Tumor-promoting Phorbol Esters Stimulate the Phosphorylation of Ribosomal Protein S6 in Quiescent Reuber H35 Hepatoma Cells*

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The addition of the tumor promoter, 12-O-tetradecanoylphorbol-13-acetate (TPA) to serum-starved quiescent Reuber H35 hepatoma cells results in a rapid 5- to 11-fold increase in the incorporation of $^{32}$P, into a $M_r = 32,000$ ribosomal protein. The $M_r = 32,000$ protein was the major phosphorylated protein extracted from isolated 80 S ribosomes and was identified as the 40 S ribosomal protein S6 based upon its migration in two-dimensional gels. Insulin, which has been demonstrated to increase the phosphorylation of S6 in a number of cell lines, caused a 10- to 20-fold increase in the incorporation of $^{32}$P, into this $M_r = 32,000$ ribosomal protein.

S6 phosphorylation was dose- and time-dependent being detected as early as 5 min following the addition of 1.6 $\mu$M TPA. Maximal phosphorylation of ribosomal protein S6 was achieved by 60 min and remained elevated for at least 90 min in the presence of TPA. The 50% effective dose for TPA was estimated to be 0.14 $\mu$M. Based upon the altered migration of S6 in pH 8.5 urea-polyacrylamide gels, it was demonstrated that the increased $^{32}$P, labeling of S6 by TPA was due to a net increase in the incorporation of phosphates into the S6 molecule. Non-tumor-promoting phorbol esters were ineffective in increasing the phosphorylation of S6. In whole cells, exogenously added 1 mM 8-bromoadenosine 3'-5'-monophosphate failed to substantially increase phosphorylation of S6 suggesting that the TPA-induced phosphorylation of S6 occurs via a cyclic AMP-independent mechanism. The S6 amino acid residue phosphorylated in response to TPA was phosphorylase. A possible role for protein kinase C in the phosphorylation of ribosomal protein S6 is discussed.

The tumor-promoting phorbol esters are noncarcinogenic agents that will induce tumor formation when administered after subthreshold doses of carcinogen (1-3). The most potent

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† The abbreviations used are: TPA, 12-O-tetradecanoylphorbol-13-acetate; 8-Br-cAMP, 8-bromoadenosine 3'-5'-monophosphate; Hepes, 4 (2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N',N'-tetraacetic acid; SDS, sodium dodecyl sulfate.
phospholipid-stimulated Ca\(^{2+}\)-dependent protein kinase (protein kinase C) (31, 32).

**EXPERIMENTAL PROCEDURES**

**Materials**

TPA, phorbol-12,13-diacetate, 4a-phorbol, and phorbol-12,13-dibutyrate were purchased from Chemical Carcinogenesis, Eden Prairie, MN. Porcine insulin was from Lilly. Carrier-free \(^{32}\)P (240-320 mCi/ml) was obtained from ICN, Irvine, CA; 4-O-methyl-TPA, phosphoserine, phosphothreonine, and 8-BrcAMP were purchased from Sigma Chemical Co.; all other reagents were purchased from Bio-Rad. Cell culture media and sera were purchased from Grand Island Biological Co.

**Methods**

**Cell Culture—**A cloned cell line (KRC7) derived from Reuber H35 rat hepatoma was kindly provided by Dr. Wesley D. Wicks, Department of Biochemistry, University of Tennessee, Knoxville, TN. Cells were grown and maintained as previously described (33). For experiments, cells were seeded onto 100-mm diameter Petri dishes at a density of approximately 5 \(\times\) 10\(^5\) cells/cm\(^2\) and cultured for 3 days in 10 ml of Dulbecco’s modified Eagle’s medium, containing 5% fetal calf serum and 5% calf serum. After this time, serum-replete medium was removed, the cell monolayer was washed 3 times with 5 ml of ice-cold Krebs-Ringer bicarbonate/Hepes buffer (120 mM NaCl, 4.78 mM KCl, 1.2 mM MgSO\(_4\), 1.2 mM CaCl\(_2\), 24 mM NaHCO\(_3\), 10 mM Hepes, pH 7.5) and scraped into 2 ml of homogenization buffer containing 50 mM Tris- HCl (pH 7.0), 10 mM benzenamide HCl, 1 mM EGTA, 100 mM NaF, 5 mM dithiothreitol, 1.5 mM MgCl\(_2\), 0.25 M sucrose, and 2% (v/v) Triton X-100. Addition of detergent resulted in rapid cell lysis. Nuclei were removed by sedimentation at 7,300 g for 60 min at 2 \(^\circ\)C in a Beckman L50 ultracentrifuge using a fixed angle Ti 60 rotor. The 120,000 \(\times\) g pellet containing total cytosolic proteins was acetone precipitated at -20 \(^\circ\)C for 16 h and resuspended in a hypotonic detergent buffer containing 1% Triton X-100 and 1% deoxycholate (35). Nuclei were sedimented, and the nuclei pellet was washed 3 times with 5 ml of ice-cold buffer and 2% (v/v) Triton X-100. Addition of detergent resulted in rapid cell lysis. Nuclei were removed by sedimentation at 7,300 g for 60 min at 2 \(^\circ\)C in a Beckman L50 ultracentrifuge using a fixed angle Ti 60 rotor. The 120,000 \(\times\) g pellet containing total cytosolic ribosomes was prepared for SDS-urea polyacrylamide gel electrophoresis by solubilizing the pellet in Laemmli’s sample buffer (34) and heating the sample to 100 \(^\circ\)C for 3 min.

Alternatively, ribosomal proteins were isolated by disrupting \(^{32}\)P-labeled cells in a hypotonic detergent buffer containing 1% Triton X-100 and 1% deoxycholate (35). Nuclei were sedimented, and the postnuclear supernatant was layered on a 1.6 M sucrose cushion in buffer containing 500 mM KCl, 5 mM MgCl\(_2\), 1 mM dithiothreitol, and 20 mM Tris- HCl, pH 7.4. The ribosomes were sedimented by centrifugation at 120,000 \(\times\) g for 18-20 h. The ribosomal pellet was resuspended in buffer containing 100 mM KCl, 5 mM \(\beta\)-mercaptoethanol, 5 mM MgCl\(_2\), 20 mM Tris- HCl at pH 7.4, and the RNA was precipitated by adding 100 mM MgCl\(_2\) and 67% glycerol acetic acid. The precipitate was removed by low speed centrifugation. The ribosome pellet was washed with 1% Triton X-100 and 2% (v/v) Triton X-100. Addition of detergent resulted in rapid cell lysis. Nuclei were removed by sedimentation at 7,300 g for 60 min at 2 \(^\circ\)C in a Beckman L50 ultracentrifuge using a fixed angle Ti 60 rotor. The 120,000 \(\times\) g pellet containing total cytosolic ribosomes was prepared for SDS-urea polyacrylamide gel electrophoresis by solubilizing the pellet in Laemmli’s sample buffer (34) and heating the sample to 100 \(^\circ\)C for 3 min.

Additionally, ribosomal proteins were isolated by disrupting \(^{32}\)P-labeled cells in a hypotonic detergent buffer containing 1% Triton X-100 and 1% deoxycholate (35). Nuclei were sedimented, and the postnuclear supernatant was layered on a 1.6 M sucrose cushion in buffer containing 500 mM KCl, 5 mM MgCl\(_2\), 1 mM dithiothreitol, and 20 mM Tris- HCl, pH 7.4. The ribosomes were sedimented by centrifugation at 120,000 \(\times\) g for 18-20 h. The ribosomal pellet was resuspended in buffer containing 100 mM KCl, 5 mM \(\beta\)-mercaptoethanol, 5 mM MgCl\(_2\), 20 mM Tris- HCl at pH 7.4, and the RNA was precipitated by adding 100 mM MgCl\(_2\) and 67% glycerol acetic acid. The precipitate was removed by low speed centrifugation. The ribosomal proteins were acetone precipitated at -20 \(^\circ\)C for 16 h and solubilized in 6 M urea in preparation for gel electrophoresis in pH 8.5 urea-polyacrylamide gels.

**Gel Electrophoresis and Autoradiography—**One-dimensional SDS-polyacrylamide gel electrophoresis was performed according to Laemmli (34) with the addition of 6 M urea. The upper electrode buffer was supplemented with 5 mM 3-mercaptoethanol to maintain the proteins in their reduced state during electrophoresis. Gels were stained overnight in 0.1% Coomassie brilliant blue R-250 in methanol-acetic acid:water (45:10:45), destained in methanol-acetic acid: water (3:1), and imbibed in 3MM paper and exposed to Kodak X-Omat x-ray film in cassettes lined with Cronex intensifier screens. Both one- and two-dimensional polyacrylamide gel electrophoresis of extracted 80 S ribosomal proteins was performed according to Del Grande and Traugh (36). Electrophoresis was performed at 4 \(\times\) 10\(^5\) A/cm for 100 V for 1 h and then increased to 200 V for 20 h.

**Quantitation of S6 Phosphorylation—**For some experiments S6 phosphorylation was quantitated by integration of the densitometry tracing obtained from the autoradiogram using a densitometer pur-

**RESULTS**

**\(^{32}\)P Labeling of Quiescent H35 Rat Hepatoma Cells—**After 3 days in serum-free DMEM, H35 rat hepatoma cells became quiescent. However, the cells remain viable and responsive to the growth stimulatory effects of both serum and insulin (30). Incubation of 3-day serum-starved H35 rat hepatoma cells with \(^{32}\)P, for 2.5 h resulted in the incorporation of \(^{32}\)P into many cytosolic proteins which was not phosphorylated in unstimulated cells. Incubation of 3-day serum-starved H35 rat hepatoma cells with \(^{32}\)P, for 2.5 h resulted in the incorporation of \(^{32}\)P into many cytosolic proteins which was not phosphorylated in unstimulated cells.

**Identification of the \(^{32}\)Pi-Labeled S6 Protein—**A 32,000 microsomal protein which was not phosphorylated in untreated control cells (Fig. 1, compare lanes 1 and 3). There were no other differences, which could be detected by one-dimensional urea SDS-polyacrylamide gel electrophoresis, in the \(^{32}\)P labeling patterns of microsomal proteins obtained from either control or TPA-treated cells (Fig. 1, lanes 1 and 3).

The TPA-stimulated increase in \(^{32}\)P labeling of the M, = 32,000 protein was not due to changes in the specific activity of intracellular [\(^{32}\)P]ATP pools. Incubation of the cells with \(^{32}\)P, for 60 min resulted in equilibration of \(^{32}\)P with ATP pools as detected by thin layer chromatography (see under "Experimental Procedures") with 0.8 fmol of \(^{32}\)P incorporated into ATP per 10\(^6\) cells. Additional incubation up to 2.5 h did not result in any further significant increase in \(^{32}\)P incorporation into ATP. Incubation of the H35 cell cultures with 1.6 \(\mu\)M TPA did not alter the total amount of \(^{32}\)P incorporated into cellular ATP. The specific activity of [\(^{32}\)P]ATP pools was probably not altered by TPA since the incorporation of \(^{32}\)P, into essentially all other detected microsomal proteins remained unchanged following the addition of TPA to the cells (Fig. 1, compare lanes 1 and 3).

**Identification of the M, = 32,000 Phosphoprotein As Ribosomal Protein S6—**The molecular weight, phosphorylation, and cell fractionation characteristics of this M, = 32,000 phosphoprotein in the hepatoma cultures suggested that it might be the 40 S ribosomal protein, S6 (see Refs. 38 and 39).
treated cells. Quantitation of the 32Pi incorporated into the pellet was subjected to centrifugation at 120,000 x g. The 120,000 x g pellet was electrophoresed in 7.5% SDS-polyacrylamide gels. The autoradiogram in Fig. 1 showed that 1 pg/ml of insulin caused the phosphorylation of a 17-fold increase in 32Pi incorporation into this protein band by densitometry scan showed a 17-fold increase in 32Pi incorporation into the 32,000 protein in the hepatoma cells was determined. Based upon these observations the M, = 32,000 microsomal protein by insulin and TPA. Monolayers of H35 cells were incubated for a total of 2.5 h with 20 μCi/ml of 32Pi. After 2 h either insulin (1 μg/ml) or TPA (1.6 μM) was added. Following incubation for 30 min, cells were lysed and the postnuclear supernatant was subjected to centrifugation at 120,000 x g. The 120,000 x g pellet was electrophoresed in 7.5% SDS-polyacrylamide gels. The 10% trichloroacetic acid-precipitable radioactivity applied to each lane was 6 x 10^6 cpm. After electrophoresis the gel was stained, dried, and exposed to Kodak X-Omat R film overnight with an intensifier screen. Lane 1, control; Lane 2, insulin-treated cells; Lane 3, TPA-treated cells. Quantitation of the 32Pi incorporated into M, = 32,000 protein band by densitometry scan showed a 17-fold increase in 32Pi, labeling following insulin treatment and an 11-fold increase in 32Pi labeling following TPA treatment.

Comparison of phosphorylation of S6 to 40 S ribosomal protein S6 in a variety of cultured cells (26, 40, 41). Since H35 cells cultured in an identical manner with that described here are exquisitely sensitive to the growth stimulatory effects of insulin (30), the ability of insulin to stimulate the phosphorylation of the M, = 32,000 protein in the hepatoma cells was determined. As shown in Fig. 1, lane 2, 1 μg/ml of insulin caused the phosphorylation of a M, = 32,000 protein which migrated identically with the TPA-stimulated phosphoprotein during electrophoresis in a 7.5% SDS-polyacrylamide gel. Integration of the M, = 32,000 peak from densitometric tracing of the autoradiogram in Fig. 1 showed that 1 μg/ml of insulin caused a 17-fold increase in 32Pi incorporation into this protein compared with an 11-fold increase in 32Pi incorporation caused by 1.6 μM TPA (see legend to Fig. 1).

To conclusively identify the M, = 32,000 phosphoprotein found in the microsomal fraction as 40 S ribosomal protein S6, 80 S ribosomes were isolated from cells preincubated with 32Pi and further incubated with 1.6 μM TPA for 30 min. 32Pi-labeled ribosomal proteins were extracted from H35 cells and subjected to two-dimensional polyacrylamide gel electrophoresis (36), as shown in Fig. 2. Carrier 80 S ribosomal protein isolated from rabbit reticulocytes was added to the H35 32Pi-labeled ribosomal proteins in order to visualize the Coomassie blue-stained protein pattern. The TPA-induced ribosomal phosphoprotein migrates in the position reported for the phosphorylated form of 40 S ribosomal protein S6 (28, 36). Based upon these observations the M, = 32,000 phosphoprotein is identified as the 40 S ribosomal protein, S6.

Comparison of the Extent of Phosphorylation of S6 Induced by TPA Compared to Control H35 Cells—The 40 S ribosomal protein S6 exists in multiple phosphorylated forms and has been reported to contain up to five phosphoryl groups (42, 43). The different phosphorylated forms of S6 can be resolved on the basis of charge by polyacrylamide gel electrophoresis in urea at pH 8.5 as described under “Experimental Procedures.” To determine whether TPA induced a net increase in the number of phosphates per S6 molecule, ribosomal proteins isolated from TPA-treated and control cells were separated by electrophoresis in urea at pH 8.5. Fig. 3, lanes 1 and 2, depict the level of S6 phosphorylation in control and TPA-treated cells, respectively, after an 18-h exposure of the x-ray film. 32Pi incorporation into S6 from control cells was too low to be detected following an 18-h exposure time (Fig. 3, lane 1). This was in contrast to the large increase in total 32Pi incorporation into S6 in the TPA-treated cells (Fig. 3, lane 2). In order to better compare possible differences in the various phosphorylated derivatives of S6 in control and TPA-treated cells, the control lane of the gel (Fig. 3, lane 1) was exposed for 4 days and is shown in Fig. 3 as lane 3. The 32Pi incorporation into control S6 was primarily into a single phosphorylated derivative of S6, presumably the monophosphorylated derivative (Fig. 3, lane 3). In addition, the S6 protein from the control cultures showed a small amount of a
polyacrylamide gels. Gels were stained, dried, and autoradiographed following treatment with TPA. Almost all of the $^{32}$Pi appeared to be incorporated into more highly phosphorylated derivatives of S6, which are absent in the control cells, can be detected in TPA-treated cells. These more highly phosphorylated derivatives of S6 probably are the triphosphorylated and tetraphosphorylated (and possibly the pentaphosphorylated) S6 derivatives (Fig. 3, lane 2). At least two more highly phosphorylated derivatives of S6, which are absent in the control cells, can be detected in TPA-treated cells. These more highly phosphorylated derivatives of S6 probably are the triphosphorylated and tetraphosphorylated (and possibly the pentaphosphorylated) S6 derivatives (Fig. 3, compare lanes 2 and 3).

Fig. 3. Separation of phosphorylated derivatives of S6 following treatment with 1.6 $\mu$M TPA. Following a 30-min exposure to 1.6 $\mu$M TPA, $^{32}$P, labeled ribosomal proteins were extracted from cells as described under “Experimental Procedures.” 250 $\mu$g of ribosomal protein from control cells and from TPA-treated cells were separated by electrophoresis toward the cathode in pH 8.5 urea-polycrylamide gels. Gels were stained, dried, and autoradiographed as described under “Experimental Procedures.” I denotes the position of the least phosphorylated derivative of S6 that could be detected by autoradiography and presumably represents monophosphorylated S6. Based upon this reference point the more highly phosphorylated derivatives of S6 have been designated diphosphorylated (2), triphosphorylated (3), and tetra (4,5), and possibly pentaphosphorylated S6. Lane 1, control cells (untreated); lane 2, TPA-treated cells (1.6 $\mu$M TPA). Lanes 1 and 2 represent an 18-h exposure of the autoradiogram. Lane 3 is a 4-day exposure of Lane 1, control. Note the intensity and alignment of the radioactive marker spots on control Lanes 1 and 3.

more highly phosphorylated species which can be seen as a faint band running just above the single major band. Treatment with TPA resulted not only in a marked increase in the amount of $^{32}$P incorporated into S6 but also retarded the migration of S6 toward the cathode, demonstrating a net increase in phosphate incorporation into the S6 molecule compared to the unstimulated cells (Fig. 3, lane 2). TPA-treated cells showed very little of the least phosphorylated S6 derivative which was detected in control cells (Fig. 3, lane 3). Almost all of the $^{32}$P appeared to be incorporated into more highly phosphorylated derivatives of S6 in TPA-treated cells (Fig. 3, lane 2). At least two more highly phosphorylated derivatives of S6, which are absent in the control cells, can be detected in TPA-treated cells. These more highly phosphorylated derivatives of S6 probably are the triphosphorylated and tetraphosphorylated (and possibly the pentaphosphorylated) S6 derivatives (Fig. 3, compare lanes 2 and 3).

Time and Dose Dependence of S6 Phosphorylation in Response to TPA—The time course of S6 phosphorylation following addition of 1.6 $\mu$M TPA was studied in 3-day serum-starved H35 cells (Fig. 4). $^{32}$P, incorporation into ribosomal protein S6 can be detected as early as 5 min after the addition of TPA. Half-maximal $^{32}$P incorporation occurred at approximately 20-25 min and was maximal by 60 min following exposure to TPA. In the presence of TPA and $^{32}$P, S6 remained maximally phosphorylated for up to 90 min.

The effect of increasing concentrations of TPA upon ribosomal protein S6 was also measured in the H35 cells (Fig. 5). Intact cells were exposed to TPA in the concentration range of 1 x $10^{-8}$ to 3 x $10^{-6}$ M for 30 min. As little as 3 x $10^{-8}$ M TPA was capable of producing a 2-fold increase in the level of S6 phosphorylation. 1.6 $\mu$M TPA caused maximal phosphorylation of ribosomal protein S6 (approximately 5- to 7-fold in this experiment). The concentration of TPA which caused half-maximal S6 phosphorylation was estimated to be 0.14 $\mu$M (Fig. 5).

Structure-Activity Relationship between Tumor-promoting and Non-tumor-promoting Phorbol Esters and S6 Phosphorylation—The relative ability of a series of phorbol esters to cause S6 phosphorylation in intact H35 cells was investigated (Table I). Those phorbol esters having tumor-promoting activity in the mouse skin were also capable of causing S6 phosphorylation in H35 rat hepatoma cells. Weakly or non-tumor-promoting phorbol esters, 4-O-methyl TPA, phorbol 12,13-diacetate, and phorbol, were incapable of stimulating S6 phosphorylation at concentrations equivalent to the effective concentration of TPA (i.e., 1.6 $\mu$M). Thus the structural requirements of the phorbol diterpenes for tumor promotion in mouse skin are similar to those for S6 phosphorylation in this rat hepatoma cell line.

Cyclic AMP has been demonstrated to cause a partial increase in the absolute level of S6 phosphorylation in rat liver (45) and HeLa cells (46). S6 phosphorylation can be
FIG. 5. Effect of increasing concentrations of TPA on \(^{32}\)P\(_{\text{incorporation}}\) into ribosomal protein S6 by intact H35 cells.

Three-day serum-starved H35 cells were washed and placed in phosphate-free medium containing 20 \(\mu\)Ci/ml of \(^{32}\)P, for 2 h. After this time those concentrations of TPA indicated (ranging from 1.6 \(\times\) 10\(^{-8}\) to 3.2 \(\times\) 10\(^{-6}\) M) were added to the cells and incubated for 30 min. Samples were prepared, electrophoresed, and autoradiographed as described in the legend to Fig. 1. The resulting autoradiogram was analyzed by densitometry, and the band corresponding to ribosomal S6 was quantitated by integrating the densitometer tracing. Data points are the average of two experiments differing by less than 10%.

TABLE I

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Relative increase in (^{32})P(_{\text{incorporation}}) into ribosomal protein S6</th>
<th>Relative potency* in promoting tumorigenesis in mouse skin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>(1.0)</td>
<td>---</td>
</tr>
<tr>
<td>TPA (1.6 (\mu)M)</td>
<td>6.7</td>
<td>+++</td>
</tr>
<tr>
<td>Phorbol-12,13-dibutrate (1.6 (\mu)M)</td>
<td>4.2</td>
<td>+++</td>
</tr>
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<td>4-O-methyl-TPA (1.6 (\mu)M)</td>
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<td>inactive</td>
</tr>
<tr>
<td>Phorbol-12,13-diacetate (1.6 (\mu)M)</td>
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<td>inactive</td>
</tr>
<tr>
<td>Phorbol (1.6 (\mu)M)</td>
<td>0.9</td>
<td>inactive</td>
</tr>
<tr>
<td>8-Br-cAMP (1 mM)</td>
<td>1.4</td>
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* Data obtained from Ref. 44.

catalyzed by cyclic AMP-dependent protein kinase in vitro but only two substrate sites for this kinase appear to exist on the S6 molecule (36, 45). As shown in Table I, 8-Br-cAMP at a concentration of 1 mM fails to substantially increase the phosphorylation of S6 in comparison to TPA or insulin in serum-starved H35 cells, resulting in only a 40–50% increase above control values. This is dramatically less than the 6.7-fold increase in S6 phosphorylation caused by TPA in the same experiment (Table I).

Phosphoamino Acid Analysis of S6—To determine the amino acid residue that is phosphorylated in ribosomal protein S6 in response to TPA, 80 S ribosomes were isolated from cells treated with \(^{32}\)P, and 1.6 \(\mu\)M TPA. Extracted ribosomal proteins were subjected to SDS-polyacrylamide electrophoresis and the 32,000-dalton phosphorylated protein band corresponding to S6 was excised and subjected to acid hydrolysis as described under “Experimental Procedures.” As shown in Fig. 6, \(^{32}\)P-lysine was the only amino acid derivative detected in TPA-treated cells. No \(^{32}\)P-threonine was detected, and \(^{32}\)P-tyrosine, which has a smaller \(R_f\) value than phosphoserine, was also not detected.

DISCUSSION

The present study demonstrates that the tumor-promoting phorbol ester, TPA, stimulates the phosphorylation of the 40 S ribosomal protein, S6, in quiescent Reuber H35 cells. The retarded mobility of the S6 phosphoproteins toward the cathode during urea polyacrylamide gel electrophoresis indicates that the TPA-induced increase in \(^{32}\)P incorporation into S6 is due to a net increase in the number of phosphates incorporated per S6 molecule. Only those phorbol-ester derivatives which were capable of promoting the formation of tumors in the mouse skin system led to an increased phosphorylation of S6 (Table I) suggesting that the covalent modification of this protein may have some relationship to the process of tumor promotion (see below). The 50% effective concentration for TPA in causing phosphorylation of S6 (0.14 \(\mu\)M, Fig. 5) in the H35 cells was similar to the amount required to cause a half-maximal increase in ornithine decarboxylase activity and polyamine biosynthesis (29) which has also been associated with the process of tumor promotion in the mouse skin. The rapidity with which phosphorylation of the S6...
molecule occurs in response to TPA (e.g., 2-fold increase in 5 min, Fig. 4) indicates that tumor-promoting phorbol esters are capable of rapidly affecting the phosphorylation activity of cells and supports the notion that protein phosphorylation is an important early mechanism of phorbol ester action.

The role of S6 phosphorylation in ribosomal function is unknown (27). A number of mitogenic agents such as epidermal growth factor, insulin, and insulin-like growth factor have been shown to stimulate S6 phosphorylation when added to quiescent cell cultures (27). It has been suggested that phosphorylation of S6 may be required for the transition of serum-deprived cells into G1 phase of the cell cycle (28). Also, it has been demonstrated that chicken embryo fibroblasts transformed by avian sarcoma virus differ from their nontransformed counterparts by being capable of phosphorylating S6 in the absence of serum. The ability of avian sarcoma virus-transformed cells to circumvent a normal cellular control mechanism by phosphorylating S6 independently of serum addition could be a factor contributing to their altered growth characteristics (47). The ability of TPA to cause S6 phosphorylation in the absence of serum mimics transformation in vitro (36). Consequently TPA-induced S6 phosphorylation could be partially mediated by cyclic AMP-dependent protein kinase. However, since cyclic AMP-dependent protein kinase can account for only a small percentage of the TPA-induced S6 phosphorylation, TPA may cause activation of another protein kinase which phosphorylates S6.

It has recently been reported by Castagna et al. (31) that phorbol ester tumor promoters can directly activate a partially purified phospholipid-dependent protein kinase (protein kinase C) in vitro, and have suggested that protein kinase C may be a membrane target for action of phorbol ester tumor promoters. Further support for this idea has been reported by Niedel et al. (32) who have demonstrated that the phorbol diester receptor from rat brain can be phosphorylated by cyclic AMP-dependent protein kinase. S6 is the substrate for protein kinase C.

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