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12-*O*-Tetradecanoylphorbol-13-Acetate Stimulates Phosphorylation of the 58,000-M_r Form of Polyomavirus Middle T Antigen In Vivo: Implications for a Possible Role of Protein Kinase C in Middle T Function

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The 58,000-M_r form (58K form) of the polyomavirus middle T antigen (mT) is a minor species distinguished by its phosphorylation in vivo on serine and by its efficient phosphorylation on tyrosine in immune complexes (B. S. Schaffhausen and T. L. Benjamin, J. Virol. 40:184–196, 1981). Here we report that the tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate (TPA), an activator of protein kinase C, rapidly stimulates phosphorylation of this mT species when added to cultures of wild-type polyomavirus-infected or polyomavirus-transformed 3T3 cells. Incubation with TPA leads to an accumulation of the 58K mT species to levels 1.5- to 5-fold higher than that in untreated cells within 15 min. TPA specifically stimulates phosphorylation of the 58K mT species without affecting that of the 56K species. Mapping by partial proteolysis shows that TPA-stimulated phosphorylation occurs at or near the site in 58K mT that is normally phosphorylated in the absence of TPA. A synthetic diacyl glycerol, 1-oleoyl-2-acetyl-glycerol, also specifically stimulates phosphorylation of 58K mT in vivo, while an inactive phorbol analog does not. TPA fails to induce phosphorylation of a 58K mT species encoded by certain nontransforming virus mutants with altered mT proteins that normally fail to undergo phosphorylation at the 58K site. These results indicate that the 58K form of mT is phosphorylated by or through the action of protein kinase C. TPA treatment of infected cells also leads to increased levels of 58K mT as measured in the immune complex kinase reaction, in which mT becomes phosphorylated on tyrosine by pp60$^{c\text{-}src}$. These results are discussed in terms of a possible role for protein kinase C in activating mT function(s), including the formation of stable complexes with pp60$^{c\text{-}src}$.

The early region of the polyomavirus genome encodes three proteins: the large, middle, and small tumor (T) antigens. Although all three polypeptides appear to function during cellular transformation, the ability of polyomavirus to transform rodent cells in culture and to form tumors in susceptible animals has been correlated most closely with the expression of the middle T (mT) protein (29, 43).

Phosphorylation reactions involving mT appear to be crucial to its function. This viral protein lacks intrinsic protein kinase activity (34, 35), but interacts with multiple cellular kinases, including pp60$^{c\text{-}src}$. Antisera against either pp60$^{v\text{-}src}$ or polyoma T antigens precipitate mT-pp60$^{c\text{-}src}$ complexes from crude extracts of polyomavirus-infected or polyomavirus-transformed cells; labeling of these immune complexes with [γ-^{32}P]ATP leads to phosphorylation of mT on tyrosine residues catalyzed by pp60$^{c\text{-}src}$ (10–12). Association of mT with pp60$^{c\text{-}src}$ enhances the tyrosine kinase activity of the latter (3). A variety of genetic and biochemical evidence supports the notion that this interaction is essential (11, 31, 32), although probably not sufficient (25, 41, 42), for cell transformation by polyomavirus.

Whether pp60$^{c\text{-}src}$ phosphorylates mT in vivo is not known. It is clear, however, that serine kinases act on mT in the cell, giving rise to two phosphorylated mT species separable by sodium dodecyl sulfate-polycrylamide gel electrophoresis (SDS-PAGE). Current evidence suggests that the 56K and 58K forms of mT are singly phosphorylated at distinct sites within a stretch of about 35 amino acids, and that each form is capable of forming complexes with pp60$^{c\text{-}src}$ (31). Only a small fraction of mT molecules is phosphorylated in vivo and forms complexes with pp60$^{c\text{-}src}$ (29). This fraction is distinguished by its preferential binding to Triton-X-100-resistant cell frameworks (35) and by its more rapid sedimentation as a high-molecular-weight complex compared with the bulk [^{35}S]methionine-labeled mT (11, 33, 44). Recent studies of rat fibroblasts expressing mT from a dexamethasone-regulated promoter have shown that it is this "kinase-active" fraction of mT molecules, and not the total level of mT, that controls various cellular parameters of transformation (29).

The ability of mT to serve as a substrate in vivo for the serine kinase that produces the minor, i.e., 58,000 M_r (58K form), is correlated with its ability to associate with pp60$^{c\text{-}src}$ as manifested by in vitro phosphorylation, and also with its transforming ability. This is best illustrated by studies of transformation-defective virus mutants. The *hr-t* mutant NG59 undergoes phosphorylation in vivo at the 56K, but not at the 58K, site (31). NG59 mT is negative in the immune complex kinase assay (31, 32); while it may interact weakly with pp60$^{c\text{-}src}$, it fails to be phosphorylated by it (2). The nontransforming mutant Py1387T encodes a truncated mT that lacks the 37 carboxy-terminal end amino acids, including a stretch of hydrophobic amino acids necessary for membrane attachment (6). Although it retains all of the serine and tyrosine phosphorylation sites, this "tail-less" mT nevertheless fails to be recognized by the 58K kinase in vivo, although phosphorylation does occur at the 56K site. This mutant mT also fails to become phosphorylated on tyrosine in vitro and thus, presumably fails to associate, at least stably, with pp60$^{c\text{-}src}$. Results with the mutant Py1387T thus suggest that the 58K kinase is a membrane-associated

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enzyme, or at least recognizes substrates only when the latter are associated with the membrane (6).

In the past several years, the work of Nishizuka and others has identified a Ca^{2+}-activated phospholipid-dependent protein kinase designated protein kinase C, which has a seemingly ubiquitous tissue distribution (21, 26). Protein kinase C meets expectations for the 58K mT kinase based on its activation by diacyl glycerol generated by phospholipid turnover in the membrane (26, 40) and also because it appears to favor membrane-associated proteins as substrates, including growth factor receptors (18, 19). It is also known that tumor promoters of the phorbol ester class bind to and activate protein kinase C (8, 24), and that this activation is associated with the recruitment of the enzyme to the membrane (20).

To investigate the possible role of protein kinase C in mT phosphorylation in vivo, we have exploited the observation that the tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate (TPA) directly activates this enzyme. We show that when added to the medium of wild-type polyomavirus-infected cells, TPA leads to a rapid and selective increase of the incorporation of $^{32}P_i$ into the 58K form of mT. This treatment also increases the amount of 58K mT recovered from cell extracts by immunoprecipitation and phosphorylation in vitro on tyrosine, indicative of complex formation with $pp60^{c\text{-}src}$.

MATERIALS AND METHODS

Materials and reagents. The phorbol esters, TPA and 4α-phorbol-12,13-didecanoate (4α-PDD), were from P-L Biochemicals, Inc. These reagents were dissolved in dimethyl sulfoxide (DMSO) (25 mg/ml), and stored at −70°C. The synthetic analog 1-oleoyl-2-acetyl-glycerol (OAG) was synthesized and kindly supplied by S. Rittenhouse, Department of Hematology, Brigham and Women's Hospital. Chymotrypsin was from Worthington Diagnostics. Protein A-Sepharose CL-4B was from Sigma Chemical Co. [^{35}S]methionine (1,300 Ci/mmol) was from Amersham Corp. $H_3{}^{32}PO_4$ (carrier free) was from New England Nuclear Corp., and [γ-^{32}P]ATP (7,000 Ci/mmol) from ICN Pharmaceuticals Inc.

Cells and viruses. Cell culture and handling of virus were as previously described (14). Wild-type virus NG59RA was derived from *hr-t* mutant NG59 by marker rescue (13). The *hr-t* mutants NG18 and NG59 (1) and the site-directed mutants Py1178T (7) and Py1387T (6) have been described before. The deletion mutant, *dl*1014 (23) and the double mutant Py1178T/*dl*1014 have been described (36). Py3T3 was Py6 derived from Swiss mouse 3T3 cells (1).

Antisera. Anti-polyoma-tumor serum (anti-T ascites) was prepared in brown Norwegian rats as described before (39). The anti-*src* monoclonal antibodies GD11 (27) and MAB327 (22) were generously provided by S. Parsons and J. Brugge. These antibodies, hereafter designated anti-*src* antibody, were diluted 1 to 10 in phosphate-buffered saline and combined in equal volumes before use. The rabbit anti-mouse serum used to precipitate MAB327 was from Dako.

T-antigen preparation and analyses. Subconfluent NIH 3T3 cells in 60-mm dishes were routinely labeled for 4 h with $H_3{}^{32}PO_4$ (500 μCi/ml) in phosphate-free Dulbecco modified Eagle medium, or for 10 h with [^{35}S]methionine (100 μCi/ml) in methionine-free modified Eagle medium supplemented with 1/10 the normal level of methionine, beginning at 20 h postinfection. The phorbol esters or OAG were dissolved in DMSO at the concentrations specified and added at 1 μl of labeling medium per ml for specified periods of time. Labeling was terminated by the addition of cold phosphate-buffered saline containing 10 mM sodium fluoride, 0.2 mM sodium vanadate, 10 mM sodium PP_i, 5 mM ATP, and 0.1 mM phenylmethylsulfonyl fluoride. The washed cell monolayers were lysed in 1 ml of Nonidet P-40 buffer containing the above inhibitors, and immunoprecipitated with 40 μl of anti-T ascites or 2 μl of the diluted anti-*src* antibody and *Staphylococcus aureus* protein A-Sepharose as described before (31). Polypeptides were resolved on discontinuous buffer SDS-polyacrylamide gels of 10 and 12.5% acrylamide and visualized by autoradiography with or without intensifying screens (Dupont Cronex Lightening Plus). Fluorography was done by the method of Bonner and Laskey (4). Alkaline hydrolysis of phosphoproteins in SDS gels was done by the method of Cooper et al. (9). For quantitation, linear exposures of autoradiographs were scanned with an LKB 2202 UltraScan densitometer interfaced to a Hewlett-Packard 3390A integrator.

In vitro kinase reactions on immune complexes were carried out as described (31) except that 5 μM cold ATP was included; cell extracts were adjusted to a final protein concentration of 400 μg/ml before immunoprecipitation. Partial proteolysis of ^{32}P-labeled mT was essentially as described before (31).

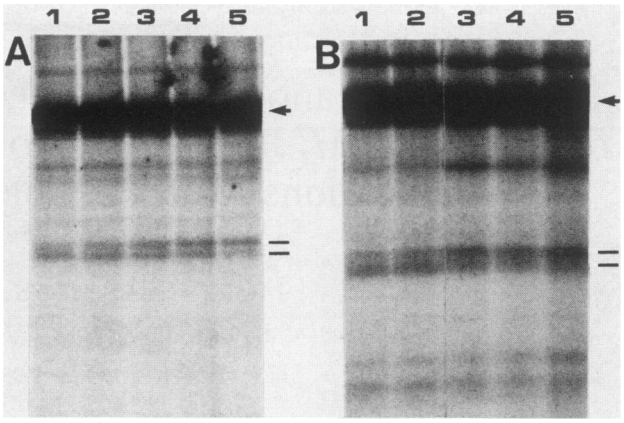

FIG. 1. Dose response and kinetics of TPA-stimulated mT phosphorylation. At 20 h postinfection, wild-type (NG59RA) polyomavirus-infected NIH 3T3 cells were incubated for 4 h in phosphate-free Dulbecco modified Eagle medium containing 500 μCi of $^{32}P_i$ per ml. (A) TPA was then added at the following concentrations (nanogram per milliliter) for 15 min: lane 1, no TPA (DMSO only); lane 2, 10; lane 3, 20; lane 4, 30; lane 5, 40. (B) TPA was added at 35 ng/ml for the following time periods: lane 1, 0 to 5 sec; lane 2, 2.5 min; lane 3, 5 min; lane 4, 10 min; lane 5, 15 min. T antigens were extracted, immunoprecipitated, separated on 10% SDS-polyacrylamide gels, and autoradiographed for 6 days. Arrows on the right of each panel indicate the position of the large T antigen; the double lines indicate the positions of the 56K and 58K forms of mT as determined from in vitro kinase and protein molecular weight markers.

RESULTS

mT phosphorylation in response to phorbol esters and a diacyl glycerol. Figure 1 shows the effects of the addition of the tumor promoter TPA on the phosphorylation of polyoma mT antigen in vivo. NIH 3T3 cells were infected by wild-type polyomavirus at a multiplicity of 10 to 20 PFU per cell. At 20 h postinfection, the cultures were incubated in phosphate-free Dulbecco modified Eagle medium supplemented with $^{32}P_i$ at 500 μCi/ml. After 4 h of incubation to allow for

the equilibration of the label with the endogenous ATP pool, TPA was added at various concentrations and for brief periods of time. Pilot experiments showed that 3 h of equilibration under these conditions was sufficient to give maximum labeling of T antigens. T antigens were then extracted, immunoprecipitated, and separated by SDS-PAGE. Panel A shows that TPA at 40 ng/ml induces preferential phosphorylation of the 58K form of mT over the 56K form after a 15-min incubation (lane 5). Concentrations of TPA in the 10- to 20-ng/ml range show a slight stimulatory effect on pp58 phosphorylation in the same time period; higher concentrations of TPA (up to 200 ng/ml) showed no greater effect than that seen at 40 ng/ml. The magnitude of the stimulatory effect on pp58 was seen to vary from experiment to experiment. In over 20 experiments, the range of stimulation was 1.5- to 5-fold, averaging about 2.5-fold. Panel B shows the kinetics of 58K mT phosphorylation in response to TPA. Exposure to TPA (35 ng/ml) for as little as 3 to 5 min results in the discernible stimulation of phosphorylation of pp58. Clear responses were seen from 5 to 15 min, with little or no further change in the ratio of pp58 to pp56 for at least 10 h in the continuous presence of TPA and $^{32}P_i$. Similar results on mT phosphorylation in response to TPA were obtained in virus-infected primary baby mouse kidney epithelial cells. Thus, TPA, at concentrations known to stimulate protein kinase C and to elicit physiological responses in other cell systems, induces a rapid and specific phosphorylation of the 58K form of mT. Forskolin (0.5 μM), a known stimulator of adenylate cyclase, had no discernible effect on mT phosphorylation in vivo under similar conditions.

The involvement of protein kinase C in the phosphorylation of pp58 mT was investigated further by using an inactive phorbol analog and a natural diacyl glycerol. The phorbol derivative, 4α-PDD, which is inactive as a tumor promoter and stimulator of protein kinase C (8), fails to stimulate mT phosphorylation. As shown in Fig. 2, TPA (lane 4), but not 4α-PDD (lane 3), gives enhanced phosphorylation of 58K mT. Concentrations of 4α-PDD up to 200 ng/ml were tried without effect. The diacyl glycerol OAG, a known stimulator of protein kinase C both in vitro and in vivo (26), when introduced into the culture medium at 10 μg/ml, stimulates 58K mT phosphorylation (lane 2). Since TPA can perturb cellular membranes leading to calcium influx, we examined whether the addition of the calcium ionophore A23187 alone could stimulate pp58 phosphorylation. At concentrations of 2×10^{-5} to 5×10^{-5} M, there was little or no effect on the ratio of pp58 to pp56, although the addition of ionophore together with TPA sometimes had a slight potentiating effect on 58K mT phosphorylation (not shown).

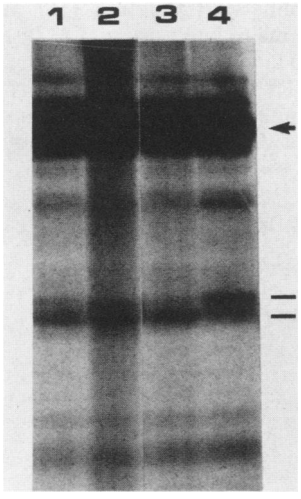

FIG. 2. Effects of an inactive phorbol ester (4α-PDD) and diacyl glycerol on mT phosphorylation in vivo. Wild-type infected NIH 3T3 cells (20 PFU per cell) were labeled with $^{32}P_i$ (500 μCi/ml) from 20 to 24 h postinfection. The cell cultures were then treated for 15 min as follows: lane 1, DMSO alone; lane 2, OAG (10 μg/ml); lane 3, 4α-PDD (35 ng/ml); lane 4, TPA (35 ng/ml). The cells were lysed and immunoprecipitated with anti-T ascites. A 7-day autoradiograph of a 10% SDS-polyacrylamide gel is shown. The arrow indicates the position of large T antigen. The positions of the 56K and 58K forms of mT determined by an internal in vitro kinase marker are indicated to the right by the double bar.

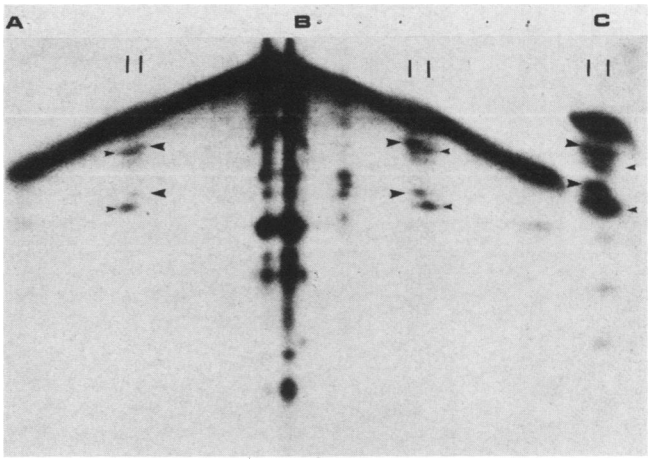

FIG. 3. Chymotryptic digestion of polyoma mT antigen labeled in vivo. Wild-type polyomavirus-infected NIH 3T3 cells (30 PFU per cell) were labeled with $^{32}P_i$ (500 μCi/ml) from 20 to 24 h postinfection. The cells were then incubated for 15 min with 35 ng of either 4α-PDD (A) or TPA (B) per ml. The T antigens were extracted, immunoprecipitated with anti-T ascites, and resolved in the first dimension on 10-cm cylindrical 10% SDS-polyacrylamide gels. Digestion was carried out with chymotrypsin (25 μg), and the cleavage products resolved on a 12.5% SDS-polyacrylamide gel in the second dimension. The gel was dried and exposed for 7 days in the presence of an intensifying screen. The cleavage pattern on the extreme right (C) is from mT labeled in vitro with [γ-^{32}P]ATP and is provided as an internal digestion standard. The positions of the 56K and 58K forms of mT are indicated at the top by double bars. The positions of the 39K/33K and 37K/31K peptides derived from the 58K and 56K forms of mT are indicated by large and small arrowheads, respectively.

Site specificity of 58K mT phosphorylation in the presence of TPA. To examine the amino acid and site specificity of TPA-stimulated mT phosphorylation, virus-infected cells were ^{32}P labeled and incubated for 15 min in the presence of 35 ng of TPA or 4α-PDD per ml. After immunoprecipitation with anti-T ascites, two-dimensional partial proteolysis peptide maps of mT were compared. Chymotrypsin and *S. aureus* V-8 protease were separately employed. Figure 3 shows the pattern of chymotryptic phosphopeptides of mT labeled in the presence of either 4α-PDD or TPA. The patterns from 4α-PDD-treated or TPA-treated cells are qualitatively the same; both profiles show the characteristic quartet of 37K/31K and 39K/33K phosphopeptides derived from 56K and 58K mT forms, respectively, as previously described (31). No new phosphopeptides are apparent in the TPA profile. Similarly, no qualitative differences were de-

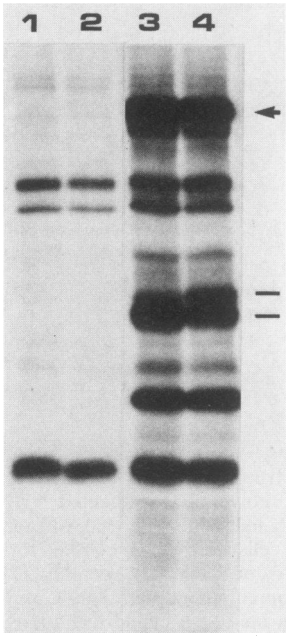

FIG. 4. T antigens labeled in vivo with [^{35}S]methionine during incubation with 4α-PDD or TPA. Mock-infected (lanes 1 and 2) or wild-type polyomavirus-infected (lanes 3 and 4) NIH 3T3 cells were labeled with [^{35}S]methionine from 18 to 28 h postinfection in the continuous presence of 35 ng of either 4α-PDD (lanes 1 and 3) or TPA (lanes 2 and 4) per ml. The cells were washed, lysed, and immunoprecipitated with anti-T ascites. The polypeptides were resolved on a 10% SDS-polyacrylamide gel and then fluorographed for 4 days. The position of large T and the 56K and 58K forms of mT determined from in vitro kinase and molecular weight markers are indicated to the right by the arrow and double bars, respectively.

tected in maps with *S. aureus* V-8 protease. The difference between the 4α-PDD and TPA mT phosphopeptide patterns is a quantitative one; namely, an increase in the 39K/33K spots derived from pp58 relative to the pp56-derived 37K/31K peptides.

The phosphate linkages in pp58 phosphorylated in the presence of TPA are fully alkali labile, as is the 58K form phosphorylated in vivo in the absence of the phorbol. Phosphoamino acid analyses of pp58 and pp56 phosphorylated in the presence or absence of TPA all show phosphoserine as the major component (over 90%) in both species, consistent with the results of an earlier report (38). Previous results have shown that these two mT species are phosphorylated at distinct sites in the C-terminal half of the molecule (31). The present results demonstrate that TPA-enhanced phosphorylation is selective for the 58K form, and suggest that it occurs at or near the serine site that is normally phosphorylated in this species.

Long-term exposure to TPA leads to accumulation of the 58K form of mT. Figure 4 shows results of long-term incubations of virus-infected cells with TPA. Infected cells were labeled with [^{35}S]methionine concurrently with exposure to TPA, from 18 to 28 h postinfection. Scanning of lanes 3 and 4 by densitometry shows a fourfold enhancement of the amount of 58K, along with a concomitant decrease in the amount of 56K; i.e., the ratio of 58K to 56K goes from 1:9 (lane 3) to 4:6 (lane 4). The 56K form remains the predominant species; only a small fraction of it is phosphorylated at the 56K site, and these molecules comigrate with the bulk unphosphorylated 56K (31). Whereas an effect of TPA in increasing the rate of phosphate turnover in pp58 could account for the results of the ^{32}P labeling experiments shown above, the results in Fig. 4 show that the tumor promoter induces a net recruitment of mT molecules from the unphosphorylated (56K) pool into the phosphorylated 58K form.

TPA fails to stimulate mT phosphorylation in virus mutants which normally fail to show a 58K species. A series of mutants previously characterized for their patterns of mT phosphorylation in vivo and in vitro have been examined for their responses to TPA. Cells infected by the mutants were ^{32}P labeled, treated for 15 min with either 35 ng of TPA or 4α-PDD per ml, and then extracted for T antigen analysis. Figure 5 shows results with four mutants. The *hr-t* mutant NG18 has a frame-shift deletion (16) and produces no immunoreactive mT-related product (37); as expected, this mutant shows no labeled material in the mT region of the gel (lanes 3 and 4). The *hr-t* mutant NG-59 produces an altered mT protein containing an isoleucine-asparagine substitution for aspartic acid at position 179 (5); its mT fails to be

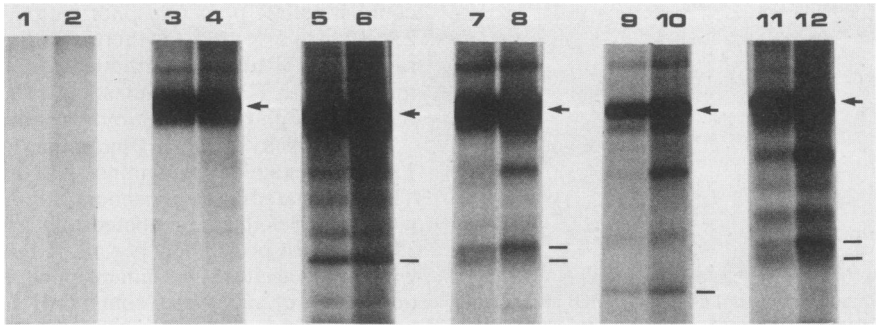

FIG. 5. In vivo labeling of T antigens of wild-type and viruses. NIH 3T3 cells were mock-infected (lanes 1 and 2) or infected with polyomavirus (20 to 30 PFU per cell; lanes 3 to 12) and labeled with $^{32}P_i$ from 20 to 24 h postinfection. The monolayers were then incubated with 35 ng of either 4α-PDD (odd-numbered lanes) or TPA (even-numbered lanes) per ml for 15 min. The T antigens were extracted, immunoprecipitated with anti-T ascites, resolved on 10% SDS-polyacrylamide gels, and autoradiographed for 6 to 10 days. The infections were as follows: lanes 1 and 2, mock infected; lanes 3 and 4, NG18; lanes 5 and 6, NG59; lanes 7 and 8, NG59RA; lanes 9 and 10, Py1387T; lanes 11 and 12, Py1178T/*dl*1014. The position of the large T antigen is indicated by an arrow. In lanes 5 and 6, the single bar denotes the position of the 56K form of mT. In lanes 7 and 8, the double bar indicates the position of the 56K and 58K forms of mT. In lanes 9 and 10, the single bar indicates the position of the truncated (51K) mT species. The double bar in lanes 11 and 12 represents the two deleted forms of mT.

phosphorylated on tyrosine in immune complex kinase reactions (2, 31, 32), and undergoes serine phosphorylation at the 56K, but not the 58K, site in vivo (31). Even in the presence of TPA, no labeled 58K species appears with this mutant (lanes 5 and 6). The wild-type virus NG59RA shows the expected TPA-specific response (lanes 7 and 8). The mutant Py1387T encodes a ~51K-tail-less mT, lacking the C-terminal membrane-anchoring sequence. Like NG59, this mutant is nontransforming, negative in the mT-immune complex kinase reaction, and undergoes serine phosphorylation in vivo only at the 56K site (6). TPA fails to induce detectable phosphorylation of a 58K mT form with this mutant (lanes 9 and 10). Finally, Py1178T/*dl*1014 is a double mutant that lacks tyrosines at the major and minor sites of phosphorylation in vitro (36). This double mutant shows a greatly reduced transforming efficiency, similar to that shown for Py1178T (7); its mT, however, shows a normal phosphorylation response to the phorbols (lanes 11 and 12). Thus, the potential for tyrosine phosphorylation of mT at the usual sites by pp60$^{c\text{-}src}$ or other tyrosine kinases is not essential for TPA-induced phosphorylation of pp58.

There is a prominent 80,000-M_r band in immunoprecipitates of extracts from wild-type virus-infected cells treated with TPA, but not in untreated or 4α-PDD-treated cell extracts. Many, but not all, preparations of anti-T ascites fluids used as a source of anti-T antibodies bring down this phosphate-labeled protein. (For example, in Fig. 5, compare lanes 2 and 4 on the one hand, with lanes 6, 8, 10, and 12 on the other; these were carried out with two different batches of ascites.) This band represents a cellular protein, presumably a substrate for protein kinase C, and is perhaps the same 80K phosphoprotein as described by Rozengurt et al. (30).

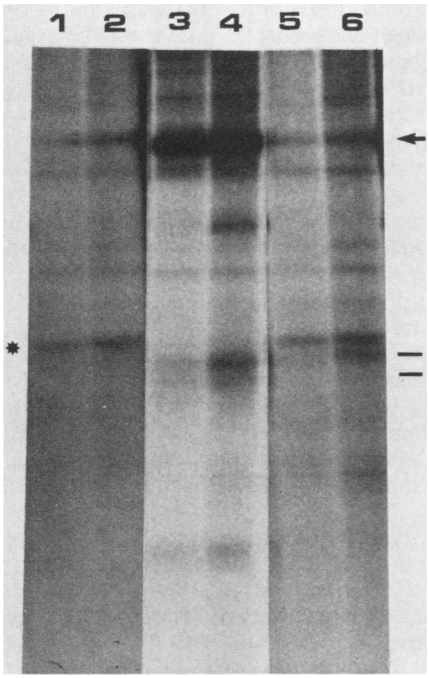

FIG. 6. Effect of phorbol ester treatment on in vivo phosphorylation of pp60$^{c\text{-}src}$. Mock-infected (lanes 1 and 2) or wild-type polyoma-infected (lanes 3 to 6) NIH 3T3 cells were labeled with $^{32}P_i$ for 4 h at 20 h postinfection. The cells were then pulsed for 15 min with 35 ng of either 4α-PDD (lanes 1, 3, and 5) or TPA (2, 4, and 6) per ml. The cells were washed, lysed, and immunoprecipitated with either anti-T ascites (lanes 3 and 4) or the anti-*src* monoclonal antibodies (lanes 1, 2, 5, and 6). The polypeptides were resolved on a 10% SDS-polyacrylamide gel and autoradiographed for 6 to 10 days. The positions of large T and the two forms of mT are indicated on the right by the arrow and double bars, respectively. The position of pp60$^{c\text{-}src}$ is indicated on the left by an asterisk.

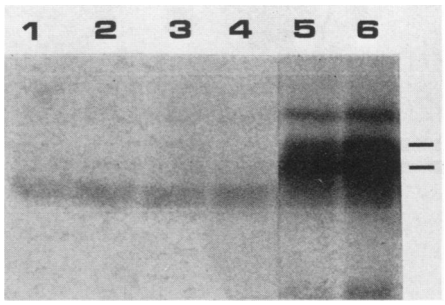

FIG. 7. In vitro labeling of mT from lytically infected cells incubated with phorbol esters. Mock-infected and polyoma-infected NIH 3T3 cells at 24 h postinfection were incubated with 35 ng of either 4α-PDD (odd-numbered lanes) or TPA (even-numbered lanes) per ml for 15 min. The T antigens were extracted and immunoprecipitated with anti-T ascites, incubated with [γ-^{32}P]ATP, resolved on 10% SDS-polyacrylamide gels, and autoradiographed for 6 days. Lanes 1 and 2, mock-infected NIH 3T3; lanes 3 and 4, NG59-infected 3T3; lanes 5 and 6, NG59RA-infected 3T3. The position of 56K and 58K mT is indicated by a double bar.

Effect of TPA treatment on association of mT with pp60$^{c\text{-}src}$. The mT molecules that are isolated as complexes with pp60$^{c\text{-}src}$ represent a minor fraction of the total mT (29). This fraction resembles the in vivo-phosphorylated mT molecules in partitioning preferentially into the Triton-insoluble framework (35). It therefore seems plausible that the in vivo-phosphorylated mT molecules are preferentially found in the fraction that is complexed with pp60$^{c\text{-}src}$.

There were two approaches undertaken to investigate whether TPA-stimulated phosphorylation of mT in vivo results in an increase in the amount of mT complexed with pp60$^{c\text{-}src}$. In the first approach, mock-infected or wild-type virus-infected cells were ^{32}P labeled, and treated with 4α-PDD or TPA. Cells were extracted and immunoprecipitated with either anti-*src* or anti-T antibody. Using anti-*src* antibody, a 60-kilodalton band is seen corresponding to the expected position of pp60$^{c\text{-}src}$ in uninfected cells (Fig. 6, lanes 1 and 2). The slight stimulation of labeling of pp60$^{c\text{-}src}$ in TPA-treated cells seen here was not evident in every experiment. However, a similar low-level stimulation of both pp60$^{c\text{-}src}$ and pp60$^{v\text{-}src}$ phosphorylation by TPA has been noted by others (15, 27). Lane 4 shows an anti-T immunoprecipitate of virus-infected cells treated with TPA; the highly labeled pp58 form of mT is clearly resolved from pp60$^{c\text{-}src}$. Lanes 5 and 6 show results with anti-pp60 monoclonal antibody. In addition to the 60-kilodalton band, there is a band that comigrates with pp58 mT. This band is also seen more prominently in extracts from TPA-treated cells (lane 6) than in extracts from 4α-PDD-treated cells (lane 5), indicating that pp58 mT phosphorylated in TPA-treated cells associates with pp60$^{c\text{-}src}$. The slight increase in level of pp60$^{c\text{-}src}$ phosphorylation in infected cells (compare lanes 5 and 6 with lanes 1 and 2) has been seen in several experiments, and may reflect mT-enhanced autophosphorylation of pp60$^{c\text{-}src}$ involving N-terminal tyrosine residues as previously described (2).

The second approach to the question of whether incubation of infected cells with TPA leads to increased levels of

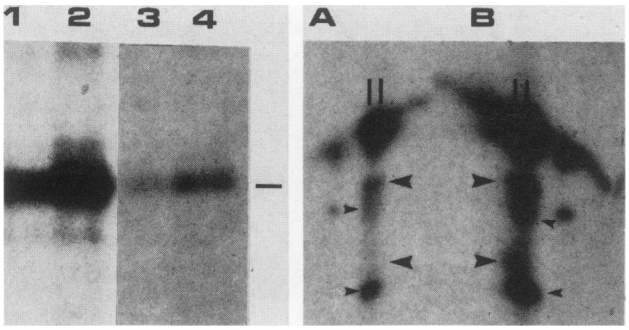

FIG. 8. In vitro labeling of mT from transformed cells incubated with phorbol esters. In the left panel, polyoma-transformed 3T3 cells were incubated with 35 ng of either 4α-PDD (lanes 1 and 3) or TPA (lanes 2 and 4) per ml. The T antigens were immunoprecipitated with anti-T ascites (lanes 1 and 2) or anti-*src* antibody (lanes 3 and 4), incubated with [γ-^{32}P]ATP, and resolved on a 10% SDS-polyacrylamide gel. The gel was alkali-treated and autoradiographed for 8 days. The position of mT is represented by a single bar. In the right panel, mT was immunoprecipitated with anti-T ascites from cells treated with either 4α-PDD (A) or TPA (B) and incubated with [γ-^{32}P]ATP. The products were resolved in the first dimension on cylindrical 10% SDS-polyacrylamide gels. Digestion was carried out with chymotrypsin, and the products resolved on a 12.5% SDS-polyacrylamide gel in the second dimension. A 10-day autoradiograph of an alkali-treated gel is shown. The positions of the 56K and 58K forms of mT are indicated at the top by double bars. The positions of the 39K/33K and 37K/31K peptides derived from the 58K and 56K forms of mT are indicated by large and small arrowheads, respectively.

mT-pp60$^{c\text{-}src}$ complexes involves immunoprecipitate kinase assays. Figure 7 shows the results of such assays of anti-T immunoprecipitates prepared from infected 3T3 cells treated with phorbols. TPA-treated wild-type virus-infected cells yield immune complexes that show a threefold enhancement of pp58 phosphorylation in vitro compared with that of 4α-PDD-treated cells (lanes 5 and 6). A similar result with polyomavirus-transformed 3T3 cells is shown in Fig. 8. The mT of this cell line has a slightly deleted mT protein that fails to resolve into distinct species. Nevertheless, by using either anti-T ascites (lanes 1 and 2) or anti-*src* antibody (lanes 3 and 4), there is a clear stimulation of phosphate incorporation into mT in vitro from the TPA-treated cells. The gels were treated with alkali before exposure to enhance the detection of phosphotyrosine. The right panel of Fig. 8 shows a partial two-dimensional chymotryptic map of the same material as that seen in lanes 1 and 2. The 58K- and 56K-derived phosphopeptides are resolved sufficiently well to show enhanced labeling of the 58K-derived peptides from TPA-treated cells. Similar results showing increased levels of 58K phosphorylation in vitro after TPA treatment have also been obtained by using polyomavirus-transformed rat cells. Stimulation by TPA of pp58 phosphorylation in vivo is thus accompanied by increased levels of pp58mT-pp60$^{c\text{-}src}$ complexes as assayed in vitro. The implications of this finding are discussed below.

DISCUSSION

Earlier results have pointed to the importance of mT-pp60$^{c\text{-}src}$ interaction in the transformation of cells by polyomavirus. This interaction is conveniently measured by kinase reactions carried out on mT-containing immune precipitates, in which mT becomes phosphorylated on tyrosine residues in a reaction apparently catalyzed by pp60$^{c\text{-}src}$ (3, 10–12, 31–33). Nontransforming virus mutants with altered mT proteins are negative in this in vitro kinase assay. Phosphate labeling of polyomavirus-infected cells shows that mT becomes labeled predominantly on serine, and not on tyrosine (38). Furthermore, two serine-phosphorylated forms of mT are distinguished by one-dimensional SDS-PAGE as 56K and 58K species and by phosphopeptide patterns after proteolytic digestion. In vitro kinase results show both 56K and 58K forms of mT to be labeled on tyrosine (31). The same mutant mTs that are negative in the in vitro kinase assay are also defective as substrates for the serine kinase that acts in vivo to produce the 58K form (6, 31). Py1387T, in particular, encoding a truncated cytoplasmic mT that fails to undergo phosphorylation at the 58K serine site, suggests that the 58K kinase may itself be membrane bound.

Additional evidence points to the existence of a "kinase-active" pool of mT molecules distinguishable from the bulk of unphosphorylated mT molecules. Extraction of infected cells with nonionic detergents shows that both the in vivo-phosphorylated mT molecules, as well as the mT molecules complexed with pp60$^{c\text{-}src}$, are preferentially bound to cell frameworks and separable from the majority of mT, which is readily extracted from the cell (35). Furthermore, sedimentation analysis has shown that the kinase-active fraction of mT, once extracted from cell frameworks, sediments faster than the bulk of metabolically labeled mT (11, 33).

These results suggest the possibility that the mT molecules that are present in complexes with pp60$^{c\text{-}src}$, and that constitute the biologically active mT population (29), are those that have been phosphorylated by serine kinases. To explore this possibility and to better understand the relationship between mT and various cellular protein kinases, we have investigated the effects of TPA, a known potent stimulator of protein kinase C. The results show that TPA rapidly and specifically induces phosphorylation of the 58K form of mT. Both the dosage and kinetic response to TPA of this phosphorylation, as well as the magnitude of the response, are in line with the effects of this tumor promoter in other systems (15, 19, 28, 30). The increased incorporation of ^{32}P into pp58 mT in the presence of TPA is not caused primarily by an increased rate of phosphate turnover in the protein, since it is paralleled by an increase in the amount of the 58K species and a decrease in the amount of the 56K form as determined from long-term metabolic labeling experiments with [^{35}S]methionine. These results provide additional evidence that the 58K form is created by a specific phosphorylation of the unphosphorylated-56K form altering its mobility in SDS-PAGE.

Phosphorylation of the 56K form of mT appears to be unaffected by TPA. This phosphorylation occurs on serine at a site distinct from that phosphorylated in the 58K mT species (31). Mutant forms of mT (NG59 and Py1387T) that are defective as substrates for the 58K kinase are still recognized by the 56K kinase (6, 31). These data suggest that two distinct cellular kinases are involved.

There are two lines of evidence supporting the conclusion that TPA-enhanced phosphorylation of pp58 mT represents a stimulation of the kinase pathway that normally produces the 58K species. The first comes from chymotryptic and V8 maps showing that the phosphopeptide patterns are qualitatively the same with and without TPA, the only difference being that the 58K-derived fragments are more heavily labeled in the presence of TPA. The second line of evidence comes from studies of virus mutants with altered mT proteins; those mutants that normally show phosphorylation at

the 58K-specific site are stimulated by TPA, while those (nontransforming) mutants that normally fail to show a 58K form show no response to the phorbol.

The data are consistent with protein kinase C acting as the 58K kinase, in keeping with the relatively rapid effect of both TPA and OAG and the absence of effect by 4α-PDD. Features of this enzyme being keyed to events in the membrane, both for its own activation and for localization of substrates like growth factor receptors (18, 19), are also consistent with it being the 58K kinase, since the latter is known to act on membrane-bound, but not soluble, mT (6). Results of in vivo labeling nevertheless cannot rule out the possibility that a second kinase, presumably one that is activated by protein kinase C, is the proximal 58K kinase. Other possibilities for an indirect effect, involving protein kinase C acting through a phosphatase or phosphatase inhibitor, are also conceivable. However, another laboratory has reported phosphorylation of mT in vitro by using purified protein kinase C and a partially purified preparation of mT (17).

The exposure of infected cells to TPA also increases the amount of mT-pp60$^{c\text{-}src}$ complexes judged by in vivo labeling and precipitation with either anti-pp60$^{c\text{-}src}$ or anti-T antibody. The incubation of lytically infected or transformed cells with TPA also leads to increased amounts of 58K mT, as seen in the tyrosine-specific in vitro kinase reaction. Thus, at least a substantial fraction of the pp58 molecules formed in the presence of TPA find their way into complexes with pp60$^{c\text{-}src}$. These findings lend further support to the suggestion that serine phosphorylation of mT, particularly of the 58K form, has a positive effect in promoting or stabilizing interactions of mT with pp60$^{c\text{-}src}$ and thus, in regulating at least some of the biological functions of mT (29). Further study is needed to confirm this directly and to better understand the interplay between mT, pp60$^{c\text{-}src}$, and protein kinase C.

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