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A TUMOR PROMOTER STIMULATES PHOSPHORYLATION ON TYROSINE

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SUMMARY: The tumor promoter 12-O-tetradecanoyl-phorbol-13-acetate is mitogenic for normal chicken embryo fibroblasts and also causes these cells to express transiently many properties of cells transformed by Rous sarcoma virus. Since some mitogenic hormones stimulate a tyrosine-specific protein kinase activity, and since the transforming protein of RSV is a tyrosine-specific protein kinase, we have examined whether TPA also stimulates protein phosphorylation on tyrosine. We report here that TPA treatment of normal cells resulted in a very rapid phosphorylation on tyrosine of a protein peak of Mr 40 to 43 kilodaltons. Thus, a similar biochemical activity (tyrosine phosphorylation) is associated with the action of polypeptide mitogenic hormones, Rous sarcoma virus and a tumor promoter. In addition, TPA treatment resulted in rapid changes in phosphorylation of proteins on serine and threonine.

When a potent tumor promoter such as 12-O-tetradecanoyl-phorbol-13-acetate (TPA) is added to cultures of chicken embryo fibroblasts, transient cellular alterations occur which are similar in some respects to the changes which accompany oncogenic transformation by Rous sarcoma virus (RSV). These changes include stimulation of cellular proliferation, increased hexose transport, increased plasminogen activator and alterations in cellular morphology (1-6). Increased proliferation and hexose transport also occur in cells treated with polypeptide mitogens. Because of the similar phenotypic effects of TPA, RSV and mitogens it is not unreasonable to suspect that these agents act at least in part through a common mechanism.

Transformation by RSV requires the activity of the viral transforming protein, $pp60^{src}$, which is a tyrosine-specific protein kinase (7,8,9), and at least some receptors for polypeptide mitogens are associated with a tyrosine-specific protein kinase activity (10-13). While one might therefore suspect the involvement of a tyrosine-specific protein kinase in the mechanism of action of

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ABBREVIATIONS: TPA: 12-O-tetradecanoylphorbol-13-acetate; RSV: Rous sarcoma virus; p-tyr: phosphotyrosine; p-thr: phosphothreonine

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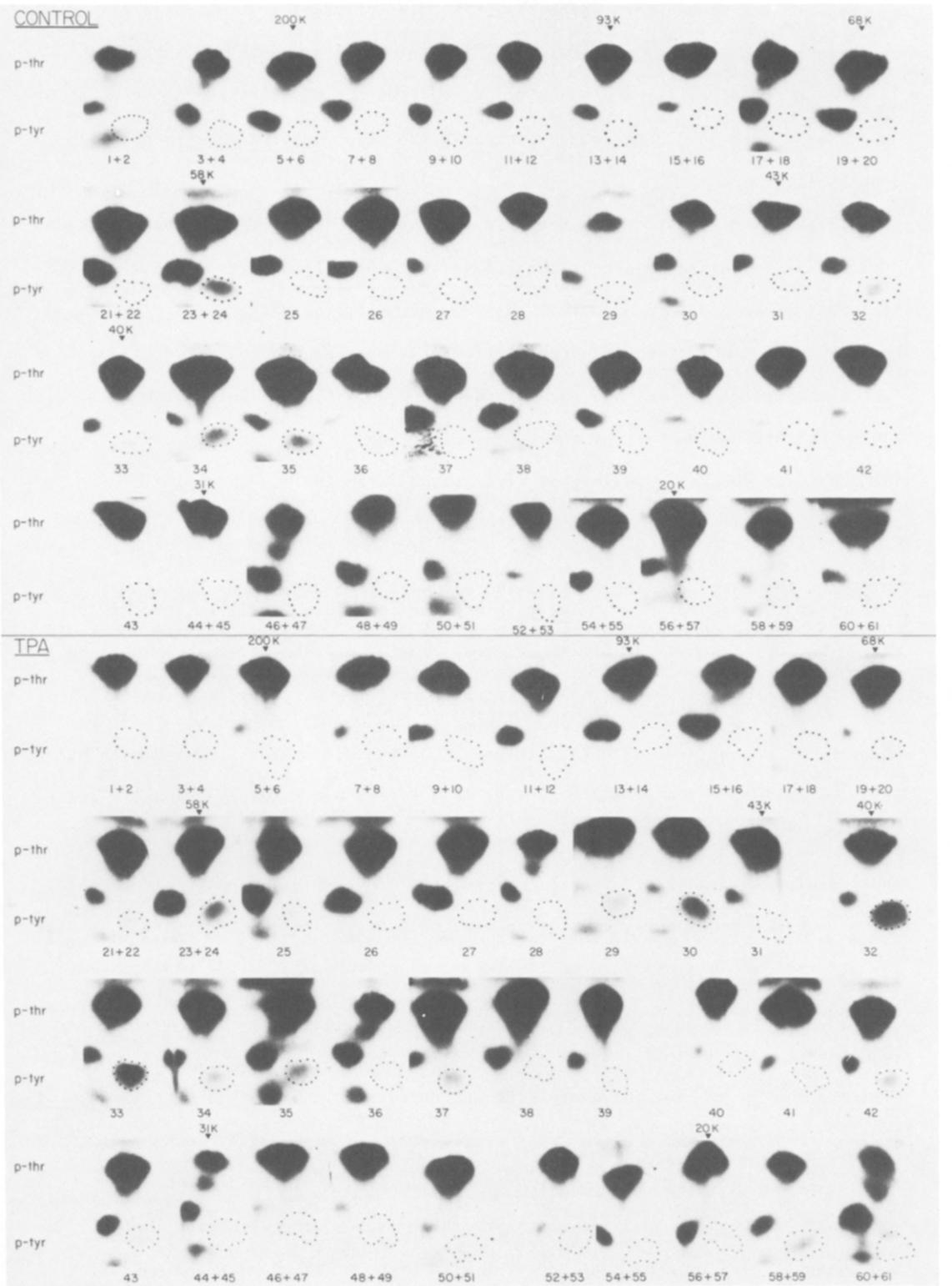
phorbol esters, it has been found that TPA does not cause a stimulation of tyrosine kinase activity of viral $pp60^{src}$ or of cellular $pp60^{c-src}$ either *in vivo* or *in vitro* (14, our unpublished results) nor does it induce the phosphorylation of a 36,000 Mr protein which is the major *in vivo* substrate for $pp60^{src}$ (15).

In this communication, we report the results of investigations making use of a sensitive technique to detect changes in protein phosphorylation (16,17). We find that after exposure of normal chicken embryo fibroblasts to TPA there is a rapid increase in tyrosine phosphorylation of a protein peak of Mr 40 to 43 kilodaltons. Similar changes have recently been found by Cooper, Sefton and Hunter, and by Gilmore and Martin (personal communications). In addition, we find that TPA treatment induces a variety of alterations in serine and threonine phosphorylation.

MATERIALS AND METHODS: TPA and 4-β-phorbol were from Sigma (St. Louis, MO). Cell culture and phosphoamino acid analysis were as described (16,17). Subconfluent cultures of chicken embryo fibroblasts were labelled with 3 mc/ml ^{32}Pi for 12-18 hrs. They were then treated with promoter, analog or solvent, washed in ice-cold phosphate-buffered saline and lysed in boiling electrophoresis sample buffer. The lysates were then boiled 5 min and electrophoresed on polyacrylamide gels.

RESULTS: Sub-confluent cultures of chicken embryo fibroblasts treated with TPA were responsive to the promoter as indicated by increased hexose transport and by a visible alteration in morphology (data not shown). Figures 1 and 2 show the phosphoamino acid content of cellular proteins of TPA-treated and control cells which were separated by electrophoresis on polyacrylamide gels. Figure 1 displays the autoradiographic exposure of the separated phosphoamino acids showing the radioactivity associated with phosphothreonine and phosphotyrosine. Quantitative results are presented in Figure 2. Our results demonstrate that several substantial changes in protein phosphorylation occurred within 5 minutes of treatment of cells with TPA in DMSO. There was a several-fold increase in tyrosine phosphorylation of a protein peak with an apparent Mr of 40-43K. There did not appear to be comparable changes in tyrosine phosphorylation of any other proteins, notably in the phosphotyrosine-containing proteins with Mr's of 32K, 36-38K and 60K which were observed in the control cells. This indicates that the 40-43K phosphoryla-


CONTROL
200K
93K
68K
p-thr
p-tyr
1+2
3+4
5+6
7+8
9+10
11+12
13+14
15+16
17+18
19+20
58K
43K
21+22
23+24
25
26
27
28
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42
31K
20K
43
44+45
46+47
48+49
50+51
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58K
43K
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31K
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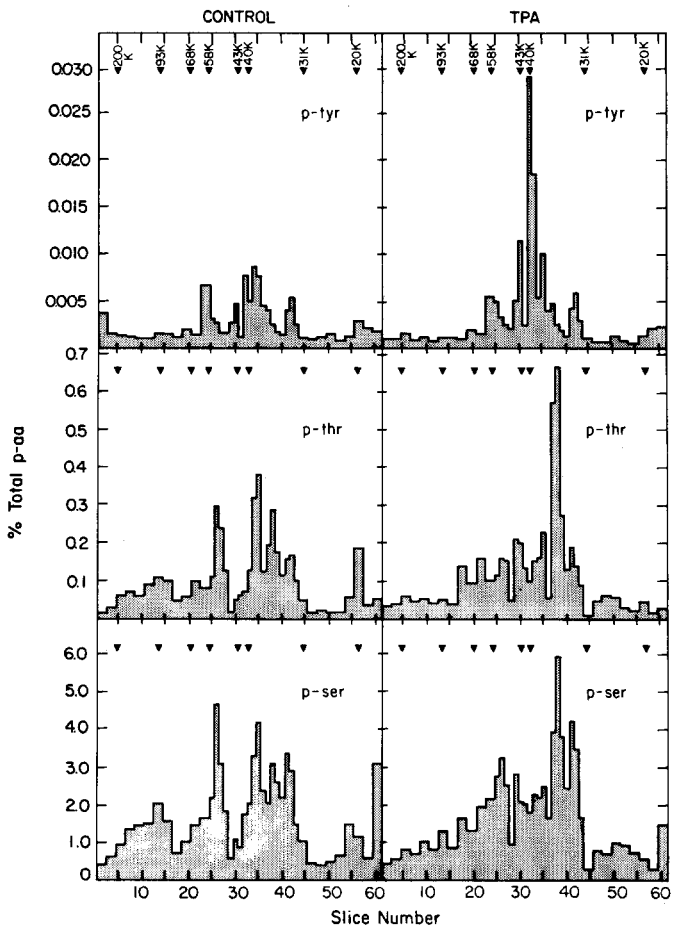

Figure 2. Effect of TPA on phosphoamino acid content of cellular proteins as a function of molecular weight. Data are expressed as % of the total phosphoamino acids in the gel, to compensate for any differences in pool or specific activity. Total cpm in phosphoamino acid for the control culture = 3,382,145 and for the TPA-treated = 2,521,610.

tions were specific protein modifications and were not part of an indiscriminate change in phosphotyrosine content.

Similar changes in phosphorylation and hexose uptake were seen with 50 and 100 ng/ml TPA, but not with the non-promoting TPA analog 4-β-phorbol (data not

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Figure 1. Effect of TPA on phosphothreonine and phosphotyrosine composition of cellular proteins as a function of molecular weight. Chicken embryo fibroblasts labeled with ^{32}Pi were treated either with TPA in DMSO (200 ng/ml TPA, 0.2% DMSO, final concentration) or with DMSO alone for 5 minutes. Cell lysates were eletrophoresed on SDS polyacylamide gels and the phosphoamino acid content of the separated proteins was determined as described (16,17). Phosphoserine content was also determined, but on a different autoradiogram. Numbers above the autoradiograms (with arrows) refer to the Mr of the molecular weight markers. Numbers below the autoradiograms refer to slice numbers. p-thr = phosphothreonine; p-tyr = phosphotyrosine.

shown). The stimulation of tyrosine phosphorylation was evident even when cells were maintained under serum-free conditions (data not shown) arguing that this change was not mediated by sensitizing the cells to serum mitogens.

The time-course of the changes in phosphotyrosine content of the 40-43K protein peak is shown in Figure 3. The phosphorylation was extremely rapid, reaching a maximum within five minutes of TPA addition. The proteins were dephosphorylated with a half-time of approximately 60-90 minutes, even though the cells were continuously exposed to TPA.

The effect of TPA on phosphorylation of serine and threonine residues appears to be more complex (Figure 2): we observed both increases and decreases in phosphorylation of various proteins. Furthermore, several of these changes occurred on

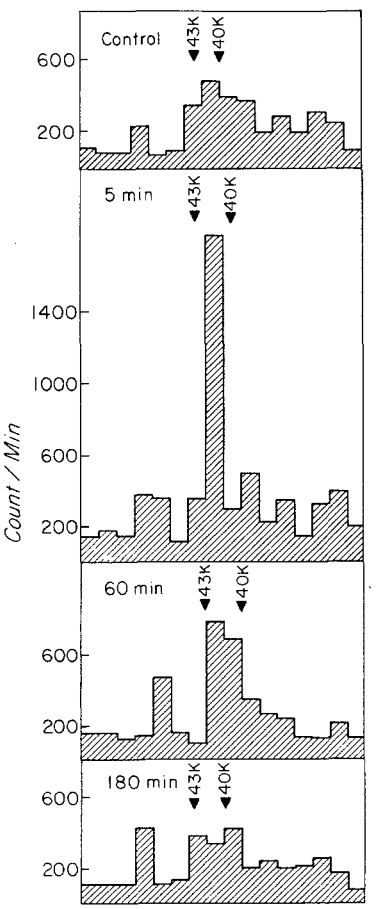

Figure 3. Time-course of phosphorylation on tyrosine following TPA treatment. Conditions are as in Figures 1 and 2. Note that only the central portion of the gel is displayed.

proteins with Mr's distinguishable from the 40-43K phosphotyrosine containing protein peak. Increases in phosphothreonine were particularly notable at Mr's of approximately 34K (a similar change has been noted by Laszlo et al (15)) and 45K. Dramatic decreases in threonine phosphorylation occurred on proteins with Mr's of 20K and 51K. Increased phosphoserine was noted at 34K.

DMSO, the solvent used in these experiments, has been reported to induce tyrosine phosphorylation in isolated membranes (18). The concentrations of DMSO required for this effect are far higher (10-20%) than those used here (0.2-1.0%). Moreover, the phosphoamino acid profile shown in Figure 2 was not detectably affected by DMSO treatment at the levels we have used, and is indistinguishable from our previously published profiles of normal cells not treated with DMSO (16,17). However, DMSO does substantially alter the rate at which the 40-43K protein peak becomes phosphorylated in the presence of TPA. In the absence of DMSO the levels of phosphorylation shown in Figures 1 and 2 were not achieved even by three hours (data not shown). This synergistic effect of DMSO and TPA may be due to effects on the permeability of the cells to TPA or (perhaps more likely) may simply be due to the insolubility of TPA in aqueous media.

DISCUSSION: This investigation was initiated with the goal of determining whether the similar phenotypic effects of TPA, RSV and polypeptide mitogens reflected similarities in the mechanism of action of these agents. We found that TPA induced dramatic changes in the phosphothreonine and phosphoserine content of cellular proteins, which were not found following transformation by RSV or treatment with polypeptide mitogens (16,17). These changes thus may be associated with activities of TPA not shared with these other agents. On the other hand, TPA treatment resulted in the tyrosine phosphorylation of a 40-43K protein peak which has been found to become similarly phosphorylated following treatment with mitogens and in RSV-transformed chick cells (16,17,19). It thus is possible that the tyrosine-specific protein phosphorylation reported here is involved in some of the phenotypic effects shared by TPA, mitogens and RSV, such as increased hexose transport or cell proliferation.

TPA is known to sensitize cells to the action of polypeptide mitogens at least some of which are able to stimulate phosphorylation on tyrosine of the 40-43,000 Mr protein peak (20). Thus, it was possible that the stimulation of tyrosine phosphorylation reported here occurred indirectly, by sensitizing the cells to polypeptide mitogens found in serum. However, we found that TPA stimulated the same tyrosine phosphorylation even in cells maintained in the absence of serum. This suggests that a TPA receptor either is, or can activate, a tyrosine-specific protein kinase.

Protein kinase C, a phospholipid-dependent protein kinase, can be activated by TPA and may in fact be a major cellular receptor for the tumor promoter (21,22). Since this enzyme has been reported to phosphorylate proteins on serine and perhaps threonine, but not tyrosine (23) the phosphoserine and phosphothreonine increases we detected may be directly due to the activity of protein kinase C, although this remains to be determined. The TPA-induced phosphorylation on tyrosine could arise from three possible sources: 1. A kinase cascade activated by protein kinase C; 2. An altered amino acid specificity for protein kinase C on certain substrates; 3. The existence of other kinase receptors for TPA, with differing specificity. The decreased phosphothreonine content following TPA treatment of proteins in the regions of 20K and 51K could be due to the activation of a phosphatase.

Genetic evidence indicates that the mitogenic effects of tumor promoters are separable from their action as promoters, and thus these two sets of phenotypic alteration may be mediated by different mechanisms (24). For example, if the mitogenic effects of TPA are mediated through tyrosine phosphorylation, it is conceivable that the ability of this phorbol ester to promote carcinogenesis is mediated through its effects on threonine and serine phosphorylation. It will be interesting to determine the phosphorylation changes induced by TPA in cells which are resistant to various of the biological effects of this agent (25). Such experiments are currently in progress, and should shed light on the functional significance of the phosphorylation changes reported here.

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