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Okadaic acid regulation of the retinoblastoma gene product is correlated with the inhibition of growth factor-induced cell proliferation in mouse fibroblasts

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ABSTRACT **Okadaic acid, a specific inhibitor of protein phosphatases 1 and 2A, was used to study the mechanism of action of transforming growth factor β (TGF-β) on cell cycle progression in C3H/10T½ mouse embryonic fibroblasts, where TGF-β exerts a growth-stimulatory effect. Concentrations of okadaic acid as low as 5 nM inhibited TGF-β (5 ng/ml)- or 10% serum-induced [^{3}H]thymidine incorporation into postconfluent, quiescent cells. Further, these inhibitory effects were observed when okadaic acid was added as late as 10 hr after TGF-β or serum stimulation. Since C3H/10T½ fibroblasts undergo the G_1/S transition at 10–14 hr after TGF-β and 8–12 hr after serum stimulation, these observations indicate that a phosphatase activity may be required for S-phase entry. In a parallel experiment, okadaic acid partially inhibited TGF-β-induced [^{14}C]leucine incorporation by 20–65%, depending upon the okadaic acid concentration. In conjunction with the effect of okadaic acid on DNA and protein synthesis, Western blot analysis indicated that okadaic acid inhibited phosphorylation of the retinoblastoma gene product and decreased its protein level, even when added 10 hr after TGF-β or 8 hr after serum stimulation. These findings strongly suggest that protein phosphatases play a pivotal role for S-phase entry in mouse fibroblasts. Moreover, protein phosphatases may be required in the intermediate steps of TGF-β or serum growth factor signal-transduction pathways for the stimulation of phosphorylation of the retinoblastoma protein, especially in late G_1.**

Phosphorylation/dephosphorylation events play a pivotal role in the regulation of signal-transduction mechanisms that control cell proliferation and differentiation. The polyether tumor promoter okadaic acid, derived from the black sponge *Halichondria okadai*, produces marked hyperphosphorylation in a manner consistent with its acting as a protein phosphatase inhibitor (1). For example, okadaic acid induces the phosphorylation of proteins such as the 27-kDa heat shock protein complex, eukaryotic translation initiation factor 4E, epidermal growth factor receptor, and cdc2 kinase substrates (c-Abl, pRb, and p53) in primary human fibroblasts (2). It was also demonstrated that the phosphorylation pattern elicited by okadaic acid, which does not bind to protein kinase C, was different from that of phorbol ester-induced activation of protein kinase C.

The mechanism of okadaic acid action on cell proliferation and differentiation is still unclear. Okadaic acid has the characteristics of a strong tumor promoter and can induce the expression of the early genes c-*fos* and c-*jun* in mouse keratinocytes, human leukemia cells, and human diploid fibroblasts (2–4). Okadaic acid is also capable of upregulating c-*fos* expression at the transcriptional and posttranscriptional level in NIH 3T3 mouse fibroblasts (5). The regulation of these early genes by okadaic acid was distinct from that observed in cells treated with phorbol 12-myristate 13-acetate. Boynton and colleagues (6) reported that okadaic acid inhibited platelet-derived growth factor (PDGF)-induced cell proliferation and decreased the number of PDGF receptors in C3H/10T½ mouse fibroblasts (6). Although these early responses may contribute, in part, to the inhibitory effects of okadaic acid on growth factor-induced DNA synthesis, they do not account for the total inhibition observed.

Transforming growth factor β (TGF-β) exerts multifunctional effects on cell proliferation, since it can either stimulate or inhibit cell growth and differentiation. Particularly, the growth-stimulatory effect of TGF-β was reported to involve the autocrine/paracrine induction of c-*sis* and subsequent production of a PDGF-like growth factor in AKR-2B mouse embryo cells and in primary human skin fibroblasts (7, 8). Although the signal-transduction mechanism of TGF-β has not been identified in fibroblasts, much evidence indicates that TGF-β may act through the retinoblastoma gene product (pRb) in Mv1Lu lung epithelial cells, where TGF-β is growth-suppressive (9, 10). pRb may be involved in the control of the G_1/S transition, or it may act as a restriction point in G_1 as cells progress to the S phase of the cell cycle (11–13). As the cell passes through the cell cycle, the phosphorylation of pRb can be observed. Reports indicate that pRb is phosphorylated by cdk2 or a cdk2-related kinase in the late G_1 and S phases and is further phosphorylated by cdc2 kinase in the S, G_2, and M phases (14–16). Okadaic acid-sensitive protein phosphatases control both the activation and the inactivation of the p34^{cdc2} kinase, depending on the cell cycle stage, as observed with different responses related to the time of okadaic acid treatment (17). In Mv1Lu cells, the growth-inhibitory effect of TGF-β was related to the prevention of pRb phosphorylation in mid to late G_1, in which TGF-β retains pRb in the underphosphorylated, growth-suppressive state (9, 10). We have previously observed that TGF-β as a growth stimulator enhances the level of *Rb* mRNA throughout the cell cycle in C3H/10T½ fibroblasts. This raises the possibility that TGF-β-induced cell cycle activation may be related to the induction of pRb and/or pRb phosphorylation. It was therefore of interest to study the effects of okadaic acid on pRb phosphorylation when TGF-β-treated cells undergo G_1/S transition. Such investigations should provide insight as to whether phosphatases are involved in the intermediate stages of the TGF-β signal-transduction mechanism, since a balance between phosphorylation and dephosphorylation reactions appears to be requisite in the progression of the cell cycle.

Here we demonstrate a possible mechanism by which TGF-β may activate protein phosphatases to stimulate intermediate cascade events involving pRb in cells exhibiting a positive effect of TGF-β on cell cycle progression.

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Abbreviations: PDGF, platelet-derived growth factor; TGF-β, transforming growth factor β; pRb, retinoblastoma gene product (protein).

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MATERIALS AND METHODS

Materials. TGF-β was kindly given by Kirk Leister (Bristol–Meyers Squibb, Syracuse, NY). TGF-β1 is synthesized in CHO cells as pre-pro-TGF-β1, which is processed at the carboxyl-terminal side of Gly-29 and Arg-278 (18). Okadaic acid was purchased from LC Services (Woburn, MA) and dissolved in dimethyl sulfoxide (Sigma). Purified mouse monoclonal antibody (G3-245) to human retinoblastoma pRb was obtained from PharMingen (San Diego). This antibody reacts specifically with an epitope between amino acids 300 and 380 on the authentic pRb (pp110–114^{Rb}).

Cell Culture. C3H/10T½ mouse embryonic fibroblasts were plated in 24-well plates for the analysis of DNA and protein synthesis and in 150-mm dishes for Western blot analysis. Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% serum [4:1 ratio of calf serum (GIBCO) and fetal bovine serum (Upstate Biotechnology, Lake Placid, NY)] and 0.001% gentamycin (GIBCO) as an antimicrobial agent was used. Growth medium was renewed every 4 days, and cells reached confluence 8–10 days after plating. Postconfluent, quiescent cells obtained 4 days after the time of confluence were used, to avoid heterogeneous cell cycle states.

Analysis of DNA and Protein Synthesis. TGF-β (5 ng/ml) was added to postconfluent, quiescent cells following medium (0.1% fetal bovine serum) change. For serum stimulation, spent medium was renewed by complete medium containing 10% serum (4:1 ratio of calf serum and fetal bovine serum). [^{3}H]Thymidine (1 μCi, 50 Ci/mmol, ICN; 1 Ci = 37 GBq) was introduced for a 2-hr pulse incorporation to measure DNA synthesis, and [^{14}C]leucine (0.1 μCi, 50 μCi/ml, DuPont/New England Nuclear) was used for analysis of protein synthesis. The reaction was stopped by the removal of medium containing either [^{3}H]thymidine or [^{14}C]leucine, followed by the addition of 5% (wt/vol) trichloroacetic acid. [^{3}H]Thymidine or [^{14}C]leucine incorporation into the acid-insoluble fraction was assayed as described (19).

Western Blot Analysis. Cell pellets were lysed in lysis buffer [250 mM NaCl/50 mM Hepes, pH 7.0/0.1% Nonidet P-40/5 mM NaF containing phenylmethanesulfonyl fluoride (0.1 mM), aprotinin (1 μg/ml), leupeptin (1 μg/ml), and β-glycerophosphate (10 μg/ml)] on ice for 20 min. Samples containing 100–140 μg of protein were mixed with SDS sample buffer and the mixtures were boiled for 5 min. After SDS/7% PAGE, Western blotting was carried out using Bio-Rad Trans-Blot cells. The transfer buffer did not contain methanol. A hydrophobic, poly(vinylidene difluoride) membrane (Gelman) was incubated for 1 min in 100% methanol and then equilibrated in transfer buffer for 20 min. The blot was blocked for 1 hr at room temperature on a shaker with 100 ml of blocking buffer [5% nonfat dry milk and 0.1% Tween 20 (Bio-Rad) in 1× TNE (10 mM Tris·HCl, pH 7.5/50 mM NaCl/2.5 mM EDTA)]. The primary antibody was G3-245 anti-pRb antibody (PharMingen) diluted in blocking buffer (3 μg/ml), and the secondary antibody was peroxidase-conjugated sheep anti-mouse IgG (1:2000 dilution, Amersham). The blot was incubated with the primary antibody for 90 min, washed, incubated with the secondary antibody for 90 min, and washed again. Each wash consisted of four rinses (10 min each) with 1× TNE on a shaker at room temperature. The enhanced chemiluminescence procedure (ECL, Amersham) and Kodak X-Omat AR film were employed.

RESULTS

Inhibitory Effect of Okadaic Acid on Mitogen-Induced Proliferation of C3H/10T½ Mouse Fibroblasts. Since okadaic acid exhibits both stimulatory and inhibitory responses on substrate phosphorylation (2), it was of interest to further study the kinetics of phosphorylation under conditions of defined cell cycle stages. To investigate the regulation of protein phosphorylation events in cell cycle progression, we studied the effect of okadaic acid (5, 20, and 50 nM) on DNA synthesis induced by TGF-β (5 ng/ml) or 10% serum in C3H/10T½ mouse fibroblasts. The addition of TGF-β to postconfluent, quiescent cells induced a 30-fold increase in [^{3}H]thymidine incorporation compared with that of the controls at t = 0 and 24 hr (Fig. 1*A*). Okadaic acid inhibition of TGF-β-induced DNA synthesis was dose-dependent. Okadaic acid at 20 nM and 50 nM completely inhibited DNA synthesis in both control cells and cells treated with TGF-β, but as observed by others (6), it was not cytotoxic. Interestingly, okadaic acid added at 0 or 10 hr after TGF-β, to cells that are in the G_1/S transition state, showed the same degree of inhibition of [^{3}H]thymidine incorporation. Similarly, inhibitory effects were observed when okadaic acid was added even 6 or 10 hr after serum stimulation (Fig. 1*B*). These results indicate that inhibition of cell proliferation by okadaic acid may not be unique to TGF-β in fibroblasts, and protein phosphatase activity may be required in late G_1.

Partial Inhibition of TGF-β-Induced Protein Synthesis by Okadaic Acid. The question arises whether the inhibitory effect of okadaic acid on DNA synthesis is due to suppression of critical protein synthesis required for S-phase entry. In an experiment parallel to the one shown in Fig. 1*A*, the effect of okadaic acid added at various time points on [^{14}C]leucine incorporation was examined. TGF-β stimulated protein synthesis by ≈20% relative to control at t = 0 and 24 hr (Fig. 2). However, the addition of okadaic acid inhibited [^{14}C]leucine incorporation by 20–65%, depending on the okadaic acid concentration, even when added 10 hr after TGF-β stimula-

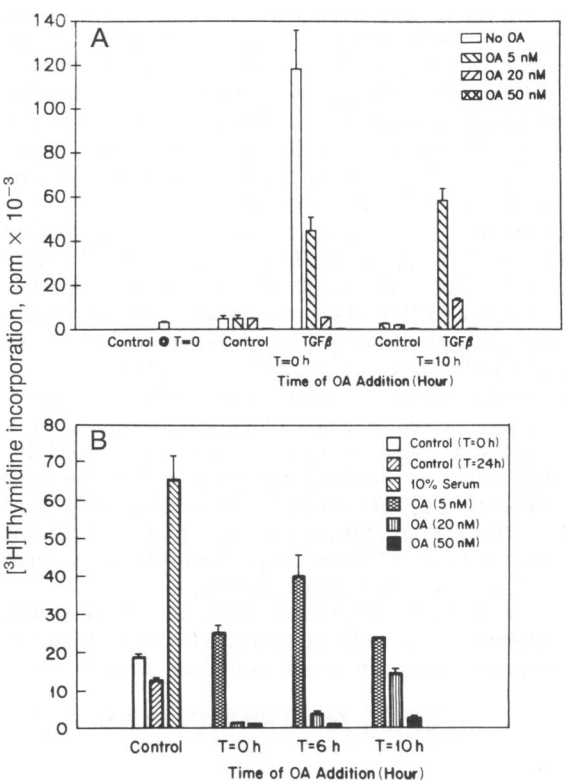

FIG. 1. Okadaic acid inhibition of mitogen-induced DNA synthesis. Postconfluent, quiescent cells obtained by growth factor depletion were treated with TGF-β (5 ng/ml) (*A*) or the medium was changed with complete fresh medium containing 10% serum (4:1 ratio of calf serum and fetal bovine serum) (*B*). Okadaic acid (OA) (5, 20, or 50 nM) was then added at the indicated times after TGF-β or serum stimulation. DNA synthesis was measured by a 2-hr pulse of [^{3}H]thymidine incorporation at t = 24 hr (or at 0 hr, for the 0-hr control). Values are means ± SD of quadruplicate samples. Control at T = 0.

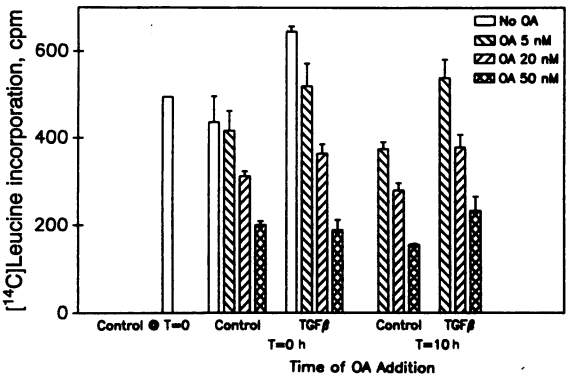

FIG. 2. Effect of okadaic acid (OA) on TGF-β-induced protein synthesis. With an experiment as shown in Fig. 1*A*, [^{14}C]leucine incorporated into the acid-insoluble fraction was measured for a 2-hr pulse at t = 24 hr. Values are means ± SD of triplicate samples.

tion. Various concentrations of okadaic acid in low-serum conditions (0.1% fetal bovine serum) showed a 10–50% inhibitory effect when compared with the 0-hr and the 24-hr controls. This partial inhibition of protein synthesis by okadaic acid may affect DNA synthesis.

Inhibitory Effect of Okadaic Acid on pRb Phosphorylation and Protein Level. It has been reported that TGF-β prevents pRb phosphorylation in keratinocytes, whose proliferation is inhibited by TGF-β (20). The observation with C3H/10T½ cells of TGF-β-induced cell proliferation gives insight into how TGF-β could modulate the pRb phosphorylation. Western blot analysis indicated that pRb became phosphorylated when cells traversed the G_1/S boundary (t = 12 hr) and hyperphosphorylated at t = 24 hr, where cells are in S phase (Fig. 3). TGF-β also increased the level of pRb. It should be noted that upregulation of *Rb* mRNA level was observed 3 hr after TGF-β stimulation of quiescent C3H/10T½ cells (data not shown). These results indicate that the growth-stimulatory effect of TGF-β in C3H/10T½ mouse fibroblasts is correlated with the induction of pRb and its phosphorylation.

Given these results, it was of interest to study the ability of okadaic acid to regulate the TGF-β-induced phosphorylation of pRb. Neither 5 nM nor 50 nM okadaic acid induced pRb phosphorylation (Fig. 4*A*), in contrast to the okadaic acid-induced pRb phosphorylation observed in human diploid fibroblasts (2). Moreover, okadaic acid appeared to decrease TGF-β-induced pRb phosphorylation. In this connection, the question was raised as to how okadaic acid added at the G_1/S transition state regulates pRb. Okadaic acid (5 and 20 nM) had little effect on pRb level compared with the controls at t = 0 and 24 hr (Fig. 4*B*). When 20 nM okadaic acid was added 10 hr after TGF-β stimulation, pRb phosphorylation was inhibited and the pRb level decreased to the same degree as when okadaic acid was added at 0 hr.

This study was further extended to the serum-stimulated environment. Postconfluent, quiescent cells were stimulated by medium change, in which complete medium contained

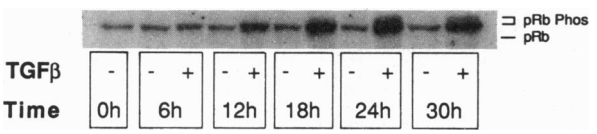

FIG. 3. Western blot analysis of TGF-β-induced pRb phosphorylation. Quiescent C3H/10T½ cells were treated with TGF-β (5 ng/ml) for 0–30 hr as indicated. One hundred micrograms of whole cell extract protein was applied to an SDS/7% polyacrylamide gel. G3-245 anti-pRb antibody (3 μg/ml; PharMingen) was used for Western analysis. Multiplets (slower migrating bands) reflect the hyperphosphorylated (Phos) pRb.

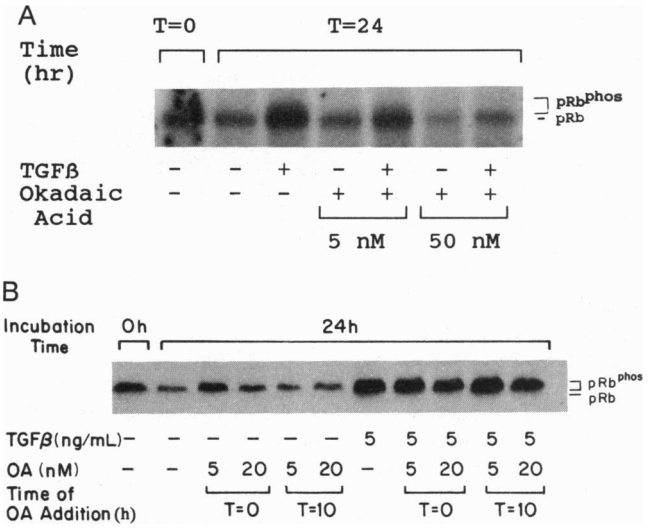

FIG. 4. Effect of okadaic acid on TGF-β-induced pRb phosphorylation. (*A*) Okadaic acid (5 or 50 nM) and TGF-β (5 ng/ml) were added to growth factor-depleted C3H/10T½ cells as indicated. (*B*) Okadaic acid (CA) (5 or 20 nM) was added to postconfluent, quiescent C3H/10T½ cells at the indicated times with or without TGF-β treatment. Cell pellets collected 24 hr after TGF-β treatment were lysed and analyzed by the same procedure used for Fig. 3.

10% serum (4:1 ratio of calf serum and fetal bovine serum). The phosphorylation status of pRb induced by serum was almost identical to that by TGF-β, showing that pRb became phosphorylated when cells passed through G_1/S phase (t = 8 hr) and hyperphosphorylated during S phase (t = 16–24 hr) (Fig. 5). In concert with its effect on serum-induced DNA synthesis (Fig. 1*B*), okadaic acid inhibited pRb phosphorylation and reduced the pRb level. The intensity of okadaic acid's inhibitory effect was dose-dependent. When cells were treated with okadaic acid 8 hr after serum stimulation, cells became less sensitive to okadaic acid, indicating that inhibition of pRb phosphorylation by 50 nM okadaic acid was very similar to that by 20 nM okadaic acid added at t = 0 hr. Our results are in accord with the idea that okadaic acid inhibition of cell cycle progression is correlated with the prevention of pRb phosphorylation and/or the decrease of the pRb level. This reflects a need for phosphatase action in the mitogenic phosphorylation cascade, especially at the G_1/S transition of the cell cycle.

DISCUSSION

Okadaic acid, an inhibitor of serine/threonine phosphatases, was used to study the role of phosphatases in TGF-β-induced cell cycle activation. Okadaic acid inhibited TGF-β-induced DNA synthesis at concentrations comparable to those reported by Dean *et al.* (6) for PDGF-stimulated C3H/10T½ fibroblasts. Okadaic acid, even when added 10 hr after TGF-β stimulation, gave similar inhibitory effects on DNA

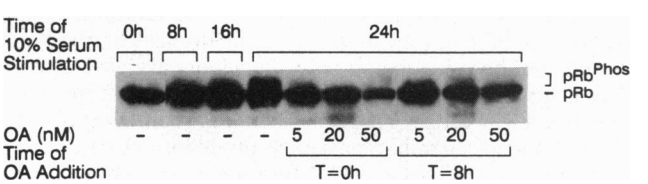

FIG. 5. Okadaic acid regulation of serum-induced pRb phosphorylation. Postconfluent, quiescent C3H/10T½ cells were stimulated by 10% serum as described for Fig. 1*B*. Okadaic acid (OA) (5, 20, or 50 nM) was added at the designated times after serum stimulation. Whole cell extracts were prepared at the indicated times. One hundred forty micrograms of total protein was applied for Western blot analysis.

synthesis as it did when added at 0 hr. This finding suggests that inhibition of TGF-β-induced DNA synthesis by okadaic acid is exerted at the G_1/S border. The inhibitory effect of okadaic acid occurring in the late G_1 phase of the cell cycle is not unique to TGF-β-induced DNA synthesis, since okadaic acid added in late G_1 exerted similar inhibitory effects on serum-induced DNA synthesis.

We examined whether okadaic acid could involve interactions which might influence pRb phosphorylation. pRb is well characterized as a tumor suppressor which is thought to negatively regulate cell proliferation (21). The physiological functions of pRb can be modified by phosphorylation during cell cycle progression. While underphosphorylated pRb is mainly detected in early G_1, it becomes phosphorylated at the G_1/S boundary (22). Cell cycle-dependent phosphorylation of pRb has been observed in cell lines as well as in primary cultures and is required for cells to enter S phase. We report here that TGF-β induced pRb phosphorylation under conditions where it stimulates DNA synthesis, and this phosphorylation preceded the G_1/S transition. Okadaic acid blocked TGF-β- and serum-induced pRb phosphorylation, indicating that protein phosphatases may be involved in the progression to the hyperphosphorylated state of pRb. Our results differ from those of Guy *et al.* (2), who showed that okadaic acid induced pRb phosphorylation. Those authors used confluent human diploid fibroblasts without assurance that cells were staged in the early G_1 phase of the cell cycle. The observed differences from our results may reflect the different species used as well as conditions selected in our studies to provide largely homogeneous cell populations.

The level of TGF-β-induced *Rb* mRNA was not altered by okadaic acid even with concentrations of 50 nM (data not shown). As we observed, okadaic acid partially inhibits TGF-β-induced protein synthesis. This may be due to events such as the inhibition of eukaryotic initiation factor eIF-2. Indeed, evidence indicated that phosphorylation of the α subunit of eIF-2 inhibited protein synthesis. Phosphatase 2A was considered to be important for the maintenance of the unphosphorylated form of eIF-2 (23). Since this phosphatase leads to dephosphorylation of eIF-2, okadaic acid could block protein synthesis by interfering with eIF-2 dephosphorylation. The possibility arises that okadaic acid inhibition of pRb phosphorylation may be influenced by a decreased level of pRb.

The two potential protein kinases for the pRb phosphorylation have been well identified as cdc2 and cdk2 (15, 16, 24). Okadaic acid sensitivity for the induction of cdc2 kinase activity has been shown to be dependent on the amount of cyclins present (17). A recent report demonstrated that *Saccharomyces cerevisiae* SIT4 protein phosphatase was required for the normal accumulation of *CLN1*, *CLN2*, and other cyclin-related RNAs during late G_1 (25). SIT4 phosphatase is 55% identical to the mammalian type 2A and 43% identical to the mammalian type 1 protein phosphatase catalytic subunit. These studies suggest that okadaic acid may regulate pRb kinases by modulating the level of cyclins.

In addition, Brautigan *et al.* (26) reported that the amount of phosphatase inhibitor 2 (I2), a specific inhibitor of phosphatase type 1, oscillated during the cell cycle in rat fibroblasts, peaking at S phase and mitosis (26). Further, those authors found that there were major changes in the abundance of I2 coincident with two critical periods—namely, DNA synthesis and mitosis. These observations provide the evidence that protein phosphatases are specifically involved in the modulation of the cell cycle. The inhibition of these phosphatases by okadaic acid may contribute to the responses we observed.

In conclusion, although inhibition of a phosphatase activity might be expected to contribute to increased pRb phosphorylation, our observations indicate that okadaic acid-induced growth suppression is correlated with an inhibition of growth factor-induced pRb phosphorylation in late G_1 phase. Therefore, protein phosphatases may be required for the indirect stimulation of pRb phosphorylation by TGF-β or other serum growth factors. Perhaps a more complex cascade mechanism may be involved in the activation of pRb kinase, such as the regulation of cyclin-dependent kinase (cdc2 or cdk2) activity and its associated cyclins by protein phosphatases. The concurrent timing of the okadaic acid-induced block of pRb phosphorylation and of DNA synthesis leads us to ask whether this correlation reflects a primary relationship.

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1. Schönthal, A. (1992) *New Biol.* **4**, 16–21.
2. Guy, G. R., Cao, X. & Tan, Y. H. (1992) *J. Biol. Chem.* **267**, 1846–1852.
3. Holladay, K., Fujiki, H. & Bowden, G. T. (1992) *Mol. Carcinog.* **5**, 16–24.
4. Adunyah, S. E., Unlap, T. M., Franklin, C. C. & Kraft, A. S. (1992) *J. Cell. Physiol.* **151**, 415–426.
5. Schonthal, A., Tsukami, Y. & Feramisco, R. (1991) *Oncogene* **6**, 423–430.
6. Dean, N. M., Mordan, L. J., Tse, K., Mooberry, S. L. & Boynton, A. L. (1991) *Carcinogenesis* **12**, 665–670.
7. Leof, E. B., Proper, J. A., Goustin, A. S., Shipley, G. D., Dicorleto, P. E. & Moses, H. L. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 2453–2457.
8. Soma, Y. & Grotendorst, G. R. (1989) *J. Cell. Physiol.* **140**, 246–253.
9. Laiho, M., DeCaprio, J. A., Ludlow, J. W., Livingston, D. M. & Massagüe, J. (1990) *Cell* **62**, 175–185.
10. Ohtsuki, M. & Massagüe, J. (1992) *Mol. Cell. Biol.* **12**, 261–265.
11. DeCaprio, J., Ludlow, J. W., Lynch, D., Furakawa, Y., Griffin, J., Piwnica-Worms, H., Huang, C. & Livingston, D. M. (1989) *Cell* **58**, 1085–1095.
12. Buchkovich, K., Duffy, L. A. & Harlow, E. (1989) *Cell* **58**, 1097–1105.
13. Chen, P. L., Scully, J., Wang, J. M. & Lee, W. H. (1989) *Cell* **58**, 1193–1198.
14. Tsai, L., Harlow, E. & Meyerson, M. (1991) *Nature (London)* **353**, 174–177.
15. Pagano, M., Draetta, G. & Jansen-Durr, P. (1992) *Science* **255**, 1144–1147.
16. Akiyama, T., Ohuchi, T., Sumida, S., Matsumoto, K. & Toyoshima, K. (1992) *Proc. Natl. Acad. Sci. USA* **89**, 7900–7904.
17. Yamashita, K., Yasuda, H., Pines, J., Yasumoto, K., Nishitani, H., Ohtsubo, M., Hunter, T., Sugimura, T. & Nishimoto, T. (1990) *EMBO J.* **9**, 4331–4338.
18. Gentry, L. E., Lioubin, M. V., Purchio, A. F. & Marquardt, H. (1988) *Mol. Cell. Biol.* **8**, 4162–4168.
19. Cutry, A. F., Kinniburgh, A. J., Twardzik, D. R. & Wenner, C. E. (1988) *Biochem. Biophys. Res. Commun.* **152**, 216–222.
20. Munger, K., Pietenpol, J. A., Pittelkow, M. R., Holt, J. T. & Moses, H. L. (1992) *Cell Growth* **3**, 291–298.
21. Weinberg, R. A. (1990) *Trends Biochem. Sci.* **15**, 199–202.
22. DeCaprio, J., Furakawa, Y., Aichenbaum, F., Griffin, J. & Livingston, D. M. (1992) *Proc. Natl. Acad. Sci. USA* **89**, 1795–1799.
23. Redpath, N. T. & Proud, C. G. (1989) *Biochem. J.* **262**, 69–75.
24. Lees, J. A., Buchkovich, K. J., Marshak, D. R., Anderson, C. W. & Harlow, E. (1991) *EMBO J.* **10**, 4279–4290.
25. Ferrandez-Sara, M. S., Sutton, A., Zhong, T. & Arndt, K. T. (1992) *Genes Dev.* **6**, 2417–2428.
26. Brautigan, D. L., Sunwoo, J., Labbe, J., Fernandez, A. & Lamb, J. C. (1990) *Nature (London)* **344**, 74–78.