EXPERIMENTAL and MOLECULAR MEDICINE, Vol. 28, No 1, 11-16, March 1996

Effect of protein kinase C, calcium and cAMP on the expression of proto-oncogenes, pRb and cell proliferation

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Accepted 8 January 1996

Abbreviations: DAG, diacylglycerol; TPA, 12-*O*-tetradecanoyl phorbol-13-acetate; db cAMP, dibutyryl cyclic AMP; IBMX, 3-isobutyl-1-methylxanthine; PKC, protein kinase C; pRb, retinoblastoma product

Abstract

The second messengers induced by growth factors are thought to have important roles in transducing the growth factor signals to nuclear events and cell proliferation. Various second messengers stimulate expression of proto-oncogenes such as c-*fos* and c-*myc* which are believed to have an important role in cell proliferation. To determine the causal relationship between these events we studied the effects of 12-*O*-tetradecanoyl phorbol-13-acetate, A23187 and dibutylyl cAMP plus 3-isobutyl-1-methylxanthine on the cell proliferation and the expression of c-*fos* and c-*myc* by using serum-deprived quiescent NIH 3T3 cells. Although A23187 stimulated the expression of these two proto-oncogenes most strongly, it stimulated cell proliferation only weakly, indicating that the expression of these proto-oncogenes are not sufficient for proliferation. Also we investgated the involvement of expression or phosphorylation of pRb in expression of c-*fos* and c-*myc*. Western blot analysis showed no significant changes in pRb expression or phosphorylation, indicating that second messengers induced expression of these proto-oncogene not through the activation of a transcription factor E2F which has been suggested to function in serum induced stimulation of these genes.

Keywords: signal transduction, second messenger systems, proto-oncogenes, cell division

Introduction

Progression of cell cycle through G_1 and S represents commitment to cell division. After this control point no more external signals are required. Although the G_1/S transition in mammalian cells is dependent on the availability of growth factors, the intracellular transduction processes resulting in the induction of DNA replication and cellular proliferation are not fully understood. After binding to specific surface receptors, polypeptide growth factors activate various isoenzymes of phospholipase C via stimulating G protein or receptor tyrosine kinase and generate second messengers diacylglycerol (DAG) and inositol (1,4,5)-trisphosphate. DAG activates and redistributes protein kinase C (PKC) (Leach *et al.*, 1989) which phosphorylates target proteins on Ser/Thr residues. inositol (1,4,5)-trisphosphate promotes Ca^{2+} release from intracellular stores via inositol (1,4,5)-trisphosphate receptor, leading the activation of several enzymes and proteins especially calmodulin-dependent kinases. Another major second messenger that regulates cell proliferation is cAMP which exerts its effects by activating cAMP-dependent kinase (PKA). cAMP generated as a second messenger after stimulation of G protein by interaction of various growth hormones with their specific receptors (Huang *et al.*, 1991). However, the role of cAMP in cell proliferation is controversial, because cAMP inhibits or stimulates cell proliferation depending on cell types (Van *et al.*, 1991). cAMP inhibits the Ras pathway in fibroblasts via phosphorylation and inhibition of the protein Ser/Thr kinase Raf-1, whereas in neurons cAMP can stimulate Ras. These observations imply the divergent effects of cAMP on cell proliferation (Karin and Smeal, 1992).

Stimulation of quiescent cell results in altered expression of proto-oncogenes, termed immediate early genes, without the need for prior protein synthesis (Mccaffrey *et al.*, 1987). Furthermore many products of activated oncogenes such as *ras*, *raf* and *src* have been shown to activate c-*fos* expression (Fujii *et al.*, 1989; Jamal and Ziff, 1990). Expression of these immediate early genes has been demonstrated to be critical for the progression through G_1 and into S phase. These inducible proto-oncogenes have been proposed to act as nuclear third messenger molecules that function in coupling growth factor signal to long change in cell proliferation by regulating the expression of target genes (Morgan and Curran, 1991). Although the signaling mechanism by which growth factor signal transduces from cytoplasm to nucleus is not known, it

is thought that transcription factors, regulated by second messenger-induced kinase cascade, play a major role.

Two of the most extensively studied immediate early genes are the c-*fos* and c-*myc*. A great number of mitogenic agents including serum, cAMP and calcium ionophore rapidly and transiently stimulate the expression of these genes during the G_0 to G_1 transition prior to the onset of DNA synthesis (Greenberg and Ziff, 1984; Moore *et al.*, 1986). Recently it has been shown that the expression of these two proto-oncogenes are also regulated by negative factors of cell proliferation.

In mammalian cells, there are negative control factors that can counter the positive signal induced by growth factors and block progression from G_1 to S (Goodrich *et al.*, 1991). These factors are encoded by anti-oncogenes or tumor suppressor genes. The first identified negative factor is retinoblastoma gene (Rb) product (pRb). Lack of its expression or mutations in its genes result in tumor formation and uncontrolled proliferation of cells. Phosphorylation state of pRb is believed important in its function and regulated during cell cycle. The hypo-phosphorylated form predominates during G_0 and G_1 period, while the phosphorylated form occurs during S, G_2, and M phase and following mitosis, pRb returns to the hypo-phosphorylated state (Ludlow *et al.*, 1990). The hypo-phosphorylated form of pRb is believed to function as a negative factors in G_0 phase and arrest the cell cycle in G_0 phase. Although the pRb activity is controlled primarily by phosphorylation, regulation of Rb expression has been also shown to be important. Epstein-barr virus-transformed lymphocytes have more pRb than do normal quiescent lymphocytes (Yen *et al.*, 1991). Furthermore, transcription of human Rb gene is positively and negatively autoregulated by its own product through ATF-2 and E2F binding sites respectively (Hamel *et al.*, 1992; Shan *et al.*, 1994). The mechanism by which pRb exerts its negative effect on the cell cycle is largely unknown. However, recently it has been shown that pRb prevents the progression of cell cycle by binding to transcription factors necessary for the expression of genes essential for DNA synthesis and cell proliferation. Among these proteins E2F is the best characterized. Hypo-phosphorylated pRb has been shown to inhibit the trans activation activity of E2F by association with it. It has been shown that the free E2F molecule is the active form and E2F in complex with pRb is the inactive form (Hiebert *et al.*, 1992). Normal physiological function of E2F is suggested to trigger the entry of cells into the S phase in response to growth factor signals through activating E2F-responsive genes, including c-*fos*, c-*myc*, dihydrofolate reductase, thymidine kinase and *cdc*2 (Mudryj *et al.*, 1990; Robbins *et al.*, 1990; Farnham *et al.*, 1993). Recently it has been shown that overexpression of E2F is sufficient to cause quiescent cells to enter S phase (Johnson *et al.*, 1993). These observation support the finding that pRb can repress c-*fos* and c-*myc* expression in serum induced 3T3 cells and adenovirus infection of quiescent cells results in the stimulation of endogeneous c-*myc* gene through E2F binding site (Hiebert *et al.*, 1989). These observations suggest that pRb may regulate cell cycle by controling the expression of c-*myc* and c-*fos*. pRb may also control c-*myc* activity posttranslationally by binding the N-terminal portion of c-*myc* protein (Rustgi *et al.*, 1991). Coinjection of pRb with c-*myc* inhibits the activity of pRb to arrest cell cycle progression (Goodrich and Lee, 1992). These observations indicate that function of c-myc and pRb is closely related antagonistically in controlling the cell cycle.

Although the importance of expression of c-*myc* and c-*fos* and pRb function in cellular proliferation is suggested, their causal relationship to the second messenger dependent transition of cells from G_1 to S phase is unclear. In this study we investigated the role of second messengers as signaling molecules that link early events generated by growth factors to nuclear events responsible for the onset of DNA replication. For this purpose, the effects of 12-*O*-tetradecanoyl phorbol-13-acetate (TPA), A23187 and dibutyryl (db) cAMP plus 3-isobutyl-1-methylxanthine (IBMX) on the expression of c-*fos* and c-*myc* and cell proliferation was studied. Also at the same experimental condition, we investigated the pRb expression by Western analysis to observe the involvement of pRb in this process.

Materials and Methods

Cell culture

NIH 3T3 cells were obtained from American Type Culture Collection (ATCC) and cultured in Dulbecco's modified Eagle medium supplemented with 5% bovine calf serum (Gibco, Gaithersburg, U.S.A.) and penicillin-streptomycin in a humidified atmosphere of 5% CO_2. Quiescent cells were obtained by serum deprivation (0.5% bovine calf serum) for two days. 100 ng/ml TPA, 50 ng/ml A23187 and 1 μg/ml db cAMP plus 1 μg/ml IBMX (all from Sigma, St. Louis, U.S.A.) were added to induce cell proliferation

Sulforhodamin B assay

Sulforhodamin B assay was performed as described (Rubinstein *et al.*, 1990). After quiescent NIH 3T3 cells were treated with various agents for two days, media was aspirated and 10% trichloracetic acid was added. After 1 h incubation at 4°C, plate was washed 5 times by using tap water and air dried. Cells were stained with 0.4% sulforhodamin B (SRB) for 30 min at room

temperature and washed 5 times by using 1% acetic acid. Bound sulforhodamin B was solubilized with 10 mM Tris and absorbance was measured at 520 nm.

RNA slot and Northern blot analyses

Total RNA from NIH 3T3 cells, incubated with each agent for 30 min or 2 h, was isolated using single step isolation method (Ausubel *et al.*, 1991). RNA was denatured in solution containing 50% formamide, 16% formaldehyde and 1X SSC by heating at 68°C for 5 minutes. After dilution of sample with 2 volume of 20X SSC, RNA was blotted to nylon membrane by using PR 600 slot blot kit (Hoefer, San Francisco, U.S.A.) and immobilized by UV-induced crosslinking. The membrane was incubated in hybridization buffer (5X SSC, 5% formamide, 0.1% N-laurylsarcosine, 7% SDS, 50 mM sodium phosphate, pH 7.0, 50 μg/ml denatured tRNA) for 6 h at 37°C. Hybridization occurred for 24 h at 37°C. The hybridization probe was prepared from gel-purified 1.0 kb fragment derived from v-*fos* gene (ATCC 41040) and labeled by random primed extension using DIG-labeled dUTP (Boeringer Mannheim, Mannheim, Germany). After hybridization the membrane was washed twice for 5 min in washing solution I (1X SSC, 0.1% SDS) at room temperature, then twice for 15 min in washing solution II (0.2X SSC, 0.1% SDS) at 68°C. Detection was performed by alkaline phosphatase-conjugated anti-DIG antibody using chemiluminescent reagent CSPD (Tropics, Bedford, U.S.A.) as a substrate. For Northern blot analysis total RNA was electrophoresed on a 0.8% formaldehyde gel and transferred to a nylon membrane. ^{32}P-labeled c-*myc* probe, a 1.4 kb *ClaI/Eco*RI fragment, containing third exon of the human c-*myc* gene (ATCC 41010) was used.

Western blot analysis

NIH 3T3 cells were lysed directly in SDS loading buffer and protein was quantitated by using an assay kit (Bio-Rad, Hercules, U.S.A.). Equal amounts of protein was resolved by SDS-PAGE. After electrophoresis, the separated polypeptides were electrophoretically transferred to nylon membrane by using electrotransfer kit (Hoefer). The blot was incubated with polyclonal pRb antibody (Ab-2, Oncogene Science, Paris, France) then followed by incubation with alkaline phosphatase-conjugated goat anti-rabbit antibody. CSPD was used as a substrate of alkaline phosphatase and the product was detected by exposing the blot on the x-ray film.

Results and Dicussion

The effects of TPA, A23187, and db cAMP plus IBMX on the proliferation of NIH 3T3 cells

Major second messengers produced after growth factor stimulation of quiescent cells were DAG, Ca^{2+} and cAMP. To observe the effects of these molecules on cell proliferation, we used tumor promoter TPA, Ca^{2+} ionophore A23187 and cell permeable db cAMP plus phosphodiesterase inhibitor, IBMX which can mimic the effects of DAG, Ca^{2+} and cAMP, respective-ly. Thus these reagents can reproduce all the downstream biological responses normally promoted by second messengers. In order to observe the role of second messengers in connecting the growth factor signal to cell proliferation, we observed the effects of TPA, A23187, db cAMP plus IBMX on the proliferation of G_0 synchronized NIH 3T3 cells by using sulforhodamin B assay. TPA and db cAMP plus IBMX stimulated proliferation 1.6-1.7 fold but A23187 stimulated only slightly (Figure 1 and 2). Optimal concentration of each agent was determined as 100 ng/ml, 50 ng/ml and 10 μg/ml for TPA, A23187 and db cAMP plus IBMX, respectively, by this experiment. This result supports the important roles of second messengers in transducing growth factor signal to cell proliferation.

The effects of TPA, A23187 and db cAMP on the amount of c-*fos* and c-*myc* mRNA

c-*fos* and c-*myc* expression is tightly associated with growth in various cell types and universally induced by mitogens and inhibited by growth inhibitory factors. The regulation of these genes is complex and is controlled not only at the level of transcription initiation but also through a block of transcription elongation, stabilization

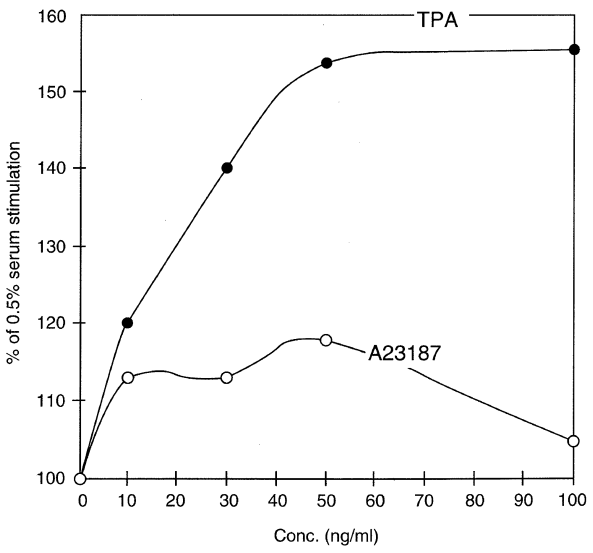


Figure 1. Effect of TPA and A23187 on the proliferation of NIH 3T3 cells. Quiescent cells were incubated for 3 h with various concentration of TPA and A23187. Proliferation was assayed by sulforhodamin B assay and presented by percent of 0.5% serum incubated cells.

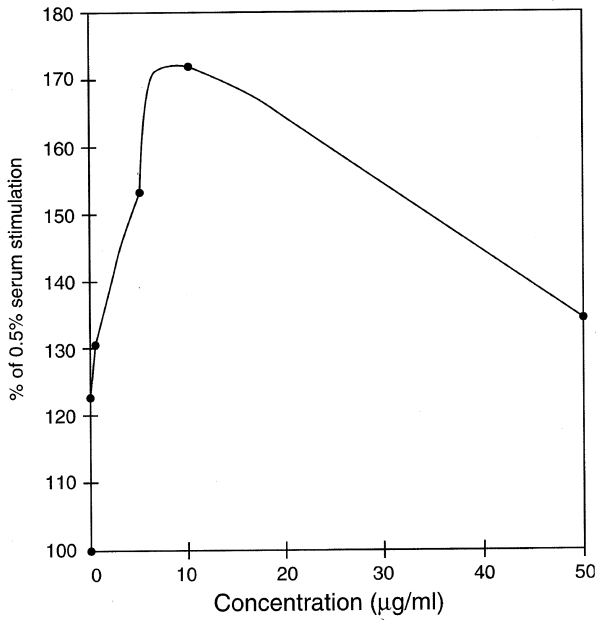
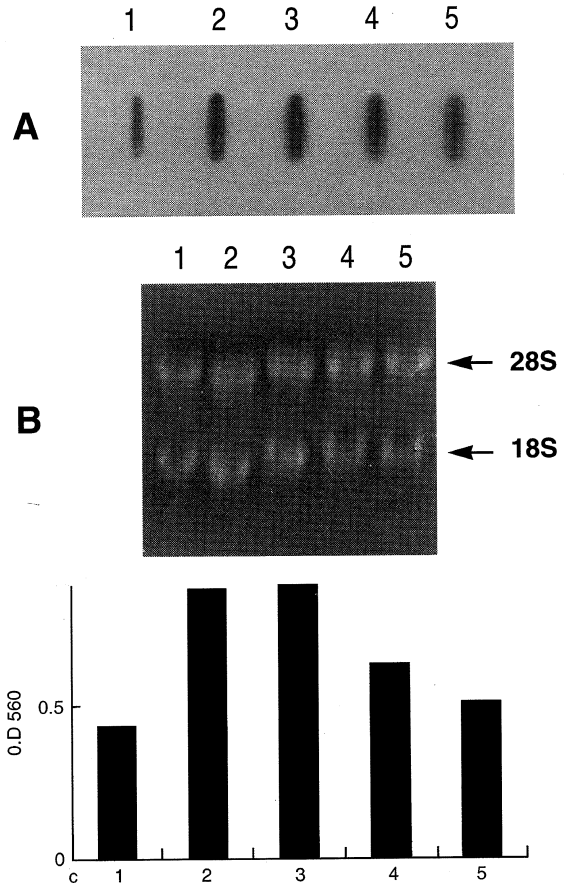


Figure 2. Effect of db cAMP plus IBMX on the proliferation of NIH 3T3 cells. Quiescent cells were incubated for 30 min with various concentration of db cAMP plus IBMX. Proliferation was assayed by sulforhodamin B assay and presented by percent of 0.5% serum incubated cells.



of mRNA and translation of transcripts (Marcu *et al.*, 1992). It is clear that transcriptional regulation of these genes is complex and that expression is controlled by both positive and negative *cis*-acting regulatory elements (Marcu *et al.*, 1992). The expression of c-*fos* and c-*myc* is induced transiently with maximal expression occurring 30 min and 2 h after stimulation respectively (Greenberg and Ziff, 1984; Moore *et al.*, 1986). Quiescent NIH 3T3 cells were treated with TPA, A23187 and db cAMP plus IBMX for 30 min or 2 h. Slot and Northern blot analysis showed that every agent stimulated the c-*fos* and c-*myc* expression (Figure 3 and 4) but the level of of stimulation was different from that of proliferation (Figure 1 and 2). Among the agents, A23187 was the strongest inducer of c-*fos* and c-*myc* expression even though it stimulated proliferation only weakly (Figure 1). Recent observation shows that the c-*fos* and c-*myc* induction is not sufficient to stimulate DNA synthesis or cell cycle progression. For example, transformed cells expressing high level of c-*fos* can be arrested in the G_0 state by serum starvation. In BER2 cells c-*myc* expression is not sufficient for mitogenic response of cells (Shibuya *et al.*, 1992). These results and ours imply that the expression of these proto-oncogenes is not sufficient for cell proliferation. It is possible that very low level of expression of these genes is sufficient to stimulate the expression of target gene such as ornithine decarboxylase (Bello-Fernandez *et al.*, 1993). Ornithine decarboxylase is known to be a key enzyme involved in the biosynthesis of polyamines that is crucial for cell proliferation (Heby and Persson, 1990). It has recently shown that overexpression of ornithine decarboxylase in NIH 3T3 cells leads to cell transformation (Auvinen *et al.*, 1992).

Effects of TPA, A23187 and db cAMP plus IBMX on the expression of pRb

Transcription initiation of c-*fos* and c-*myc* genes is regulated by several transcription factors including serum response factor (SRF), AP-1, cAMP response element binding protein (CREB) (Graham and Gilman, 1991; Karin and Smeal, 1992). Many phosphorylation events regulated by second messengers are being identified as being involved in regulating the action of these transcription factors. Thus these transcription

Figure 3. Slot blot analysis of c-*fos* mRNA. After 30 min stimulation of quiescent NIH 3T3 cells with each agent, total RNA was prepared. **(A)** 20 μg of total RNA was blotted on the nylon membrane and hybridized with DIG-labeled 1.0 kb v-*fos* DNA probe. 1, 0.5% serum media; 2, 100 ng/ml TPA; 3, 50 ng/ml A23187; 4, 10 μg/ml db cAMP plus 10 μg/ml IBMX; 5, 10% serum media. **(B)** The same amount of RNA was electrophoresed and the gel was stained by ethidium bromide, showing the analysis of equivalent amounts of total RNA. **(C)** Densitometer scanning of (A)

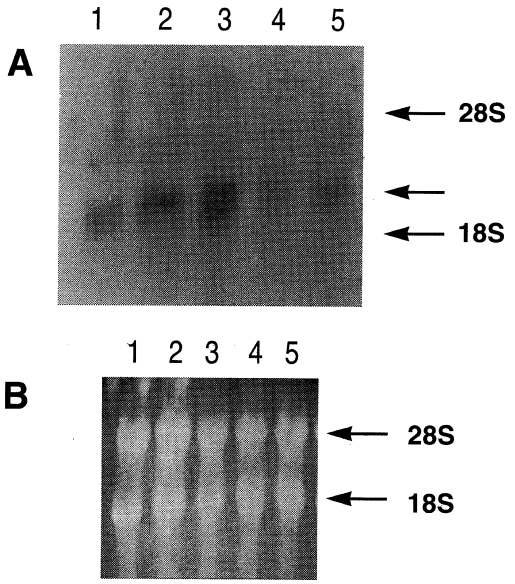


Figure 4. Northern blot analysis of c-*myc* mRNA. **(A)** After 2 h stimulation of quiescent NIH 3T3 cells with each agent, Northern blot analysis of 30 μg of total RNA was performed. 1, 0.5% serum media; 2, 100 ng/ml TPA; 3, 50 ng/ml A23187; 4, 10 μg/ml db cAMP plus 10 μg/ml IBMX; 5, 10% serum media. **(B)** Ethidium bromide stained gel used for Northern blot, showing the analysis of equivalent amounts of total RNA.

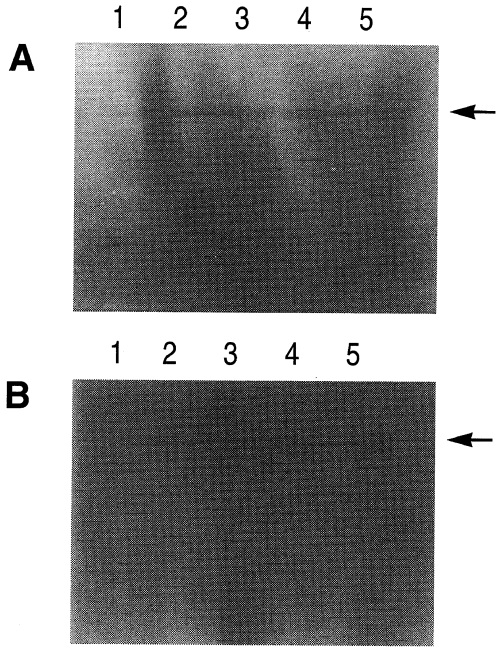


Figure 5. Western bot analysis of pRb expression. NIH 3T3 cells were treated with each agent, as shown in Figure 3 for 30 min **(A)** or 2 hours **(B)** and cellls were lysed directly. Equal amount of protein was electrophoresed by 10% SDS PAGE, transferred to nitrocellulose paper and probed with anti-pRb antibody as described in Materials and Methods. The arrow indicates the 105 kDa pRb protein.

factors should be regarded as nuclear messengers that mediate the actions of signal transduction pathways stimulated by growth factors.

Recently it has been shown that pRb also regulates the transcription of these proto-oncogenes which contain E2F binding site, by association with E2F. E2F binding sites are required for a transcriptional activation after serum stimulation of quiescent cells (Mudryj *et al.*, 1990; Robbins *et al.*, 1990). Recent experiments have demonstrated that E2F levels increase by stimulation of quiescent NIH 3T3 cells with kinetics similar to that shown for the activation of c-*myc* transcription. These observation imply that second messengers of growth factor can regulate pRb expression and function may control the expression of c-*fos* and c-*myc*.

To elucidate the relationship between second messengers and expression of pRb, we investigated the effects of TPA, A23187 and db cAMP plus IBMX on the pRb expression by Western analysis (Figure 5). Western blot analysis revealed the amount of pRb and also the relative amounts of the hyper- and hypo-phosphorylated forms because unphosphorylated pRb migrated faster than the phosphorylated pRb during gel electrophoresis (Yen and Chandler, 1992). Figure 5 shows that these three agents do not cause any significant changes in the amount of pRb and the phosphorylated state of this protein. This result suggests that pRb is not involved in the regulation of c-*fos* and c-*myc* by second messengers. However it could not be excluded that Western blot analysis may not be sensitive enough to detect minor changes in phosphorylation of pRb which could activate E2F.

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