Effect of Tumor Promoters on src Gene Expression in Normal and Transformed Chick Embryo Fibroblasts

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ABSTRACT

The effects of 12-O-tetradecanoylphorbol-13-acetate (TPA) on the src protein kinase of normal chick embryo fibroblasts (CEF) and of the src kinase of cells transformed by a temperature-sensitive mutant of avian sarcoma virus (CEF-tsASV) were studied and compared with the known effects of TPA on cell morphology and plasminogen activator (PA) activity. One hr after the addition of TPA to normal CEF, there was a 3- to 8-fold stimulation of kinase activity when compared to control cultures; during the subsequent 24 hr, TPA produced less than a 2-fold stimulation. Although TPA induced levels of PA in CEF which were equivalent to those produced by CEF-tsASV grown at 36°, the latter cells contained much higher levels of kinase activity than those of CEF plus TPA. In addition, TPA failed to enhance the kinase activity of CEF-tsASV at either 36° or 40°, even though at both temperatures TPA induced morphological changes and markedly enhanced PA activity. These results suggest that the effects of TPA on morphology and PA are not due to an effect on these protein kinases.

INTRODUCTION

A remarkable property of the tumor promoter TPA and related phorbol ester tumor promoters is that at nm concentrations they induce normal cells to acquire phenotypic properties characteristic of cells transformed by oncogenic viruses or chemical carcinogens. This mimicry of transformation includes: synthesis of plasminogen activator, altered morphology and growth control, and structural and functional changes in several cell surface properties (for review, see Refs. 29 and 30). A corollary to mimicry is the phenomenon of enhancement, i.e., when TPA is added to cells that are already transformed, it induces further expression of markers of transformation (13, 29, 30). Cell transformation by ASV results from the expression of a single viral gene termed src (15, 28). The src gene product is a cyclic adenosine 3':5' monophosphate-independent protein kinase whose activity is easily detected in immunoprecipitates by its ability to transfer radiolabel from [γ-32P]ATP to the heavy chain of rabbit IgG (7, 12, 19, 24). Normal avian cells contain in their genome a src-related DNA sequence termed src which is transcribed into polyadenylated RNA (25–27). In addition, a phosphoprotein pp60src that has kinase activity similar to the ASV pp60src has been detected in normal avian and mammalian cells (5, 20). In view of the ability of TPA to induce mimicry of transformation in normal CEF (10) and enhancement in ASV-transformed CEF (23, 31, 32), it seemed possible that TPA might act by increasing the amount or activity of these protein kinases. The present results provide evidence that this is not the case and suggest that the effects of TPA on the expression of markers of transformation are mediated by other mechanisms.

RESULTS AND DISCUSSION

Prior to examination of these kinases, it was necessary to establish experimental conditions under which TPA clearly displayed mimicry of transformation in normal CEF and enhancement in ASV-transformed CEF. The transformed cells CEF-tsASV were obtained by infecting CEF with the tsNY68 mutant of ASV since such cells are temperature sensitive with respect to expression of transformation (15). Thus, we could study the effects of TPA in virus-infected cells at both the permissive and nonpermissive temperatures. Two markers of transformation, morphology and PA activity, were used. Within 18 hr after the addition of TPA (30 ng/ml; ~10⁻⁸ M) to normal CEF, there was disruption of the normal parallel cell-cell orientation; individual cells also contracted from the growth surface and became more refractile (Fig. 1, A and B). At the permissive temperature of 36°, the CEF-tsASV cells showed marked loss of cell-cell orientation, extensive overlap, and some retraction from the growth surface (Fig. 1C). TPA caused marked retraction of these cells and the formation of very large cell clumps (Fig. 1D). At the nonpermissive temperature of 40°, CEF-tsASV had a morphology which was intermediate between that of CEF and CEF-tsASV grown at 36° (Fig. 1E). The addition of TPA to CEF-tsASV at 40° caused retraction of the cells, loss of the normal cell-cell orientation, and the appearance of numerous spherical forms (Fig. 1F). At 40°, however, TPA did not induce the formation of the large cell clumps seen in CEF-tsASV cultures exposed to TPA at 36°. Similar types of effects of TPA on the morphology of CEF and CEF-tsASV have been described previously (23, 32).

The addition of TPA to normal CEF led to a marked induction of PA activity which was apparent within 3 to 6 hr and persisted for at least 24 hr (Chart 1). At 36°, CEF-tsASV produced levels of PA which were comparable to those of normal CEF fully induced by TPA. The addition of TPA to CEF-tsASV at 36°
further enhanced PA production, and this was apparent within 3 hr (see also Ref. 23). At 40°C, CEF-tsASV produced only TPA. Thus, by the criteria of PA activity, morphology, and to a level which was comparable to that of normal CEF plus small amounts of PA; the addition of TPA induced PA synthesis.

We found that a 1-hr exposure to TPA resulted in a severalfold increase in kinase activity when we used either the TBR or the CR-TBR antisera (Table 1). When normal CEF were exposed to TPA for 24 hr, there was less than a 2-fold difference in kinase activity between the control and TPA-treated cells (Table 1). As described previously (2, 4), the CR-TBR antiserum was much more active with extracts of normal CEF than the TBR antiserum.

In the absence of TPA, the kinase activity of CEF-tsASV grown at 36°C was much higher than that of normal CEF (Table 1), reflecting the activity of the viral src gene (7). Although, the addition of TPA to normal CEF altered their morphology and induced PA activity to levels as high as those seen in CEF-tsASV grown at 36°C (Fig. 1; Chart 1), the levels of pp60src kinase activity in CEF plus TPA were less than one-tenth those of CEF-tsASV at 36°C with the TBR antiserum and less than one-fifth with the CR-TBR antiserum. Furthermore, although the addition of TPA to CEF-tsASV at 36°C caused further alterations in morphology and a further increase in PA activity (Fig. 1; Chart 1), these effects were generally associated with a decrease in kinase activity (Table 1). A lack of correlation between TPA-induced effects and kinase activity was also apparent when CEF-tsASV were studied at the nonpermissive temperature of 40°C. In studies with the TBR antiserum, exposure to TPA caused about a 2-fold decrease in kinase activity; with the CR-TBR antiserum, there was no consistent or significant change (Table 1). This is despite the fact that, under the conditions used, TPA induced PA synthesis to a level which was comparable to that of normal CEF plus TPA. Thus, by the criteria of PA activity, morphology, and response to TPA, CEF-tsASV at the nonpermissive temperature closely resemble but are not identical to normal CEF.

Having established conditions under which TPA induced mimicry and enhancement, we then examined whether these effects were paralleled by changes in either the host or viral-coded pp60src protein kinases. Cell cultures that were near confluence were exposed to TPA (30 ng/ml) or the dimethyl sulfoxide solvent (0.003%) for either 1 or 25 hr, and the cells were washed and harvested as described in Chart 1. Cell pellets were solubilized in lysis buffer [0.5% NP-40:0.5% sodium deoxycholate:0.1 M NaCl:0.01 M Tris-HCI (pH 7.2):0.001 M EDTA] containing 100 kallikrein inactivator units of aprotinin per ml. The lysates were then clarified at 100,000 X g for 30 min, and 0.4-ml aliquots of the supernatants were incubated with 5 p of antiserum. After 30 min at 4°C, the immune complexes were precipitated with 0.15 M NaCl in 0.05 M Tris-HCI, pH 7.2. To detect protein kinase activity, the immunoprecipitates were resuspended directly in the reaction mixture. The 20 μl reaction mixture contained: 20 mm Tris-HCl (pH 7.2); 5 mm magnesium acetate; and 6 to 1.0 μm [γ-32P]ATP (New England Nuclear: 2000 Ci/mmol). Reactions were incubated at 20°C for 10 min. One ml of 0.15 M NaCl:0.05 M Tris-HCI (pH 7.2) was added. The mixtures were centrifuged, the supernatants were discarded, and the pellets were resuspended in 20 μl of sample buffer [0.07 M Tris-HCl (pH 6.0):11% glycerol:3% sodium dodecyl sulfate:0.1% bromophenol blue:5% 2-mercaptoethanol]. After heating in boiling water for 3 min, the mixtures were centrifuged, and the supernatants were analyzed by gel electrophoresis.

Table 1

<table>
<thead>
<tr>
<th>Cells</th>
<th>Growth temperature</th>
<th>With TBR antisera</th>
<th>With CR-TBR antisera</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 hr</td>
<td>25 hr</td>
<td>1 hr</td>
</tr>
<tr>
<td>CEF</td>
<td>36</td>
<td>+</td>
<td>3.7</td>
</tr>
<tr>
<td>CEF-tsASV</td>
<td>36</td>
<td>-</td>
<td>23.4</td>
</tr>
<tr>
<td>CEF-tsASV</td>
<td>40</td>
<td>-</td>
<td>65.9</td>
</tr>
<tr>
<td>CEF-tsASV</td>
<td>40</td>
<td>+</td>
<td>28.9</td>
</tr>
</tbody>
</table>

Phosphorylating activity (fmol 32P incorporated/mg protein)
identical conditions, TPA exposure altered the morphology and PA activity in CEF-tsASV (Fig. 1; Chart 1). It should be noted that in the present study when CEF-tsASV were grown in identical conditions, TPA exposure altered the morphology and closely resembled that of normal CEF; however, growth of these cells at 40° did not cause a decrease in vitro pp60src kinase activity when compared to growth at 36° (Table 1). This is in contrast to previous studies on the effects of temperature on the pp60src kinase of this temperature-sensitive mutant (12, 24). The reasons for this discrepancy are not apparent, although we did use a different extraction procedure.

During the course of these studies, we observed that, even in the absence of TPA, extracts obtained from CEF or CEF-tsASV at 3, 6, or 25 hr usually had higher levels of in vitro pp60src kinase activity than did those obtained from 1-hr cultures (Table 1). Presumably, this reflects the fact that the fresh medium, most likely the serum, added at Time 0 contains factors that enhance the kinase activity. The apparent stimulation by TPA in the 1-hr samples may reflect a similar effect since previous studies indicate that TPA can mimic or act synergistically with serum growth factors (1, 9).

We have also studied possible effects of TPA on the extent of in vivo phosphorylation of these M.W. 60,000 proteins. Cell cultures were incubated with 32P after 24 hr in the absence or presence of TPA (30 ng/ml), lysates were prepared and immunoprecipitated, the immunoprecipitates were gel electrophoresed, and the gels were radioautographed (7). With normal CEF, a faint band of radioactivity was detected in the M.W. 60,000 region with the CR-TBR antisera. This band did not differ significantly in intensity when cultures were labeled in the presence or absence of TPA. With CEF-tsASV, a very intense band was detected in the M.W. 60,000 region, but no major differences were seen when cultures were labeled with 32P in the absence or presence of TPA, at either 36° or 40°.

Similar experiments were performed in which cell cultures were incubated at 36° with [35S]methionine after a 24 hr exposure to TPA and the labeled M.W. 60,000 proteins detected in immunoprecipitates by gel electrophoresis (21) (Fig. 2). These gels were intentionally overexposed to maximize sensitivity. With normal CEF, a very faint band was seen in the M.W. 60,000 region. This was more apparent with the CR-TBR than the TBR antisera. The intensity of this band was not affected by exposure of the cells to TPA (30 ng/ml) during the 35S labeling period. With CEF-tsASV, an intense band of radioactivity in the M.W. 60,000 region was seen with both the TBR and CR-TBR antisera (Fig. 2). No major differences in the intensity of this band were seen when CEF-tsASV were 35S labeled in the presence of TPA.

At the present time, the cellular mechanisms responsible for control and coordination of the multiple phenotypic markers characteristic of transformed cells are poorly understood. Our results indicate that, although TPA is a potent inducer of morphological changes and PA synthesis in normal CEF and enhances the expression of both of these markers in ASV-transformed cells, these effects are not linked to the in vitro protein kinase activities of the host-coded pp60sec kinase or the ASV-coded pp60src kinase. During the preparation of this manuscript, Goldberg et al. (14) reported studies that led to a similar conclusion, although in our studies we looked at earlier time points (1 to 25 hr), whereas they studied cell culture extracts for in vitro kinase activity 3 days after exposure to TPA. In addition, we have utilized a temperature-sensitive mutant of ASV to further explore the action of TPA under permissive and nonpermissive conditions. To avoid possible in vitro artifacts, we also examined the effects of TPA on the in vivo phosphorylation and in vivo synthesis of pp60src and pp60sec proteins, again with negative results.

The in vitro assay of these kinases is only semiquantitative and utilizes an unnatural substrate, the IgG molecule. Nevertheless, this assay does demonstrate marked differences between normal CEF and CEF transformed by ASV. We would assume, therefore, that if TPA induces PA through the same mechanism as ASV, then the in vitro kinase assay would be sufficient to demonstrate this. Further studies are required to determine whether TPA mimics the action of ASV by inducing a different protein kinase or by influencing the expression of markers of transformation through entirely different mechanisms. Recent studies indicate that certain morphological revertants of ASV-transformed cells retain high levels of the pp60src protein (6). Thus, it would appear that, although pp60src may play an essential role, the phenotype of transformed cells is controlled by a complex interaction between host genes and virus-related sequences. The present results suggest that TPA achieves its effects by modulating the expression of host genes unrelated to the src gene and, therefore, may provide a useful probe of these functions.

ACKNOWLEDGMENTS

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Fig. 1. Effect of TPA on morphology of normal and ASV-transformed CEF. CEF were prepared from 11-day-old embryos (Spafas, Inc., Norwich, Conn.) and maintained in culture in Dulbecco's medium, supplemented with 10% tryptose phosphate broth, 5% calf serum, and 1% chicken serum. CEF-tsASV were obtained by infecting CEF with the temperature-sensitive mutant of SR-ASV, ASV tsNY66, obtained from H. Hanafusa (16). Cells were grown at 36° or 40° for 18 hr in the absence or presence of 30 ng TPA per ml. A, CEF at 36°; B, CEF at 36° plus TPA; C, CEF-tsASV at 36°; D, CEF-tsASV at 36° plus TPA; E, CEF-tsASV at 40°; F, CEF-tsASV at 40° plus TPA.
Fig. 2. Immunoprecipitation of $^{35}$S-labeled pp60$^{c-src}$ proteins from CEF and CEF-tsASV incubated with or without TPA. Cells were incubated at 36° for 24 hr in the absence or presence of 30 ng TPA per ml and then with 25 μCi of $[^{35}S]$methionine per ml (New England Nuclear; 792 Ci/mmol) for an additional 2 hr. Immunoprecipitates were then prepared and analyzed directly by gel electrophoresis as described in the legend to Table 1. Arrows, M.W. 60,000 region. 1, CEF samples: a, immunoprecipitate with nonimmune serum; b, immunoprecipitate with CR-TBR; c, immunoprecipitate with TBR. 2, CEF plus TPA samples: a, immunoprecipitate with nonimmune serum; b, immunoprecipitate with CR-TBR; c, immunoprecipitate with TBR. 3, CEF-tsASV samples: a, immunoprecipitate with nonimmune serum; b, immunoprecipitate with CR-TBR; c, immunoprecipitate with TBR. 4, CEF-tsASV plus TPA samples: a, immunoprecipitate with nonimmune serum; b, immunoprecipitate with CR-TBR; c, immunoprecipitate with TBR.
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