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Differential Effects of Overexpression of PKCα and PKCδ/ϵ on Cellular E2F Activity in Late G1 Phase

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Introduction of a reporter gene containing E2F binding sites linked to the luciferase gene permitted us to detect transient cellular E2F activity in late G1 phase rat 3Y1 fibroblasts. Overexpression of three major protein kinase C (PKC) isozymes expressed in 3Y1 cells caused differing effects on E2F activity depending on the isozymes overexpressed. Overexpression of PKCα inhibited E2F activity while the overexpression of PKCδ or PKCϵ enhanced it, suggesting that these PKC isozymes play different roles in the regulation of E2F activity. Consistent with previous findings that the activation of PKC by TPA in late G1 phase results in the inhibition of DNA synthesis (Huang, C., and Ives, H.E., 1987, *Nature* **329,** 849–850), the addition of TPA in late G1 phase specifically inhibited E2F activity. Overexpression of PKC isozymes resulted in an enhancement of the TPA-induced inhibition of E2F in late G1 phase. This enhancement was observed for all three PKC isozymes examined, suggesting that these PKC isozymes all are potent mediators of the TPA-induced inhibition of E2F activity in late G1 phase.

Several lines of evidence suggest the involvement of Protein kinase C (PKC) at an early phase of the cell cycle. One example is the synthesis of diacylglycerols, endogenous activators of PKC, upon stimulation of a variety of growth factors (2). Consistent with this is the activation of some PKC members upon growth factor treatment of cells (3, 4). On the other hand, the effect of PKC activators such as TPA on the progression of the cell cycle depends on the cell type examined. For example, TPA stimulates DNA synthesis in some cells such as Swiss 3T3 cells and lymphocytes (5, 6), whereas it inhibits DNA synthesis in vascular smooth muscle cells and rat 3Y1 cells (1, 3). However, the reason for the difference in the effect of TPA remains completely to be clarified. One study on vascular smooth muscle cells revealed that treatment of the cells with TPA in late G1 phase inhibits DNA synthesis triggered by growth factors (1). In vascular endothelial cells, the treatment of cells with TPA in late G1 phase inhibits DNA synthesis whereas it induces DNA synthesis when added in early G1 phase (7, 8). The forced activation of PKC by TPA in late G1 phase suppresses the cdk activity as well as Rb phosphorylation (7, 8). Thus, it is possible that the inhibition of DNA synthesis by TPA might involve the action of PKC in late G1 phase of the cell cycle. These observations suggest the intriguing possibility that PKC acts at at least two independent points in the cell cycle progression, early G1 and late G1 phase.

Among the events implicated to be involved in the progression of G1 Phase, E2F is of particular interest. E2F was first found as a nuclear protein that binds to the adenovirus E2 Promoter (9, 10). The E2F-binding sequence is commonly found in the promoter/enhancer region of cellular genes that play roles in G1/S progression. These include the gene for dihydrofolate reductase, thymidine kinase, DNA polymerase α, ribonucleotide reductase, c-myc, N-myc, and c-myb(11, 12, 13). In early G1 phase, E2F forms an inactive complex with the retinoblastoma susceptibility gene product

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Abbreviations: PKC, protein kinase C; TPA, 12-*O*-tetradecanoyl phorbol 13-acetate.

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(pRb) and its transcriptional activity is suppressed (14). In late G1 phase, pRb is phosphorylated, and loses its ability to bind to E2F. Consequently, E2F is released from the complex resulting in the induction of genes required for DNA synthesis (15, 16).

Molecular cloning and biochemical studies have revealed that PKC comprises a family of proteins consisting of at least 11 members among which 9 have been shown to be cellular mediators of the action of TPA (17). The overexpression of PKCδ in NIH 3T3 cells results in growth retardation as measured by cell number. On the other hand, the overexpression of PKCϵ results in growth stimulation (18). Although these observations raise the possibility of different roles of PKC isozymes in growth control, there has been no further demonstration of the roles of various PKC isozymes on the progression of the cell cycle. Stimulation of quiescent rat 3Y1 fibroblasts by serum or growth factors results in the acute activation of all three major PKC isozymes, PKCα, PKCδ, and PKCϵ(3). This suggests that all of these PKC isozymes might be involved in events occurring during early G1 phase of the cell cycle. In the present study, we addressed the question of how these PKC isozymes are involved in the regulation of cell cycle progression in late G1 phase. For this purpose, we constructed a reporter gene containing E2F binding sites and examined the effects of the overexpression of various PKC isozymes. We show that the overexpression of PKCα inhibits E2F activity while the overexpression of PKCδ or PKCϵ enhances it in rat 3Y1 fibroblasts.

MATERIALS AND METHODS

Plasmid construction. The reporter plasmid, E2P-luc., containing E2P oligonucleotides corresponding to −71/−34 of adenovirus E2 Promoter (AGTTTTCGCGCTTAAATTTGAGAAAGGGCGCGAAACTA) was inserted upstream of a human interferon β gene promoter (−55/+19) followed by a structural gene encoding luciferase (10). The control reporter plasmid, mE2P-luc., includes mutant E2P oligonucleotides (AGTTTTCAGATTTAAATTTGAGAAAGG-GATCGAAACTA) instead of the E2P oligonucleotides. PKCα (YK504), PKCδ(M241), and PKCϵ(YK529) expression plasmids contain a PKC coding sequence in an expression vector SRD, which is driven by the SRα-promoter as described elsewhere (19).

Transfection. Rat fibroblast 3Y1 cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS). Cells were seeded at a concentration of 3×10^5 cells/60 mm dish and transfected 24 hours after changing to fresh medium. Transfection was done by calcium phosphate co-precipitation with 7 μg DNA including 3 μg of reporter plasmid, 2 μg of each PKC expression vector and various amounts of empty expression vector, SRD, to make a total of 7 μg DNA. After transfection, the cells were made quiescent by culturing in DMEM containing 0.5% FCS for 48 hours.

Luciferase assay. Cell lysates for luciferase assay were prepared at appropriate times after stimulation of quiescent cells by 10% fetal calf serum. 3Y1 cells in 6 cm dish were washed three times with PBS and harvested with 150 μl PBS. The cell pellet was suspended in 30 μl of 0.1 M KPO_4, pH7.8, and lysed by repeated freezing and thawing. After the lysate was clarified by centrifugation, 10 μl was used for luciferase assay according to the method described elsewhere (3). Values shown represent means of at least three independent transfections using different lot of DNA.

Western blotting. Cells prepared for luciferase assay were lysed in SDS-PAGE sample buffer and cellular proteins were analyzed by SDS-PAGE followed by immunoblotting using antibodies specific to the PKC isozymes (3).

RESULTS

Transient activation of E2F reporter gene in late G1 phase. In order to monitor E2F activity in intact cells, we constructed a reporter plasmid in which E2F binding sites are linked upstream of the structural gene for luciferase. After introduction of this reporter into rat 3Y1 fibroblasts, the cells were made quiescent by serum starvation and were then stimulated by the addition of 10% serum to start the cell cycle. Fig. 1 shows the time course of luciferase activity after serum stimulation, indicating that reporter expression is transient to 15 hours at a maximum, whereas the control reporter containing the mutant E2F binding sequence shows no activity. This clearly indicates that the reporter can monitor cellular E2F activity. DNA synthesis under these conditions starts after about 12 hours and peaks at around 15 to 18 hours (data not shown). Considering that time required for the synthesis of luciferase protein is several hours, the luciferase activity peak at 15 hours also supports the notion that reporter expression reflects cellular E2F activity.

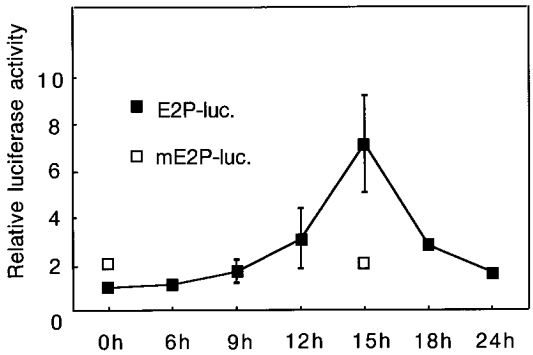

FIG. 1. Time course of E2P reporter expression in 3Y1 cells. E2P-luc. (closed squares) or mE2P-luc. (open squares) (3 μg) and empty vector (4 μg) were transfected (60 mm dish). Cells were kept in DMEM supplemented with 0.5% FCS for 48 hours and became quiescent. Then, the cells were stimulated with 10% FCS, and cell lysates were obtained by a freeze-thaw method in a total of 30 μl of 100 mM potassium phosphate buffer (pH 7.8) at indicated time points.

Overexpression of PKCδ or PKCϵ enhances the E2F reporter expression while overexpression of PKCα suppresses it. We next examined the effect of the transient overexpression of three PKC isozymes on E2F reporter expression. The three PKC isozymes, PKCα, PKCδ, and PKCϵ, are the major PKC isozymes expressed in 3Y1 cells as well as in many other cells. As shown in Fig. 2A, the overexpression of PKCα suppressed reporter expression 15 hours after serum stimulation. On the other hand, the overexpression of PKCδ or PKCϵ resulted in an enhancement of reporter expression. The expression levels of both endogeneous and exogenous PKC isozymes are shown in Fig. 2B. Note that the transient system permits the introduction of transfected DNA into a portion

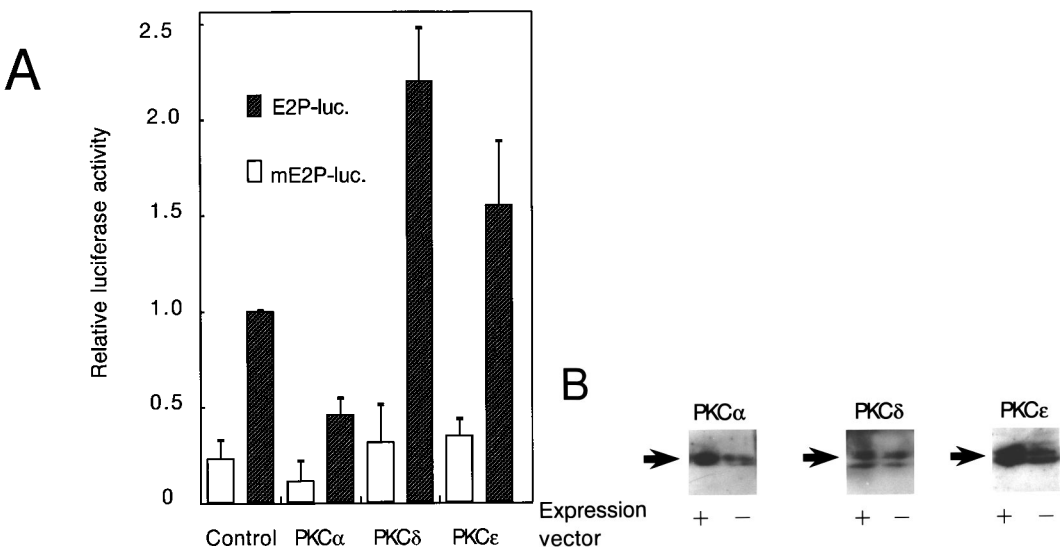

FIG. 2. (A) Different effects of PKC subspecies on E2F reporter expression. PKC overexpression vector (2 μg) and E2P-luc. (hatched bars) or mE2P-luc.(open bars) (3 μg) were transfected (60 mm dish) and total DNA was adjusted to 7 μg with empty vector. Cells were kept in DMEM supplemented with 0.5% FCS for 48 hours and became quiescent. The cells were then stimulated with 10% FCS. Samples were prepared 15 hours after serum stimulation. Values were normalized to the luciferase activity of E2P-luc. reporter in vector-transfected control cells (taken as 1). (B) Western blot analysis of the overexpression of PKC subspecies. PKC overexpression vector (2 μg) and E2P-luc.(3 μg) were transfected (60 mm dish) and total DNA was adjusted to 7 μg with empty vector. Cells were kept in DMEM supplemented with 0.5% FCS for 48 hours and became quiescent. The cells were then stimulated with 10% FCS. Samples were prepared 15 hours after serum stimulation and analyzed by SDS-PAGE followed by immunoblot using antibodies specific for the PKC isozymes.

of the cell population; under our conditions roughly 5 to 20% of cells incorporated DNA (data not shown).

Forced activation of PKC in late G1 phase by TPA results in the specific inhibition of E2F reporter expression. Fig. 3 shows the effect of TPA treatment for 30 min. on E2F reporter expression 15 hours after serum stimulation. TPA treatment was started 0, 6, and 12 hours after serum stimulation. Interestingly, only when treatment began 6 hours after stimulation was there any inhibition of reporter expression, most likely reflecting the inhibition of E2F activity. This is consistent with previous observations in vascular smooth muscle cells and vascular endothelial cells that the addition of TPA in late G1 phase affects DNA synthesis (1, 7, 8). This further confirms that E2F reporter expression is a marker for cell cycle progression occurring in late G1 phase.

Overexpression of any of the three PKC isozymes results in the enhancement of the TPA-induced suppression of E2F reporter expression. The effect of TPA in late G1 phase on E2F reporter expression was then examined using PKC overexpressing cells. As shown in Fig. 4, cells overexpressing any of the three PKC isozymes showed the enhancement of TPA-induced suppression of E2F reporter expression.

DISCUSSION

In the present study, we show that a reporter gene containing the E2F-binding site is transiently expressed in late G1 phase (Fig. 1). This permitted us to directly monitor cellular E2F activity by means of gene expression in intact cells. The effect of TPA on reporter expression was observed only when TPA was added 6 hours after the start of the cell cycle (Fig. 3). This is consistent with the earlier observation that the addition of TPA in late G1 phase specifically inhibits cellular DNA synthesis (1), and further suggests that the inhibitory effect of TPA (added in late G1) on cell cycle progression is also applicable to 3Y1 cells.

The second point of the present study is that the overexpression of PKCα severely inhibits E2F

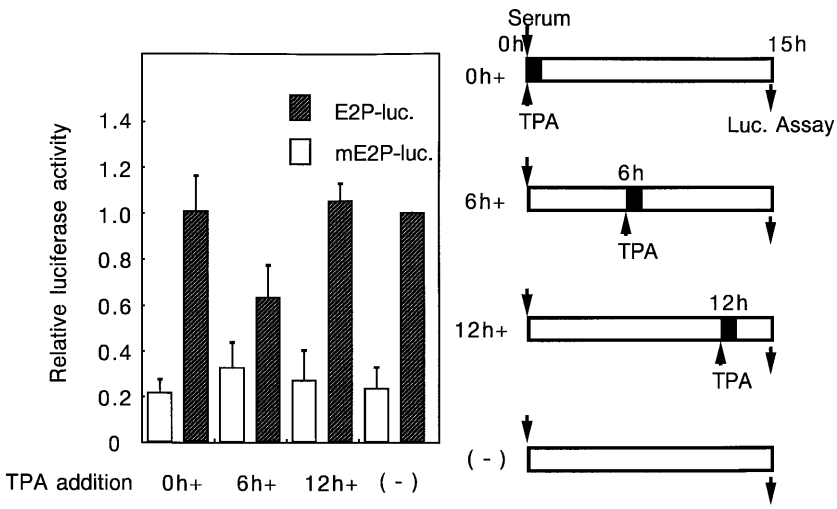

FIG. 3. Inhibition of E2F reporter expression by TPA treatment. E2P-reporter (hatched bars) or mutant-E2P-reporter (open bars) (3 μg) and empty vector (4 μg) were transfected (60 mm dish). Cells were kept in DMEM supplemented with 0.5% FCS for 48 hours and became quiescent. The cells were then stimulated with 10% FCS, and TPA (100 ngml^{-1}) was added for 30 minutes starting 0, 6, or 12 hours after serum stimulation. Samples were prepared 15 hours after serum stimulation. Values were normalized to that of E2P-luc. reporter in vector-transfected control cells without TPA treatment (taken as 1). The four kinds of cell-treatment protocol are shown on the right. Black boxes indicate the 30 min. TPA treatment.

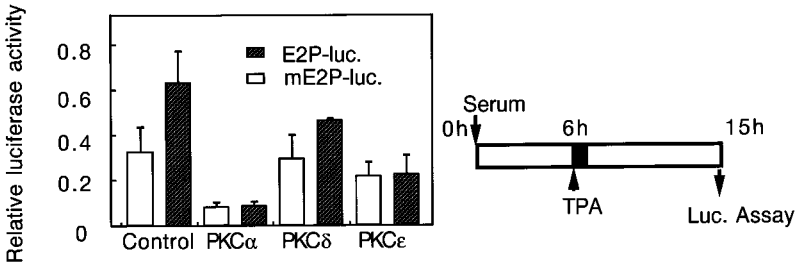

FIG. 4. Overexpression of PKC isozymes results in an enhancement of the TPA-induced suppression of E2F reporter expression. PKC overexpression vector (2 μg) and E2P-reporter (hatched bars) or mutant-E2P-reporter (open bars) (3 μg) were transfected (60 mm dish) and total DNA was adjusted to 7 μg with empty vector. Cells were kept in DMEM supplemented with 0.5% FCS for 48 hours and became quiescent. The cells were then stimulated with 10% FCS and TPA (100 ngml^{-1}) was added for 30 minutes starting 6 hours after serum stimulation. Samples were prepared 15 hours after serum stimulation. Values were normalized to that of E2P-luc. reporter in vector-transfected control cells without TPA treatment (taken as 1). The cell-treatment protocol is shown on the right. The black box indicates the 30 min. TPA treatment.

activity, whereas the overexpression of PKCδ or PKCϵ enhances it (Fig. 2). Considering that all three PKC isozymes are activated in early G1 phase (3, Mizuno in preparation), the enhancing effect of PKCδ and PKCϵ overexpression could mainly reflect their positive role in the progression of early G1 phase. Furthermore, the inhibitory effect of PKCα suggests its negative role on cell cycle progression in late G1 phase.

Earlier studies have shown that PKCα is the PKC isozyme whose overexpression causes retardation of cell proliferation. Overexpression of PKCα in R6 cells but not PKCβI or PKCϵ results in growth retardation (20, 21). Similar observations were also reported for CHO cells (22). Interestingly, differentiation of mouse B16 melanoma cells by retinoic acid results in an increase in PKCα and forced overexpression of PKCα causes differentiation without retinoic acid (23). An increase in PKCα has also reported for human K562 erythroleukemia cells treated with TPA to differentiate into megakaryocyte (24). Although these observations do not simply implicates the role of PKCα in cell cycle progression, these are consistent with the idea that PKCα is one of the candidate PKC isozyme which is involved in the inhibition of DNA synthesis at late G1 phase of the cell cycle.

The involvement of these three PKC members in the TPA-induced inhibition of E2F activation in late G1 phase can be estimated from the results shown in Fig. 4, where overexpressed PKC was activated by TPA in late G1 phase. Interestingly, PKCα showed the most striking enhancing effect on the TPA-induced inhibition of E2F activity, while both PKCδ and PKCϵ also showed some enhancing effect. This suggests that all three PKC isozymes are mediators of the TPA-induced inhibition of E2F, and with PKCα the most. This is consistent with the difference observed between PKCα and PKCδ/ϵ (Fig. 2), that the overexpression of PKCα, but not PKCδ or PKCϵ, inhibits the E2F reporter expression.

Earlier experiments have shown that the forced activation of PKC by TPA in late G1 phase suppresses cdk activity as well as the phosphorylation of Rb (7, 8). Thus, our observation of the TPA-induced inhibition of E2F seems to involve the inhibition of cdk activity and Rb phosphorylation. However, how the activation of PKC leads to the inhibition of these processes in late G1 remains totally unknown.

The TPA-induced inhibition of cell cycle progression such as through E2F activation suggests a mechanism involving PKC in late G1 phase. Our present demonstration suggests that PKCα is the major PKC isozyme involved in this process. If such PKC-mediated regulation of late G1 phase operates during the normal cell cycle, PKC (PKCα for example) must be activated at this specific phase. Since the activation of PKC has only been analyzed in early G1 phase, the timing and duration of the activation of each PKC member during cell cycle progression, including late G1, are among the most important points to be clarified.

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