with Fig. 1*A* is simply an increase in the number of amplification cycles. G_0 populations of 3T3 cells always contain a small fraction of cycling cells (2–4%) and, with RT-PCR, an increase in the number of cycles or in exposure time will bring out a positive signal for growth-regulated genes.

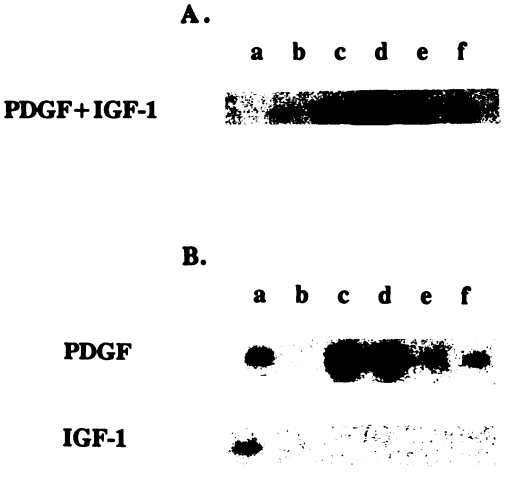

Fig. 1. Expression of cyclin D1 mRNA in BALB/c 3T3 fibroblasts stimulated with different growth factors. In *A*, cells were made quiescent and stimulated with growth factors as described in "Materials and Methods". *Lane a*, BALB/c 3T3 quiescent; *Lane b*, stimulated with PDGF + IGF-1 for 1 h, 4 h (*Lane c*), 8 h (*Lane d*), 16 h (*Lane e*), and 24 h (*Lane f*). Hybridization to amplification products obtained in 20 cycles of PCR is shown (for amplimers and probe, see "Materials and Methods". *B*, BALB/c 3T3 quiescent (*Lane a*) and stimulated with either PDGF or IGF-1 for 1 h (*Lane b*), 4 h (*Lane c*), 8 h (*Lane d*), 16 h (*Lane e*), and 24 h (*Lane f*). Hybridization to products obtained in 25 cycles of PCR is shown in parallel for PDGF and IGF-1.

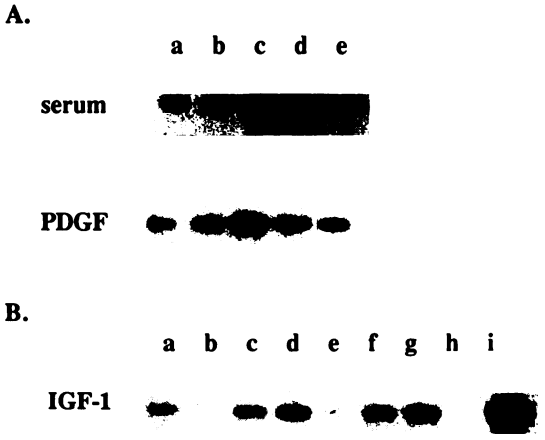

Fig. 2. Expression of cyclin D1 mRNA in WI-38 fibroblasts stimulated with growth factors. *A*, WI-38 quiescent (*Lane a*) and stimulated with serum or PDGF for 4 h (*b*), (*c*) 8 h, (*d*) 16 h, and (*e*) 24 h. Hybridization to products obtained in 15 cycles of PCR is shown. *B*, WI-38 quiescent (*Lane a*) and stimulated with IGF-1. *Lane b*, empty; *Lane c*, 8 h; *Lane d*, 16 h; and *Lane e*, 24 h. *Lane f*, WI-38 stimulated with serum in the presence of missense IGF-1R oligonucleotides for 8 h; *Lane g*, WI-38 stimulated with serum in the presence of antisense to IGF-1R RNA for 8 h; *Lane h*, 24 h with missense; *Lane i*, 24 h with antisense.

Another way to show that IGF-1 (by itself) is not required to induce cyclin D1 mRNA is to use the antisense strategy on the IGF-1 receptor, in order to decrease the amount of IGF-1 receptor in cells subsequently stimulated with serum. We could not do that in BALB/c 3T3 cells because the sequence of the murine IGF-1 receptor RNA is not known, and even a single mismatch in an antisense oligodeoxynucleotide sequence could compromise the experiment. We therefore used for this purpose WI-38 human diploid fibroblasts. Cells were plated and made quiescent as previously described (19). Fig. 2 shows that stimulation with either serum or PDGF produces an increase in the levels of cyclin D1 mRNA, 7-fold and 6-fold, respectively (repeated twice with the same results). With IGF-1 only, there was a slight increase (1.3- and 1.5-fold increase in 2 separate experiments which could be due to variations in the RT-PCR reaction) at 16 h; more significantly, Fig. 2*B* shows that when WI-38 cells are stimulated with serum, an antisense oligodeoxynucleotide to the IGF-1R RNA does not inhibit the increase in cyclin D1 RNA levels. At 8 h after stimulation, cyclin D1 RNA is about the same in controls (Fig. 2*B*, *Lane c*), cells treated with sense oligomer (Fig. 2*B*, *Lane f*), and cells treated with antisense oligomer (Fig. 2*B*, *Lane g*). At 24 h, when the amount of cyclin D1 RNA actually declines (Fig. 2*A*, *Lane e* and 2*B*, *Lane h*), an antisense oligomer to the IGF-1R produces a paradoxical increase (Fig. 2*B*, *Lane i*). Treatment of WI-38 cells with the same antisense oligonucleotide directed against the IGF-1 receptor inhibited the proliferation of these cells in response to both serum and purified growth factors [epidermal growth factor (25 ng/ml), IGF-1 (100 ng/ml), and dexamethasone (55 ng/ml)]. In the case of growth factor stimulation the cell number increased in 24 h from $8.15 \times 10^4 \pm 1.6 \times 10^3$ (SD)/cm^2 to $1.06 \times 10^5 \pm 2.8 \times 10^2$/cm^2 while the antisense-treated cultures showed no increase in cell number ($7.5 \times 10^4 \pm 2.8 \times 10^2$/cm^2). The sense oligonucleotide had no effect ($1.11 \times 10^5 \pm 1.9 \times 10^3$/cm^2).

Essentially the same result was obtained when serum was used to stimulate these cells (final cell numbers: control, 4.7×10^4/cm^2; antisense, 3×10^4/cm^2; sense, 4.7×10^4/cm^2). The specificity of the antisense oligodeoxynucleotide to the IGF-1R RNA and its ability to decrease the number of IGF-1 binding sites have already been documented in previous papers (20, 21).

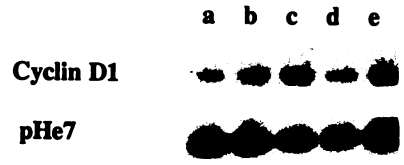

Fig. 3. Effect of oligodeoxynucleotides anti-c-*myb* or anti-hIGF-1R RNA on the expression of cyclin D1 mRNA in HL-60 cells. *Lane a*, untreated cells; *Lane b*, HL-60 treated with sense oligodeoxynucleotides to c-*myb* mRNA; *Lane c*, cells treated with antisense to c-*myb* mRNA; *Lane d*, cells treated with missense to hIGF-1R mRNA; *Lane e*, cells treated with antisense to hIGF-1R mRNA. Treatment with oligodeoxynucleotides was for 48 h. Hybridizations to products obtained in 20 cycles of PCR for cyclin D1 and in 10 cycles of PCR for *pHe7* are shown in parallel.

We confirmed these results in HL-60 cells, a human promyelocytic leukemia cell line (15). We first tested the efficacy of the antisense oligodeoxynucleotide against the IGF-1 receptor RNA. We confirmed the results of Pietrzkowski *et al.* (20) that an antisense oligomer to the IGF-1 receptor RNA inhibits cell proliferation, with the percentage of [^{3}H]thymidine-labeled cells decreasing from 77 to 19%.

Fig. 3 shows that an antisense oligodeoxynucleotide to the IGF-1 receptor RNA has no effect on the levels of cyclin D1 mRNA (compare Fig. 3., *Lanes a* and *e*). Incidentally, an antisense to c-*myb* also has no effect (Fig. 3, *Lane c*), although we used the same antisense sequence to c-*myb* RNA that has been shown to inhibit cellular proliferation (16), as well as the expression of IGF-1 receptor RNA (21). Since the antisense to the IGF-1 receptor RNA has been shown to dramatically decrease the number of IGF-1 binding sites and since HL-60 cells were growing in serum (which contains large amounts of IGF-1), these experiments confirm those of Fig. 2: *i.e.*, that the activation of the IGF-1 receptor by its ligand is not necessary for the maintenance of cyclin D1 RNA levels.

DISCUSSION

Although G_1 cyclins are known to play an important role in cell cycle progression (see Ref. 22 for a review), little is known about the environmental factors (especially growth factors) that regulate their expression. Matsushime *et al.* (7) have reported that in macrophages, cyclin D1 RNA is induced by treatment with colony-stimulating factor 1, the sole growth factor necessary for these cells to proliferate. Lu *et al.* (23) have noted induction of cyclin D mRNA levels in regenerating rat liver after partial hepatectomy; however, the possible role of growth factors in this stimulation has not been considered. We show here that cyclin D1 RNA levels in fibroblasts are increased by serum or PDGF but not by IGF-1. This is in contrast to the regulation of another RNA, the product of which is often associated with cyclins, the product of the *cdc2* gene. In fibroblasts, the levels of *cdc2* mRNA are increased by serum or IGF-1 but not by PDGF (14). This difference is consistent with the dual growth factor requirement in the control of cell proliferation of fibroblasts in culture.

We have measured cyclin D1 RNA levels by RT-PCR (17). When properly controlled, this technique is highly specific and reasonably quantitative. We always use various cycles of amplification (although here we show only the number of cycles that gives the best photographic results) and a control RNA expressed in constant amounts throughout the cell cycle, *pHe7*, and we repeat the experiments several times. In both 3T3 and WI-38 cells, both serum and PDGF reproducibly increase cyclin D1 mRNA levels. IGF-1 fails to do so (the 30% increase in WI-38 cells is not significant), as confirmed by the finding that cyclin D1 RNA levels are not affected by an antisense oligodeoxynucleotide to IGF-1R RNA. In fact, treatment with an antisense oligomer to IGF-1R RNA (capable of inhibiting growth of WI-38 cells) induces a paradoxical increase in cyclin D1 mRNA levels, for which, at the moment, we have no explanation. The specificity of this antisense has been documented in previous papers from this laboratory (20, 21). It decreases the number of IGF-1 binding sites and the same effect can be produced with another antisense to IGF-1R RNA to a sequence downstream from the sequence used in this paper. These antisense oligomers to the IGF-1R RNA consistently inhibit the expression of DNA synthesis genes, such as *PCNA*, *cdc2*, etc., while having no effect on upstream genes, such as c-*myc* or c-*myb* (21).

Our conclusion is that the regulation of cyclin D1 RNA expression seems to be temporally located upstream of the IGF-1R, as also indicated by the fact that its expression is PDGF inducible. The mechanism(s) by which PDGF increases cyclin D1 RNA levels (whether directly or indirectly through other PDGF-inducible genes) remains to be elucidated.

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