MOLECULAR AND CELLULAR BIOLOGY, June 1997, p. 3418–3428 Vol. 17, No. 6
0270-7306/97/$04.00+0

Tumor Promotion by Depleting Cells of Protein Kinase Cδ

ZHIMIN LU,[1] ARMAND HORNIA,[1] YOU-WEI JIANG,[1]† QUN ZANG,[1]
SHIGEO OHNO,[2] AND DAVID A. FOSTER[1]*

Department of Biological Sciences, Hunter College of The City University of New York, New York, New York 10021,[1] and Department of Molecular Biology, Yokohama City University School of Medicine, Yokohama, Japan[2]

Received 18 October 1996/Returned for modification 17 November 1996/Accepted 20 March 1997

Tumor-promoting phorbol esters activate, but then deplete cells of, protein kinase C (PKC) with prolonged treatment. It is not known whether phorbol ester-induced tumor promotion is due to activation or depletion of PKC. In rat fibroblasts overexpressing the c-Src proto-oncogene, the phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate (TPA) induced anchorage-independent growth and other transformation-related phenotypes. The appearance of transformed phenotypes induced by TPA in these cells correlated not with activation but rather with depletion of expressed PKC isoforms. Consistent with this observation, PKC inhibitors also induced transformed phenotypes in c-Src-overexpressing cells. Bryostatin 1, which inhibited the TPA-induced down-regulation of the PKCδ isoform specifically, blocked the tumor-promoting effects of TPA, implicating PKCδ as the target of the tumor-promoting phorbol esters. Consistent with this hypothesis, expression of a dominant negative PKCδ mutant in cells expressing c-Src caused transformation of these cells, and rottlerin, a protein kinase inhibitor with specificity for PKCδ, like TPA, caused transformation of c-Src-overexpressing cells. These data suggest that the tumor-promoting effect of phorbol esters is due to depletion of PKCδ, which has an apparent tumor suppressor function.

Carcinogenesis is a multistep process involving successive rounds of mutation (initiation) and selected amplification (promotion) of mutated cells. Eventually, mutated cells acquire an appropriate complement of genetic changes such that the cells divide without proper control, giving rise to a tumor (10). This process can be accelerated by stimulating the replication and amplification of mutated cells, increasing the numbers of partially transformed cells subject to further mutation to a more cancerous state. Substances that stimulate the division of incompletely transformed cells are known as tumor promoters, and while not inducing directly the genetic changes that ultimately result in a tumor, they can dramatically speed up the process (44).

The best-studied class of tumor promoters are the phorbol esters, which exert their effects on protein kinase C (PKC). The PKC isoforms, of which there are no fewer than nine that are responsive to the tumor-promoting phorbol esters, are encoded by a multigene family. Upon phorbol ester treatment, PKC isoforms become associated with the cell membrane and active (27). However, upon prolonged phorbol ester treatment, PKCs are proteolytically degraded (43). The time course for PKC depletion upon phorbol ester treatment varies substantially for different cell types, from a few hours to a few days. Tumor promotion requires repeated long-term exposure to phorbol esters, suggesting that depletion rather than activation of PKC is important for tumor promotion. However, it has been pointed out that even though PKC is depleted by prolonged phorbol ester treatment, newly synthesized PKC would be brought to the membrane, where there would be a short-lived, but potentially significant, phorbol ester-induced activation of PKC (22). Thus, it is not clear whether activation or depletion of PKC is important for the tumor-promoting effects of phorbol esters.

* Corresponding author. Phone: (212) 772-4075. Fax: (212) 772-5227. E-mail: foster@genectr.hunter.cuny.edu.

† Present address: Memorial Sloan-Kettering Cancer Center, New York, NY 10021.

We have found that the tumor-promoting phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate (TPA) stimulates anchorage-independent growth of rat fibroblasts overexpressing the c-Src proto-oncogene. Thus, TPA is able to induce amplification of cells that have an initiating mutation (c-Src overexpression) and therefore functions very much like a tumor promoter in this cell culture model. We have exploited the promoter-like properties of TPA on c-Src-overexpressing rat fibroblasts to investigate the role of PKC in tumor promotion.

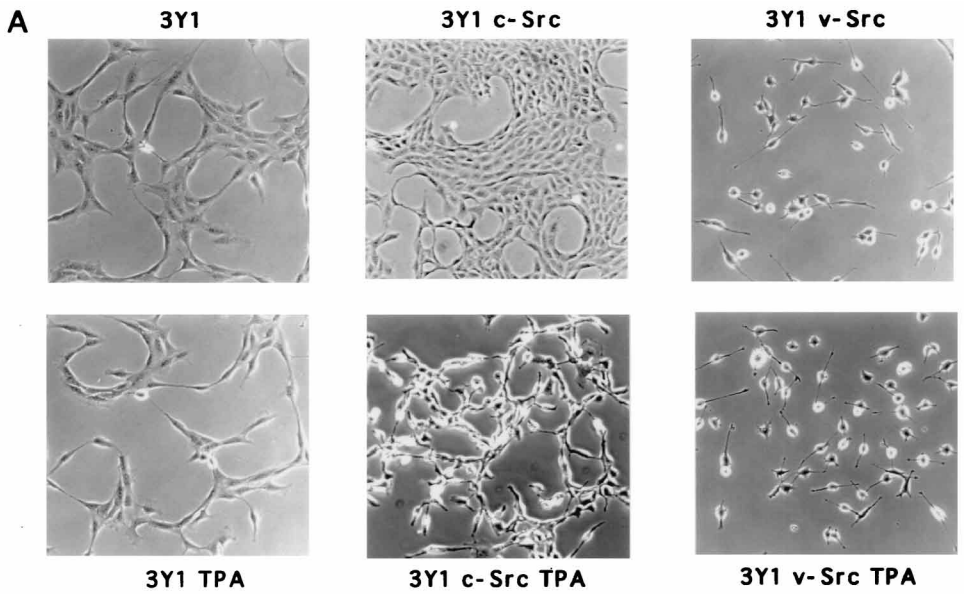
MATERIALS AND METHODS

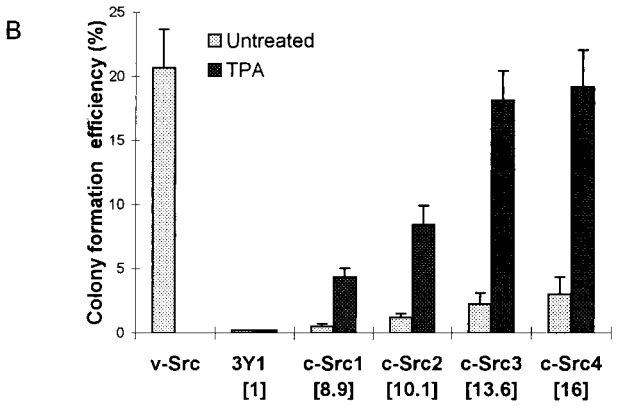
Cells and cell culture conditions. Rat 3Y1 cells or rat 3Y1 cells expressing either v-Src or c-Src were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% bovine calf serum (HyClone). Cell cultures were made quiescent by growing them to confluence and then replacing the medium with fresh medium containing 0.5% newborn calf serum for 1 day. For growth of cells in soft agar, 10^3 cells were suspended in top agar (DMEM, 20% calf serum, 0.38% agar) and overlaid onto hardened bottom agar (DMEM, 20% calf serum, 0.7% agar) as described previously (32).

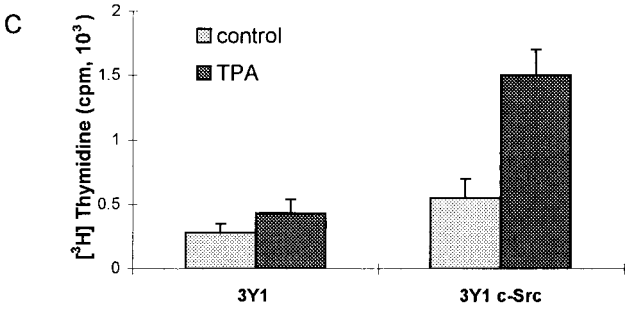
Transfection and G418 selection. Cells were plated at a density of 10^5 cells/100-mm-diameter dish 18 h prior to transfection. Transfections were performed by using Lipofectamine reagent (GIBCO) according to the vendor's instructions. Transfected cultures were selected in 400 μg of G418 per ml for 10 to 14 days at 37°C. At that time, G418-resistant colonies were picked and expanded for further analysis under selective conditions.

Materials. [^{3}H]thymidine, [γ-^{32}P]ATP, and [^{3}H]myristate were obtained from New England Nuclear. The PKC inhibitors staurosporine, calphostin C, chelerythrine chloride, bisindolylmaleimide II, rottlerin, and Go 6976 were obtained from Calbiochem. Bryostatin 1 was obtained from LC Biochemicals. A monoclonal antibody to PKCα was obtained from Upstate Biotechnology; polyclonal antibodies to PKCδ and PKCε were obtained from GIBCO. An antiphosphotyrosine monoclonal antibody (PY20) was obtained from Transduction Laboratories. Monoclonal antibody 327, used to immunoprecipitate Src proteins, was obtained from Oncogene Science.

Western analysis. Extraction of proteins from cultured cells was performed as previously described (37). Equal amounts of protein were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis with an 8% acrylamide separating gel. Transfer to nitrocellulose was performed as described by Joseph et al. (19). After blocking overnight at 4°C with 5% nonfat dry milk–isotonic phosphate-buffered saline (PBS [136 mM NaCl, 2.6 mM KCl, 1.4 mM KH_2PO_4, 4.2 mM Na_2HPO_4]), the nitrocellulose filters were washed three times for 5 min in PBS and then incubated with antibodies as described in the figure legends. Depending upon the origin of the primary antibodies, either anti-mouse or anti-rabbit immunoglobulin G was used for detection with the ECL system (Amersham).







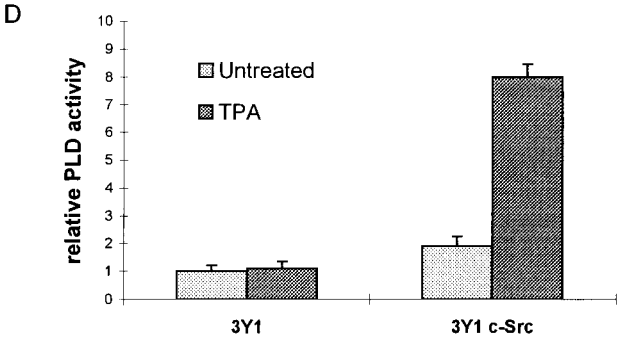


FIG. 1. Cells overexpressing c-Src display a transformed phenotype upon treatment with TPA. (A) 3Y1 cells and 3Y1 cells overexpressing either c-Src or v-Src were either left untreated or treated with TPA (400 nM) for 24 h, at which time the morphology of the cells was examined. The untreated cells were given an equivalent volume of dimethyl sulfoxide, which was the solvent for TPA. (B) Anchorage-independent growth was examined in 3Y1 cells, v-Src-transformed cells, and four independent c-Src-expressing 3Y1 cell lines in the presence or absence of TPA (400 nM). Cells (10^3) were suspended in soft agar, and the percentage of cells forming colonies was determined. The relative levels of c-Src expression, as determined by Western blot analysis, are shown in brackets. (C) 3Y1 cells and 3Y1 cells overexpressing c-Src were grown to confluence and then placed under low-serum growth conditions (0.5%) for 20 h. TPA (400 nM) was then added and DNA synthesis was determined by measuring the incorporation of [^{3}H]thymidine during a 1-h pulse 24 h later. Error bars represent the standard errors for triplicate samples from a representative experiment. (D) PLD activity in 3Y1 cells and 3Y1 cells overexpressing c-Src in the presence and absence of TPA (400 nM) was determined as described previously (36). Error bars represent the ranges of duplicate samples from a representative experiment after normalization to the PLD activity in the untreated parental 3Y1 cells.

PLD assays. Confluent 35-mm-diameter culture dishes were prelabeled for 4 h with [^{3}H]myristate, 3 μCi (40 Ci/mmol) in 3 ml of medium containing 0.5% newborn calf serum. Phospholipase D (PLD)-catalyzed transphosphatidylation in the presence of 1% butanol was performed as described previously (35, 36). Extraction and characterization of lipids by thin-layer chromatography were performed as previously described (35).

DNA synthesis. Confluent cells were placed in 0.5% serum for 24 h in 24-well tissue culture dishes. DNA synthesis was measured by a 1-h pulse with [^{3}H]thymidine (1 μCi/ml; 20 Ci/mmol). After the 1-h pulse, the cells were collected and trichloroacetic acid-precipitable counts were determined by scintillation counting.

Protein kinase assays. Protein kinase assays were performed according to procedures described by Clark and Brugge (3).

RESULTS

Cells overexpressing c-Src display a transformed phenotype upon long-term treatment with TPA. Our laboratory has been examining the role of PKC in the transduction of intracellular signals initiated by the tyrosine kinase activity of v-Src (31, 37, 45, 46). These studies were extended to include cells overexpressing the nontransforming c-Src proto-oncogene. We observed that upon prolonged exposure to TPA (400 nM, 24 h), 3Y1 rat fibroblasts overexpressing c-Src displayed a morphology that resembled that displayed by v-Src-transformed 3Y1 cells (Fig. 1A). TPA did not have this effect upon parental 3Y1 cells, nor did the TPA solvent dimethyl sulfoxide affect the morphology (Fig. 1A). When the TPA was removed, the cells

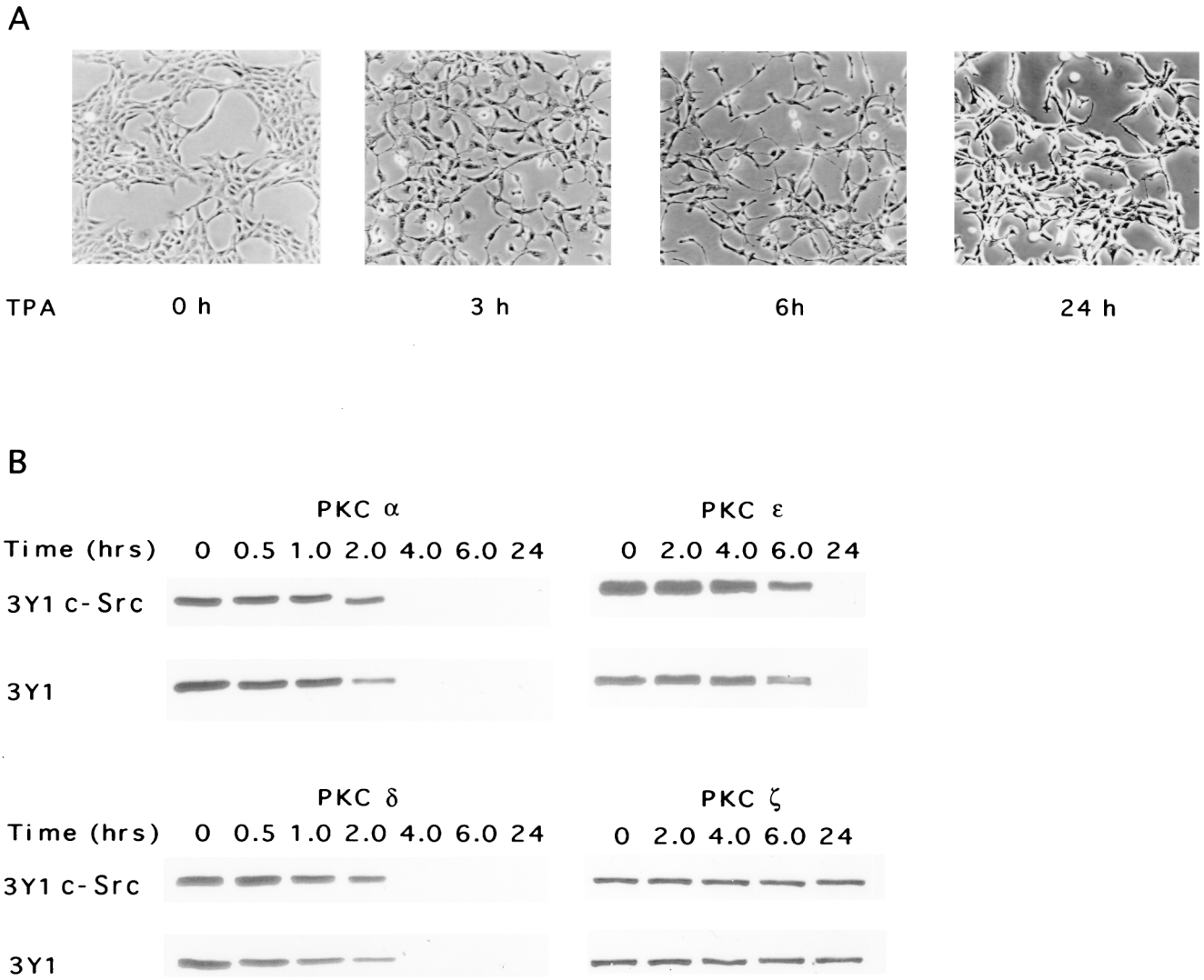


FIG. 2. The effects of TPA administration correlate with the disappearance of PKC. (A) The kinetics of the transformed morphology shown in Fig. 1 was determined by examining the morphology at the times shown. (B) The levels of PKC isoforms α, δ, ε, and ζ were determined at the times shown after TPA (400 nM) addition.

reverted to a flat shape (data not shown), suggesting that the effect was reversible. To determine whether the morphological changes observed in response to TPA actually represented a transformed phenotype, we examined the ability of the TPA-treated c-Src-expressing cells to form colonies in soft agar. As shown in Fig. 1B, the c-Src-overexpressing cells that were treated with TPA formed colonies in soft agar, whereas the parental 3Y1 cells did not. The efficiency of colony formation correlated with the levels of c-Src expression in several independent clones of c-Src-overexpressing 3Y1 cells (Fig. 1B). When soft-agar colonies were picked and grown in the absence of TPA, they were flat and nontransformed, although a few transformants did arise from some of the colonies (data not shown). We next looked at the effect of TPA treatment on cell cycle regulation in 3Y1 cells and in 3Y1 cells overexpressing c-Src. Cells were grown to confluence and then placed in medium containing low serum (0.5%) for 24 h, which minimized DNA synthesis as measured by the incorporation of [^{3}H]thymidine. When TPA was added to the confluent c-Src-overexpressing 3Y1 cells, substantial increases in DNA synthesis levels were detected 12 h after TPA treatment, and DNA synthesis reached peak levels by 20 h after TPA treatment (Fig. 1C). TPA did not significantly affect DNA synthesis in parental 3Y1 cells (Fig. 1C). We previously demonstrated that both v-Src and v-Ras activate PLD activity (18, 35). The activation of PLD did not require PKC; in fact, depleting cells of PKC actually appeared to enhance v-Src-induced PLD activity (36). Therefore, we examined the effect of TPA treatment upon PLD activity in c-Src-overexpressing cells. In Fig. 1D, it is shown that upon prolonged treatment with TPA (24 h, 400 nM), PLD activity in c-Src-overexpressing cells was elevated to levels observed previously in v-Src-transformed cells (35, 36). The long-term TPA treatment had no effect upon PLD activity in the parental 3Y1 cells (Fig. 1D). These data are consistent with TPA having promoter-like effects on cells overexpressing c-Src by allowing these partially transformed cells to be amplified under the restricted growth conditions of soft agar suspension, which in turn allows cell cycle progression and induction of a biochemical phenotype characteristic of v-Src-transformed cells.

The transforming effect of TPA on c-Src-overexpressing cells correlates with the depletion of PKC isoforms. To deter-

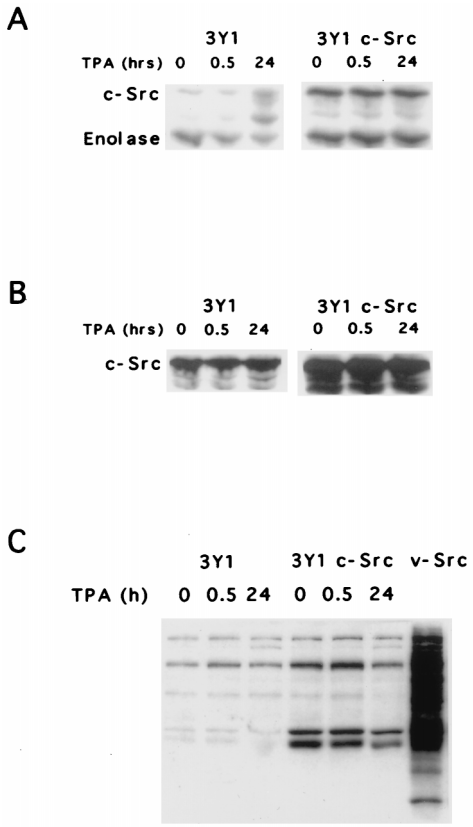


FIG. 3. TPA treatment does not affect the kinase activity of c-Src (A), c-Src levels (B), or total cellular phosphotyrosine (C). Treatment (400 nM, 24 h) was carried out as described in Materials and Methods.

mine whether the effects of TPA were due to the activation or downregulation of PKC, the kinetics of appearance of morphological transformation was compared with the kinetics of PKC isoform depletion. The kinetics of appearance of the transformed morphology induced by TPA in c-Src-expressing cells is shown in Fig. 2A. The round shape and refractile properties characteristic of transformed cells were detected by 6 h after the addition of TPA. We next examined the kinetics of PKC isoform depletion. We previously reported that in 3Y1 cells, PKC isoforms α, δ, ε, and ζ are the predominant isoforms expressed (45). As shown in Fig. 2B, the levels of isoforms α and δ were dramatically reduced by 4 h and were not detectable by 6 h after TPA treatment; isoform ε was substantially reduced by 6 h but was depleted noticeably more slowly in response to TPA. As expected, the TPA-resistant isoform ζ was unaffected by this treatment. These data reveal a correlation between the appearance of morphological transformation and the downregulation of PKC in response to TPA, with morphological transformation detectable within 2 h after depletion of isoforms α and δ.

TPA treatment does not affect the level or kinase activity of c-Src. We next examined the effect of PKC depletion on c-Src protein levels and kinase activity. We first looked at the effect of TPA treatment on the kinase activity of c-Src, and as shown in Fig. 3A, neither c-Src autophosphorylation nor the ability to phosphorylate enolase was affected by TPA treatment of c-Src-overexpressing cells. Similarly, there were no changes in either the levels of c-Src protein (Fig. 3B) or the levels of cellular tyrosine phosphorylation (Fig. 3C) in response to TPA treatment. Thus, the effect of TPA-induced enhancement of transformation does not appear to be due to any obvious direct effects upon either c-Src protein levels or kinase activity.

Inhibiting PKC induces transformed phenotypes in c-Src-expressing cells. The data presented above are consistent with a model in which the downregulation of PKC, specifically PKCδ, is responsible for the promoter-like effects of TPA on c-Src-overexpressing 3Y1 cells. To further implicate downregulation of PKC as being responsible for the tumor-promoting effects of TPA, we examined the effects of compounds that inhibit PKC on 3Y1 cells and 3Y1 cells overexpressing c-Src. We first examined the effects of the PKC inhibitors on DNA synthesis. c-Src-overexpressing and parental 3Y1 cells were treated with several compounds that inhibit PKC by different mechanisms. These included staurosporine (4), bisindolylmaleimide II (40), calphostin C (17), and chelerythrine chloride (17). Staurosporine and bisindolylmaleimide II are ATP analogs, calphostin C competes with the physiological activator of PKC diacylglycerol, and chelerythrine chloride competes with PKC substrates. As shown in Fig. 4A, all four inhibitors mimicked the effect of TPA treatment on DNA synthesis shown in Fig. 2. The effects of the inhibitors were detected within a few hours after treatment with the inhibitors, suggesting that inhibiting PKC facilitated release from a late G_1 block. We also investigated the effect of the PKC inhibitors on PLD activity: as observed with long-term TPA treatment, each of the inhibitors led to increased PLD activity in c-Src-overexpressing cells (data not shown). We next examined the effects of the PKC inhibitors on the morphology of 3Y1 cells and 3Y1 cells overexpressing c-Src. While the effects of most of the PKC inhibitors on cell morphology were subtle and inconclusive, the effects of staurosporine, which was the most potent stimulator of DNA synthesis, were quite striking. Staurosporine treatment resulted in a pronounced refractile morphology characteristic of transformation in c-Src-overexpressing 3Y1 cells but not in parental 3Y1 cells. The transformed morphology was detected between 2 and 4 h after treatment (Fig. 4B). This time course is similar to that for the appearance of the observed transformed morphology in response to the depletion of PKC, in which PKCα and PKCδ were gone between 4 and 6 h and the transformed phenotype was first detected between 2 and 4 h later (Fig. 2). Like long-term TPA treatment, staurosporine treatment had no effect upon the kinase activity of c-Src over the time course used, although longer-term treatments were slightly inhibitory (data not shown). Because of the toxicity of these compounds, we were unable to examine the effects of the inhibitors upon anchorage-independent growth. While it is difficult to ascertain the precise effects of the PKC inhibitors upon the cells, all of the PKC inhibitors, which inhibit PKC by different mechanisms, induced the transformation-related phenotypes only in c-Src-overexpressing cells and, importantly, none of them activated PKC. These data further suggest that depletion, rather than activation, of PKC is responsible for the promoter-like effects of TPA on c-Src-overexpressing cells.

Bryostatin 1 blocks the tumor-promoting ability of TPA. Bryostatin 1, a compound that stimulates membrane association and activation of PKC much like TPA does (14, 38, 39), has been reported to prevent tumor promotion in mouse skin by TPA (14). We therefore examined the effects of bryostatin 1 on TPA-induced transformed morphology in c-Src-overexpressing 3Y1 cells. As shown in Fig. 5A, bryostatin 1 completely blocked TPA-induced refractile morphology. Bryostatin 1 also blocked the TPA-induced colony formation in soft agar observed in c-Src-overexpressing cells (Fig. 5B). These inhibitory effects of bryostatin 1 were not due to toxic effects of this compound, since we were able to culture cells in bryostatin 1 at the concentrations used here for several weeks with no

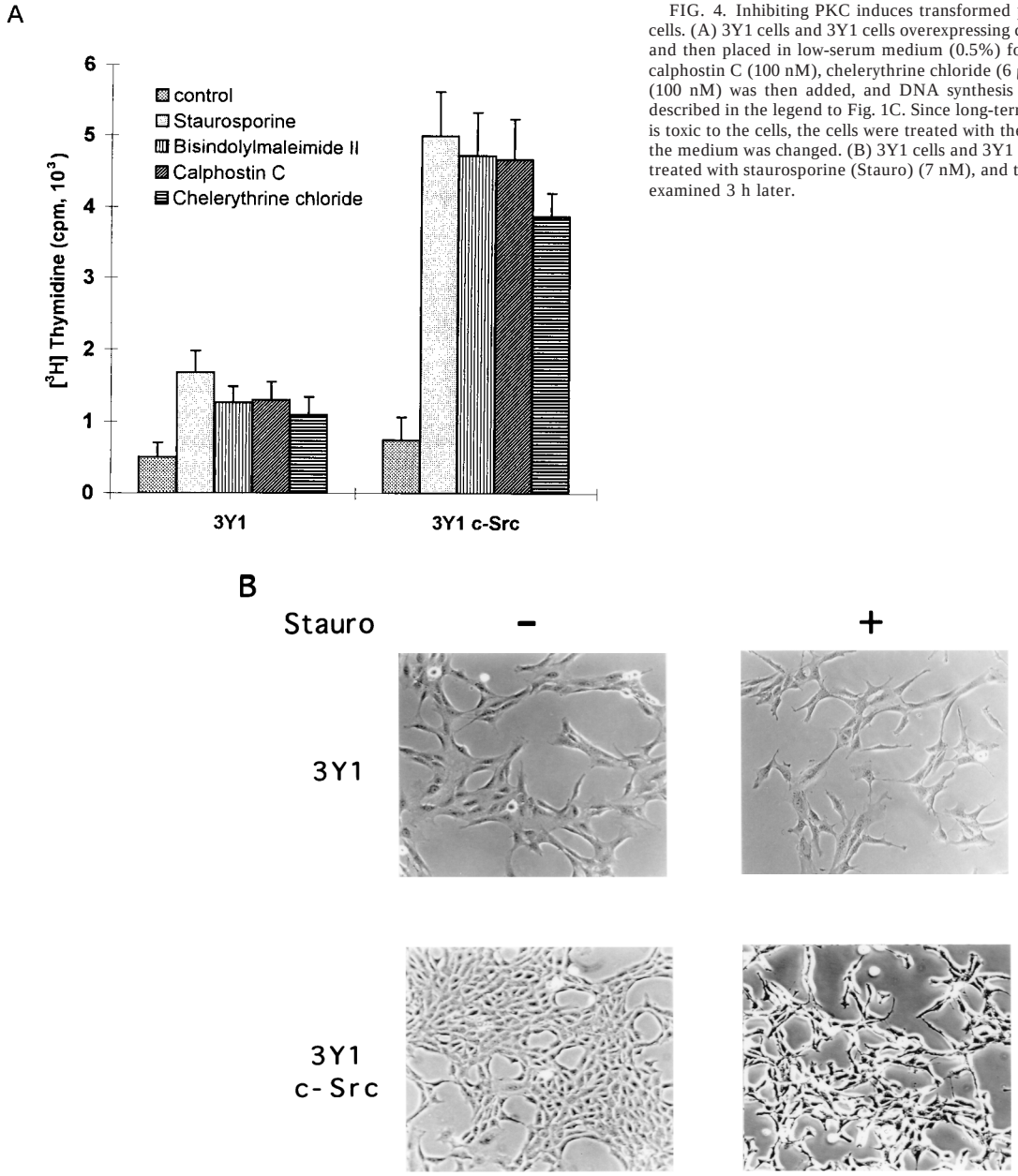
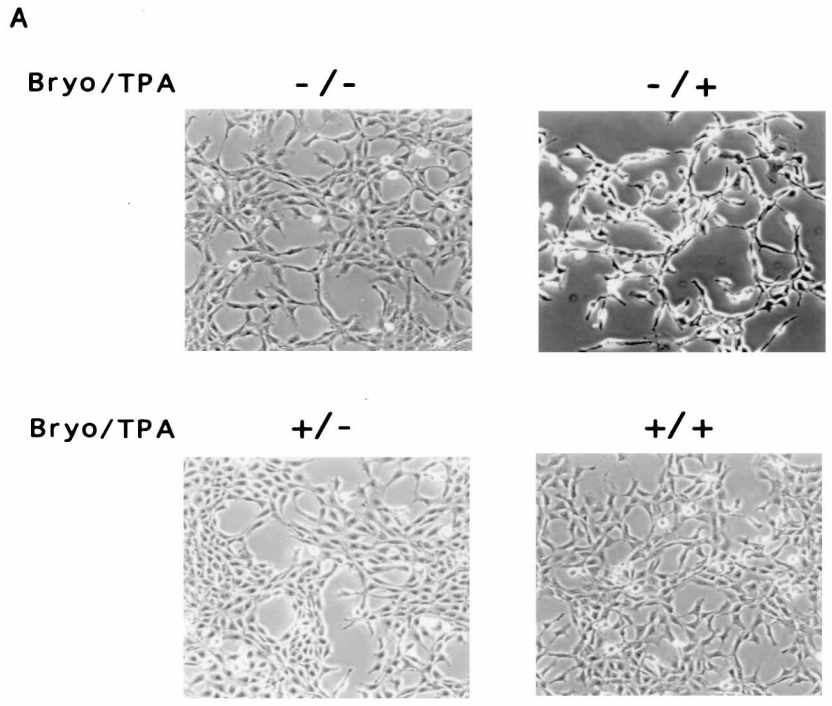


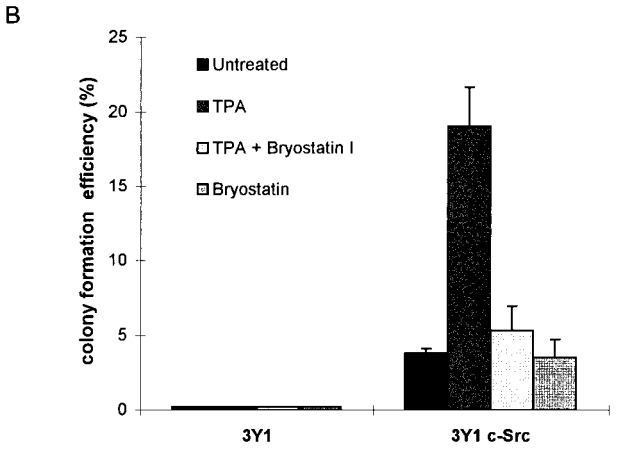
FIG. 4. Inhibiting PKC induces transformed phenotypes in c-Src-expressing cells. (A) 3Y1 cells and 3Y1 cells overexpressing c-Src were grown to confluence and then placed in low-serum medium (0.5%) for 24 h. Staurosporine (7 nM), calphostin C (100 nM), chelerythrine chloride (6 μM), or bisindolylmaleimide II (100 nM) was then added, and DNA synthesis was determined 10 h later as described in the legend to Fig. 1C. Since long-term treatment with the inhibitors is toxic to the cells, the cells were treated with the inhibitors for 2 h, after which the medium was changed. (B) 3Y1 cells and 3Y1 cells overexpressing c-Src were treated with staurosporine (Stauro) (7 nM), and the morphology of the cells was examined 3 h later.

detectable toxicity or changes in growth properties (data not shown). Bryostatin 1 also blocked TPA-induced increases in PLD activity (Fig. 5C) and DNA synthesis (data not shown). Thus, as reported previously for TPA-induced tumor promotion in mouse skin, bryostatin 1 inhibits the tumor promoter-like effects of TPA on c-Src-overexpressing cells.

Bryostatin 1 prevents TPA-induced downregulation of PKCδ but not PKCα or PKCε. Differential regulation of PKC isoforms by TPA and bryostatin 1 has been reported (38, 39). We therefore examined the effect of bryostatin 1 on the levels of the PKC isoforms in c-Src-overexpressing cells that were both treated with TPA and left untreated. As shown in Fig. 6A, bryostatin 1 prevented the TPA-induced downregulation of PKCδ but not PKCα or PKCε. Figure 6A also shows that bryostatin 1 by itself led to downregulation of PKCα and PKCε but not PKCδ, and as shown in Fig. 4A, this treatment did not induce a transformed morphology. In addition to preventing TPA-induced morphology changes and PKCδ downregulation, bryostatin 1 also stimulated membrane association of PKC isoforms α, δ, and ε (Fig. 6B), which correlates with activation of PKC isoforms. Thus, bryostatin 1, much like TPA, is capable of both activating and depleting cells of PKC isoforms, with the only detectable differences between bryostatin 1 and TPA treatment being that bryostatin 1 is unable to downregulate PKCδ or to induce the transformed phenotypes induced by TPA. And, since bryostatin 1 is able to inhibit the effects of TPA, the data suggest that downregulation of PKCδ accounts for the promoter-like effects of TPA on c-Src-overexpressing 3Y1 cells.

The effects of bryostatin 1 on TPA-induced transformation in c-Src-overexpressing cells correlate with the level of PKCδ. To further establish the relationship between the presence of





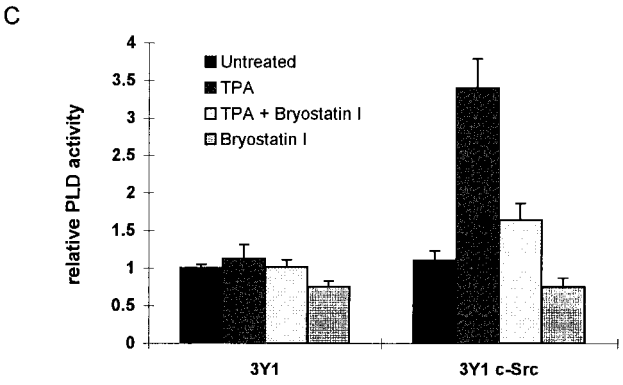


FIG. 5. Bryostatin 1 blocks the promoter activity of TPA. (A) 3Y1 cells overexpressing c-Src were either left untreated or treated with TPA (400 nM), bryostatin 1 (Bryo) (1 μM), or both, as shown, and the morphology of the cells was examined 24 h later. (B) The ability to form colonies in soft agar in the presence of TPA (400 nM) and bryostatin 1 (1 μM), as shown, was determined as described for Fig. 1. (C) The effect of bryostatin 1 on the TPA-induced increase in PLD activity was investigated as described in the legend to Fig. 1.

PKCδ and the TPA-induced transformed phenotype, we examined the dose response to bryostatin 1 in c-Src-overexpressing cells. Figure 7A shows that even at 1 nM bryostatin 1, cells begin to look less transformed, and at 100 nM, the cells are essentially flat and resemble cells that had not been treated with TPA. The effects of increasing concentrations of bryostatin 1 on the levels of PKCδ are shown in Fig. 7B, in which it can be seen that inhibition of the transformed morphology shown in Fig. 7A correlates strongly with the appearance of PKCδ.

A dominant negative PKCδ mutant and a PKCδ-specific inhibitor induce transformation of cells overexpressing c-Src. The data presented above implicate PKCδ as the basis for the tumor promoter-like effects reported here. To establish that PKCδ inhibition was sufficient for the tumor promoter-like effects in c-Src-overexpressing cells, we inhibited the effects of PKCδ specifically. We first introduced a dominant negative PKCδ mutant into c-Src-overexpressing cells. This mutant (DK376A), which has a Lys-to-Ala mutation in the conserved ATP-binding site in PKCδ, was shown previously to act as a dominant negative mutant for PKCδ (16), as was a similar mutant in which Lys 376 was converted to Arg (21). Upon transfection of a plasmid expressing the dominant negative PKCδ mutant into c-Src-overexpressing cells, we selected several clones and then examined their ability to form colonies in soft agar. As shown in Fig. 8A, these cells formed colonies in soft agar and there was a correlation between colony numbers and the levels of PKCδ. Expression of the dominant negative PKCδ mutant also caused morphological transformation (Fig. 8B).

We next examined the effect of compounds reported to

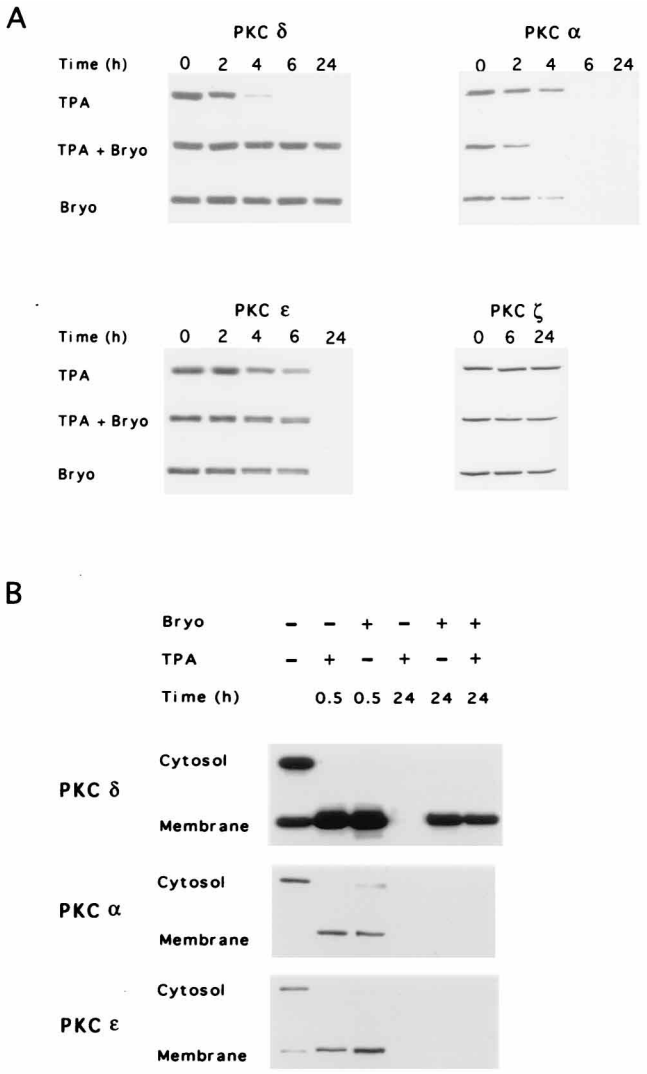


FIG. 6. Bryostatin 1 prevents TPA-induced downregulation of PKCδ but not PKCα or PKCε. (A) The effects of bryostatin 1 on PKC isoforms α, δ, ε, and ζ in c-Src-overexpressing 3Y1 cells were determined by Western analysis. The cells were either left untreated or treated with TPA (400 nM) and bryostatin 1 (Bryo) (1 μM) for the times shown. (B) The effects of bryostatin 1 on the cellular distribution of PKC isoforms α, δ, and ε were determined by isolating membrane and cytosolic fractions from c-Src-overexpressing 3Y1 cells and determining the levels of these PKC isoforms in the two fractions by Western analysis as described previously (45).

inhibit PKCα and PKCδ preferentially. Unlike the less specific PKC inhibitors described above (Fig. 4), these inhibitors were not toxic to the cells and could therefore be used to examine anchorage-independent growth. Go 6976 is highly specific for Ca^{2+}-dependent PKC isoforms, including PKCα but not PKCδ or PKCε (23), and rottlerin inhibits PKCδ more than it does isoforms α and ε (13). The effects of these compounds on colony formation in soft agar are shown in Fig. 8C. The PKCα inhibitor had no effect upon colony-forming ability; however, the PKCδ inhibitor induced colony formation to levels that were higher than those induced by TPA. The effects of rottlerin occurred in the presence of either bryostatin 1 or Go 6976. Rottlerin also induced a transformed morphology in c-Src-overexpressing cells (Fig. 8B). Interestingly, rottlerin induced colony formation of parental 3Y1 cells; however, the colony-forming efficiency was half that observed for c-Src-overexpressing cells, and the colonies grew to only about 20% of the size of the colonies of c-Src-overexpressing cells (data not shown). Thus, downregulation of PKCδ may promote tumor formation in systems other than the c-Src-overexpressing system described here. These data demonstrate that inhibition of PKCδ is sufficient for induction of anchorage-independent growth of cells overexpressing c-Src.

DISCUSSION

In this study, we have shown that the tumor-promoting phorbol ester TPA induces a transformed phenotype in 3Y1 rat fibroblasts overexpressing c-Src. The appearance of a transformed morphology in response to TPA treatment correlated with the downregulation of PKC, especially isoforms α and δ. Transformation-related phenotypes were also induced by compounds that inhibit PKC. Bryostatin 1 prevented the TPA-induced transformed phenotype in c-Src-overexpressing cells, and the only detectable difference between the PKC isoforms was that PKCδ was not downregulated by TPA when bryostatin 1 was present. Consistent with PKCδ downregulation being responsible for the effects of TPA, a specific inhibitor of PKCδ and a dominant negative PKCδ mutant both induced anchorage-independent growth in cells overexpressing c-Src. These data are consistent with a model in which the tumor-promoting effects of phorbol esters are due to the downregulation of PKCδ.

Several groups have investigated the effects of PKC isoforms on cell growth, and PKC isoforms have been reported to have both positive and negative effects upon cell division. Isoforms β (1) and ε (25) were reported to stimulate cell growth and transformation. Conversely, isoforms α (1) and δ (16, 25) have been reported to inhibit growth. In contrast, Liao et al. (22) reported that overexpression of a truncated PKCδ containing only the regulatory domain inhibited anchorage-independent growth. In their report, the authors proposed that their PKCδ regulatory domain could have acted as a dominant negative mutant of PKCδ and concluded that PKCδ has a positive role in cell growth. An alternative hypothesis is that the overexpressed PKCδ regulatory domain could have behaved like wild-type PKCδ and acted to suppress anchorage-independent growth. Such a mechanism has been demonstrated for PKCα, in which a regulatory domain was shown to stimulate PLD activity (34). This alternative interpretation is consistent with the inhibitory role for PKCδ observed here and by others (16, 25). It may also be of significance that, unlike the other PKC isoforms, PKCδ can be phosphorylated by tyrosine (8, 20). Tyrosine phosphorylation of PKCδ has been reported to be either stimulatory (20) or inhibitory (8, 9, 46). Thus, PKCδ may have different functions in different cellular contexts. The ability to downregulate PKCδ function through tyrosine phosphorylation may be another way to induce the promoter-like effects described here. In this regard, it has been reported that in both v-Ras-transformed cells (8) and v-Src-transformed cells (46), PKCδ is tyrosine phosphorylated with a corresponding downregulation of kinase activity.

It is not clear how downregulation of PKCδ might contribute to transformation. PKC has been reported to downregulate several receptors with tyrosine kinase activity, including the epidermal growth factor (EGF) (6, 11, 12, 24) and insulin (2) receptors, and a role for PKC in the downregulation of receptor tyrosine kinase signals has been proposed (33). Thus, it is possible that depleting cells of PKCδ could help sustain an active growth factor-induced signal by preventing downregulation of an activated receptor. Constitutively active tyrosine

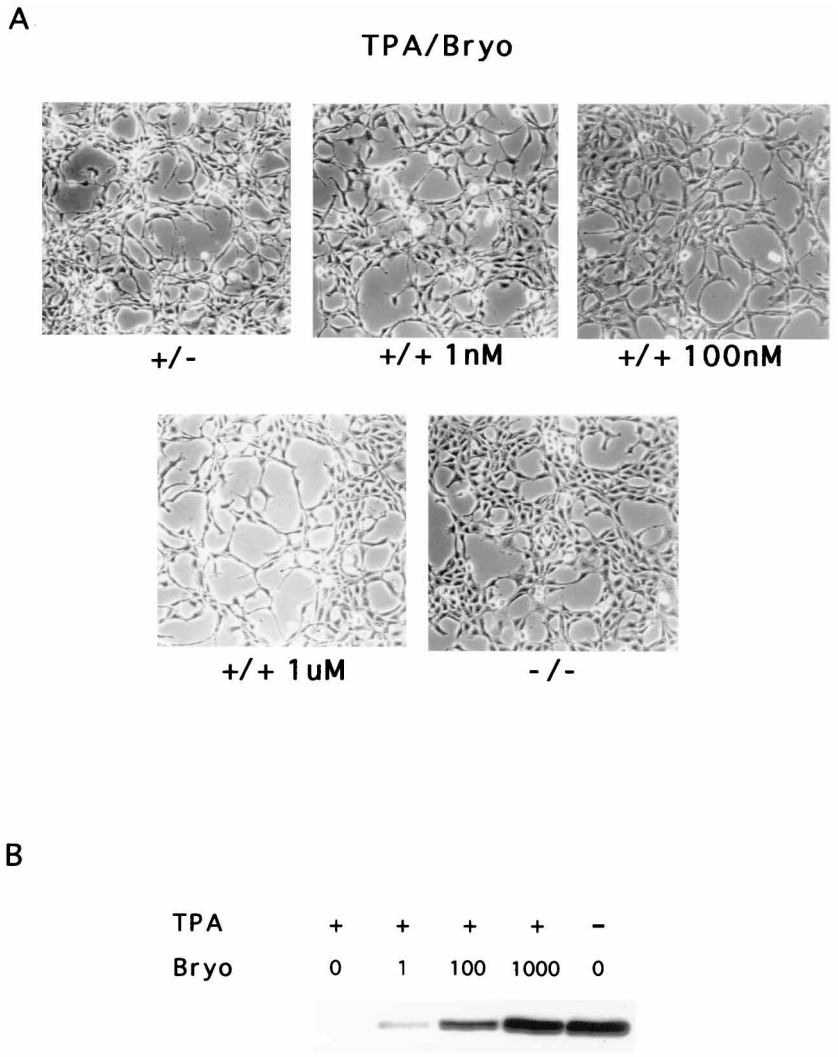


FIG. 7. The effects of bryostatin 1 on TPA-induced transformation in c-Src-overexpressing cells correlate with the level of PKCδ. c-Src-overexpressing 3Y1 cells were treated with TPA (400 nM) and increasing nanomolar concentrations of bryostatin 1 (Bryo) as shown, and the morphology of the cells (A) and PKCδ levels (B) were examined 24 h later.

kinases that lead to transformation have been postulated to function through the transmodulation of receptors with tyrosine kinase activity, resulting in ligand-independent activation of these receptors and in the generation of cell division signals (5, 30, 42). Thus, a possible molecular mechanism to explain the results presented here is that c-Src activates a receptor tyrosine kinase that is downregulated by PKCδ. Depleting cells of PKCδ would enhance this signal, which is consistent with the lack of any dramatic effect of TPA treatment upon the kinase activity of c-Src.

The hypothesis that downregulation rather than activation of PKC is important in tumor promotion is consistent with several studies that have examined the effects of disrupting the interactions between receptor tyrosine kinases and phospholipase Cγ. The platelet-derived growth factor (PDGF), EGF, and fibroblast growth factor receptors all interact with and activate phospholipase Cγ (28). Mutations to these receptors that disrupted phospholipase Cγ activation, and presumably the subsequent activation of PKC in response to the generated diacylglycerol, did not abolish the mitogenic effects of PDGF, EGF, and fibroblast growth factor (7, 26, 29, 41). Additionally, it has been demonstrated previously that PDGF was able to stimulate DNA synthesis in the absence of phospholipase C activation (15). These studies all suggested that the activation of PKC in response to tyrosine kinase activity is not required for the mitogenic response to these growth factors.

It is not clear to what extent the cell culture model for tumor promotion used here resembles the tumor-promoting effects of phorbol esters originally described for mouse skin. However, the effects of TPA on c-Src-overexpressing cells, i.e., the stimulation of anchorage-independent growth and amplification, are completely consistent with the classical description of a tumor promoter, where initiated cells are amplified by treatment with the tumor promoter. In this regard, it is significant that bryostatin 1, which blocked the promoter-like effects of TPA on c-Src-overexpressing cells, also prevented the tumor-promoting effects of TPA on mouse skin (14). Thus, the promoter-like effects observed here have much in common with tumor promotion in an animal model and suggest that downregulation of PKCδ may also be important for tumor progression.

The ability to stimulate anchorage-independent growth with

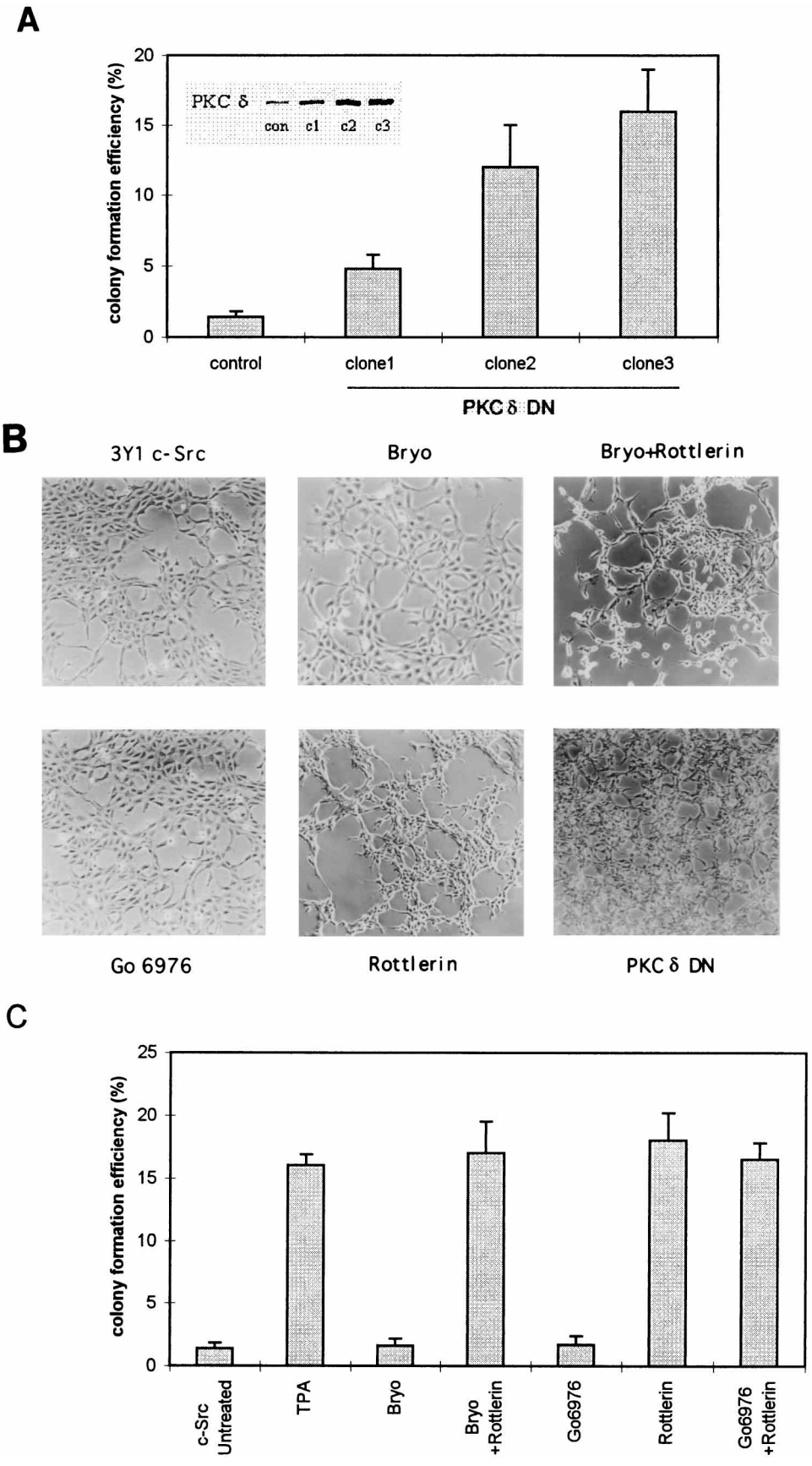

A
colony formation efficiency (%)
20
15
10
5
0
PKC δ
con c1 c2 c3
control
clone1
clone2
clone3
PKC δ DN
B
3Y1 c-Src
Bryo
Bryo+Rottlerin
Go 6976
Rottlerin
PKC δ DN
C
colony formation efficiency (%)
25
20
15
10
5
0
c-Src Untreated
TPA
Bryo
Bryo +Rottlerin
Go6976
Rottlerin
Go6976 +Rottlerin

FIG. 8. A dominant negative (DN) PKCδ mutant and a PKCδ-specific inhibitor induces transformation of cells overexpressing c-Src. (A) c-Src-overexpressing cells were transfected with a plasmid expressing a dominant negative PKCδ mutant. Several clones were picked, and Western analysis was used to examine PKCδ expression levels. The ability of parental c-Src-overexpressing cells and three clones expressing the dominant negative mutant to form colonies in soft agar was examined as described in the legend to Fig. 1. con, control. (B) Cells overexpressing c-Src were treated with bryostatin 1 (Bryo) (1 μM), rottlerin (30 μM), or Go 6976 (0.5 μM) as shown, and the morphology of the cells was examined 24 h later. The morphology of c-Src-overexpressing cells that were stably transfected with a dominant negative PKCδ mutant (clone 3) is also shown. (C) c-Src-overexpressing 3Y1 cells were examined for the ability to form colonies in soft agar in the presence of TPA (400 nM), bryostatin 1 (Bryo) (1 μM), rottlerin (30 μM), and Go 6976 (0.5 μM) as shown.

the dominant negative PKCδ mutant and the PKCδ-specific inhibitor suggests that PKCδ has the ability to suppress transformation. In this regard, it is of interest that the PKCδ-specific rottlerin could also stimulate anchorage-independent growth of parental 3Y1 cells, albeit to a substantially reduced level, suggesting that PKCδ may suppress transformation of many cells. These data implicate PKCδ as a tumor suppressor and suggest that PKCδ or downstream substrates of PKCδ could be targets for therapeutic intervention.

ACKNOWLEDGMENTS

We thank Phil Kaplan, Robert Krauss, and Neil Rosen for comments on the manuscript. We thank Harold Mischak for many helpful suggestions.

This investigation was supported by grants from the National Institutes of Health (CA46677), the Council for Tobacco Research (3075), and the American Cancer Society (BE-243) (to D.A.F.) and by a Research Centers in Minority Institutions (RCMI) award from the Division of Research Resources, National Institutes of Health (RR-03037), to Hunter College.

ADDENDUM IN PROOF

We have now stably expressed a dominant negative PKCα mutant in 3Y1 cells that overexpress c-Src and find that this PKCα mutant does not induce transformation as the analogous dominant negative PKCδ mutant does. This further establishes the specificity of the effects reported for the PKCδ isoform.

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