Specific Stimulation by Phorbol Esters of the Phosphorylation of Histones H2B and H4 in Murine Lymphocytes

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ABSTRACT

The effect of phorbol diesters on histone phosphorylation in BALB/c mouse lymphocytes, cells which do not respond to these agents with cell division, but with other biochemical and biological changes, was investigated. A technique for fractionating the proteins was used which was more powerful than those used previously in similar studies of phorbol diester effects on the metabolism of these proteins. Exposure of lymphocytes to tumor-promoting phorbol esters resulted in a rapid and specific increase in phosphorylation of the nuclear histone proteins H2B and H4. Within 2 hr, the phosphorylation of these two proteins rose to levels 6- to 8- and 2- to 4-fold greater, respectively, than those in control cells, when lymphocytes were exposed to 800 nm 12-O-tetradecanoylphorbol-13-acetate. Lower levels were observed with other phorbol analogues commensurate with their relative tumor-promoting abilities. Lymphocyte mitogens did not increase phosphorylation under the conditions used. The potential ability of the cell system used for defining early in vivo and in vitro phorbol diester effects, and those which are independent of cell division, is discussed.

INTRODUCTION

Multistage models of chemical carcinogenesis have been described for many tissues. In the mouse skin, papilloma and subsequently carcinoma formation may be affected by certain phorbol diesters, such as 12-O-tetradecanoylphorbol-13-acetate TPA, when applied repeatedly after a single subeffective dose of a complete carcinogen. An obstacle to understanding the events occurring in the later (tumor promotion) stages of carcinogenesis completed by TPA has been the complexity of the epidermal response to this type of agent (5). In particular, cellular biochemical changes specific to promotion have been difficult to identify due to concurrent induction of cell proliferation and inflammation, events which appear necessary, but possibly insufficient for tumorigenesis.

Over the last few years, we have been investigating the responses to phorbol esters of BALB/c mouse splenocytes, which are quiescent cells (48) containing greater than 90% of their nucleated cells as lymphocytes. We reported previously that these agents do not precipitate cell division in this cell type in the absence of lymphocyte mitogens (11). Phorbol esters have differentiation-modulating and other effects on many cell types (24, 43, 52), including lymphocytes (4, 9, 27), and cells of this type have also been shown to exhibit several responses which are also expressed following topical application of these agents to mouse skin. The former do not respond with cell division, however. Examples of positive responses include induction of ornithine decarboxylase activity (18, 31), stimulation of phospholipid metabolism (28, 38), histone phosphorylation (3, 23, 36), and cyclic nucleotide concentrations (5, 10). The first phenomenon is inhibited in both systems by retinoids, moreover (18). In addition, splenocytes (42) and other lymphocytic cells (13, 39) have a high density of specific phorbol diester receptors, identifiable with protein kinase C (2, 8, 19, 22, 29, 40, 51).

One aspect of the activity of phorbol diesters proposed in vivo, as well as in vitro, has been modulation of gene expression (5, 7, 17, 24, 35). A biochemical process which has been implied to play a role in gene expression in several systems is phosphorylation of the histone nuclear proteins (6, 12, 14, 25, 33, 34). We, therefore, wished to determine if this process played a role in phorbol diester activity in vitro and eventually in vivo. It has been well established previously that phosphorylation of specific amino acid residues on certain histones is associated with specific cell cycle-related events. Studies on phosphorylation which relate specifically to changes in gene expression must therefore be done in a nondividing system, a requirement which is met by BALB/c splenocyte cultures. Previous studies on phorbol ester-induced changes in histone phosphorylation have been limited by inadequate methods for separation of individual histone protein species. Therefore, we set out to examine phorbol ester effects using a high-resolution 2-dimensional gel electrophoresis method recently established in our laboratory.

MATERIALS AND METHODS

Reagents. The phorbol esters TPA, phorbol-12,13-didecanoate, phorbol-12,13-dibenzoate, and 4-O-methyl TPA were purchased from CRC, Eden Prairie, MN. Pokeweed mitogen was obtained from Grand Island Biological Company (Grand Island, NY). Carrier-free 32P, was obtained from either New England Nuclear (Boston, MA) or ICN Radiochemical Division (Irvine, CA).

Cell Culture. Cells were cultured in RPMI Medium 1640 (Grand Island Biological Co.) which was supplemented with sodium bicarbonate (0.2%) and gentamicin (50 mg/liter) (Garamycin; Schering Corp., Kenilworth, NJ). Fetal bovine serum (Reheis Chemical Co., Phoenix, AZ, or MA Bioproducts, Walkersville, MD) was added to a final concentration of 10%. Phosphate-free RPMI Medium 1640 was prepared according to the same formula as that supplied by Grand Island Biological Co., except that phosphate salts were omitted. Salts were all reagent grade, and the amino acids were obtained from Sigma Chemical Co., St. Louis, MO. Spleens were removed from 9- to 17-week-old female BALB/c mice (Charles River Breeding Laboratories, Inc., Wilmington, MA). A cell suspension was made either by teasing the spleens apart with forceps or by pressing them through a No. 80 mesh stainless-steel screen. The resulting suspension was allowed to stand for 5 min in a test tube to allow large particles to settle and then centrifuged (5 min,
1200 x g, 20°), and the pellet was resuspended in 5 ml of medium. The number of cells in this suspension was determined using a hemocytometer, and cell viability was assessed by trypan blue exclusion. Viability was always greater than 95%. The suspension was diluted to the appropriate cell concentration by the addition of medium, and an aliquot added to polystyrene tubes to produce a final concentration of 2 x 10^5 cells/ml in a total volume of 0.5 ml. 32P, was added to a final concentration of 500 μCi/ml. For the majority of experiments, TPA and radiolabel were added concurrently; however, for those in which TPA was compared to pokeweed mitogen, 2 x 10^5 cells, along with the test agent, were placed in 35-mm plastic Petri dishes in a final volume of 2 ml of medium. At the end of the incubation period, the cells were scraped from the plates using a Teflon policeman, transferred to plastic tubes, and centrifuged at 1200 x g for 5 min at 20°. The cell pellet was resuspended in 0.5 ml of medium containing 32P, (500 μCi/ml). All incubations were carried out in an incubator at 37° in a humidified atmosphere of 7% carbon dioxide in air.

At the end of the incubation with 32P, 1 ml of ice-cold PBS was added to the tubes, which were then centrifuged (1200 x g, 5 min, 0°). The cells were washed twice more by resuspending them in 1 ml of PBS and centrifuging under the same conditions. The supernatant was discarded, and either the pellet of cells was frozen at -20° or the nuclei were isolated directly.

**Preparation of Nuclei and Extraction of Histones.** The following procedures were conducted at 0°. The cell pellet was suspended in 1 ml of a sucrose buffer containing 5 mM Tris, 250 mM sucrose, 3 mM calcium chloride, 45 mM Tris-HCl, and 50 mM SDS-Triton (containing 1% Triton X-100) and homogenized with 10 strokes in a 7-ml Pyrex ground-glass tissue homogenizer in order to break up cell aggregates. In order to burst the plasma membranes, the suspension was drawn in and out of a 25-gauge needle 4 times (20). The nuclei were then pelleted by centrifugation (1200 x g, 5 min), resuspended in 1 ml of SBS by vortexing, and then repelletted (1200 x g, 5 min). The resulting pellet was resuspended in 1 ml of SBS containing 30 mM sodium chloride and 20 mM EDTA and centrifuged (2000 x g, 5 min). The histones were extracted twice in 0.25 N HCl, first with 1 ml for 30 min, followed by centrifugation (2000 x g, 10 min), then with 0.5 ml for 10 min. The histones were precipitated from the combined extracts by adding 0.5 ml of 100% trichloroacetic acid and allowing the mixture to stand overnight at 4°. The precipitate was recovered by centrifugation (2000 x g, 10 min), first washed with 1 ml of freshly prepared 1% HCl (v/v) in acetone, and then washed twice more with acetone, and the histones were dried under a stream of nitrogen.

**Gel Electrophoresis.** A modification of a 2-dimensional system of polyacrylamide gel electrophoresis described by Hardison and Chalkley (16) was used to fractionate the histones. The gel used in the first dimension contained 0.9 M acetic acid, 2.5 M urea, 15% acrylamide, 0.09% w acetic acid, 2.5 M urea, 15% acrylamide, 0.09% N,N',N''-tetramethylethylendiamine and was poured as a slab (14.5 x 10 x 1.2 mm). Both the upper and lower electrophoresis buffers consisted of 0.9% acetic acid. Following polymerization, the gel was preelectrophoresed at 70 V for about 15 hr, the gel stained with Coomassie blue for 30 min at 37°, and then destained in 1 part methanol/1 part 7% acetic acid. Radioactivity was detected either by autoradiography or liquid scintillation counting as specified in the text. Autoradiography was performed by first drying the gel onto Whatman 3MM Chromatography Paper with heat under a vacuum. The gel was placed in contact with a piece of Kodak X-Omat-AR film and a Dupont Cronex-lighting-plus intensifying screen. Exposure was carried out at -70° for an appropriate length of time. Liquid scintillation spectrometry was carried out by cutting the stained spots from the gel, which had been frozen on dry ice, placing them in 4.5 ml of ScintiVerse liquid scintillation cocktail (Fisher Scientific Co., Pittsburgh, PA), and counting for 20 min. Protein was quantified using the method of Lowry et al. (26) modified for small volumes of protein solution, with calf thymus core histone as the protein standard. Each experiment shown was representative of several run under identical conditions. Values shown were the average of at least duplicates, and varied by 15% or less from respective means.

**RESULTS**

In preliminary experiments using only one-dimensional acid-urea polyacrylamide gels for histone fractionation, we had found an apparent increase in the phosphorylation of histone H2B following a 2-hr treatment of splenocyte cultures with TPA* (1600 nm). Highly phosphorylated histone H2A can comigrate with histone H2B in this gel system (15, 35), however. In order to confirm the identity of this phosphorylated species, the histones were therefore separated using 2-dimensional gels, as described in "Materials and Methods." This method provided an excellent separation of all 5 of the major groups of histones, as well as separating subtypes of histones H2A, H3, and H2B (Fig. 1). The stained proteins were identified in the gel using 3 criteria. The first was the characteristic pattern of migration in the first-dimension gel. The second was the use of individual mouse spleen histones purified by the method of Oliver et al. (32). The identity of H2B was further firmly established by a characteristic change in mobility in the first-dimension on changing the urea concentration in the gel (16). An autoradiogram of histones run on this 2-dimensional gel system showed clearly that histone H2B was phosphorylated in cells given a 2-hr treatment with TPA (1600 nm) (Fig. 1). A stimulation of H4 phosphorylation was also observed. Treatment of the histones with alkaline phosphatase liberated the radiolabel, indicating that the nature of the bound radioactivity was a phosphate ester. Analysis of the phosphoamino acids by paper electrophoresis (47) of an acid hydrolysate of unfractionated histone revealed the presence of phosphoserine and phosphothreonine but not phosphotyrosine (data not shown). Since test agents were found in subsequent experiments to affect the subtypes in the same qualitative and quantitative manner, the radioincorporation data for the subtypes of histones H3, H2A, and H2B were combined to give a single value for each overall histone type.

Treatment of cell cultures for 2 hr with increasing concentrations of TPA resulted in a dose-dependent stimulation of the phosphorylation of histones H2B and H4 (Chart 1). At a 1600 nm concentration, the stimulation of H2B was 667% and the stimulation of H4 was 247% of the controls. The phosphorylation of the other histones was affected to a much lesser degree, with changes of no more than 62% compared to controls being observed. Similar findings were made over a series of 20 experiments.

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STIMULATION OF HISTONE PHOSPHORYLATION BY PHORBOL DIESTERS

Chart 1. Effect of TPA concentration on histone phosphorylation. Spleen cell suspensions (4 x 10^7 lymphocytes/ml) were incubated in the presence of MP and increasing concentrations of TPA for 2 hr. Each point shows the mean of duplicate cultures. The average incorporations of radiolabel (cpm/mg ± S.E.) for controls were: H1, 5840 ± 300; H3, 3140 ± 240; H2A, 5220 ± 660; H2B, 280 ± 80; and H4, 320 ± 0.

Table 1

<table>
<thead>
<tr>
<th>Histone fraction</th>
<th>4-O-MeTPA</th>
<th>PDB</th>
<th>PDD</th>
<th>TPA</th>
</tr>
</thead>
<tbody>
<tr>
<td>H1</td>
<td>156</td>
<td>146</td>
<td>143</td>
<td>120</td>
</tr>
<tr>
<td>H3</td>
<td>155</td>
<td>184</td>
<td>172</td>
<td>106</td>
</tr>
<tr>
<td>H2A</td>
<td>143</td>
<td>92</td>
<td>97</td>
<td>64</td>
</tr>
<tr>
<td>H2B</td>
<td>167</td>
<td>233</td>
<td>320</td>
<td>880</td>
</tr>
<tr>
<td>H4</td>
<td>121</td>
<td>186</td>
<td>200</td>
<td>214</td>
</tr>
</tbody>
</table>

*4-O-MeTPA, 4-O-methyl TPA; PDB, phorbol-12,13-dibenzoate; PDD, phorbol-12,13-didecanoate.

Programs. A series of phorbol esters with different abilities to promote tumors in mouse skin was tested to examine the relationship between tumor promotion in vivo and histone phosphorylation in vitro. For the compounds (1600 nm) which were tested (TPA, a potent tumor promoter; phorbol-12,13-didecanoate, a moderate promoter; phorbol-12,13-dibenzoate, a weak promoter; and 4-O-methyl TPA, a nonpromoter) the in vitro phosphorylation of histone H2B correlated well with promoting ability (Table 1). The changes in H4 phosphorylation followed those of H2B, but the stimulation relative to controls was much less. Only small, inconsistent changes were observed in the phosphorylation of the other histones, although phosphorylation of histone H2A was found to decrease somewhat in some experiments. Results shown are from a single experiment, although the same qualitative findings were made from several repetitions of this study.

Because the stimulation of H2B and H4 phosphorylation was quite evident after 2 hr of treatment, we wished to see how rapidly the change took place. In order to observe responses after short treatments, the labeling protocol was modified. In this way, qualitatively similar, but quantitatively larger effects were observed. In these experiments, the cells were preincubated for 1 hr in phosphate-free medium containing ^32P (500 μCi/ml) prior to the addition of TPA (800 nm) or solvent alone, and the incubation was terminated by the addition of 2 volumes of ice-cold PBS after 10, 20, 45, or 120 min. Chart 2 shows that TPA increased the phosphorylation of histones H2B and H4 after only a 10-min exposure. Consistent with other experiments, the response for H2B was stronger than that for H4. Over 3 replicates of the same experiment, similar qualitative results were obtained.

In order to compare the effects of TPA with a lymphocyte mitogen, we examined the effects of pokeweed mitogen, an agent which is active toward both B and T lymphocytes. The concentration used stimulated the uptake of ^3H]thymidine to value 6-fold that of controls after 48 hr of treatment under these culture conditions (data not shown). Compared with TPA, a 2-hr treatment with this agent produced no substantial changes in the phosphorylation of any of the histones (Chart 3). There was no detectable phosphorylation of H4, furthermore. In an experiment where incorporation of radiolabel was determined using autoradiography, the T-lymphocyte mitogen phytohemagglutinin likewise did not stimulate the phosphorylation of either H2B or H4 (data not shown).

DISCUSSION

The pattern of histone phosphorylation in untreated spleen cells was found in this study to be quite similar to that observed

Chart 2. Effect of brief treatment with TPA on H2B and H4 phosphorylation. Cell suspensions (4 x 10^7 lymphocytes/ml) were incubated for 1 hr in phosphate-free medium containing ^32P (500 μCi/ml). TPA (800 nm) or solvent alone was then added, and the cells were harvested after 10, 20, 45, or 120 min of treatment. O, control; x, TPA. S.E.s were within 15% of values indicated within experiments, a representative example of which is depicted.
STIMULATION OF HISTONE PHOSPHORYLATION BY PHORBOL DIESTERS

Chart 3. Effect of treatment with TPA or pokeweed mitogen on histone phosphorylation. Spleen cell cultures (1 x 10⁷ lymphocytes/ml) were incubated for 2 hr with either TPA (800 µg) or pokeweed mitogen (PWM) (10 µg stock/ml). The cells were then collected, resuspended in medium containing [³²P], and incubated for 2 more hours. Bars, average of duplicate cultures ± S.E.

in other cells in culture and in various tissues. There was a high degree of phosphorylation of H1 and H2A, moderate phosphorylation of H3, and a low degree of phosphorylation of H2B and H4. Following treatment with the potent tumor promoter TPA, the most striking change was a rapid stimulation of H2B phosphorylation. This increase brought the level of H2B phosphorylation up to that observed for H3 in control cultures. Critical to these analyses was the clear separation of H2B from the other histones using 2-dimensional polyacrylamide gel electrophoresis. Other more commonly used methods for histone fractionation, such as one-dimensional acid-area polyacrylamide gel electrophoresis were found not to clearly separate H2B from the other histones, making quantitation of changes in its degree of phosphorylation difficult. TPA treatment also increased H4 phosphorylation, although to a lesser extent compared to H2B. The phosphorylation of histones H1, H3, and H2A exhibited only small changes in response to tumor promoter treatment. This lack of substantial stimulation provided indirect evidence that the phosphorylation of H1 and H3 is related to chromatin condensation. H2A phosphorylation is believed to be involved in gene regulation (33). Little is known concerning the biological significance of H2B or H4 phosphorylation, however; in trout testis, only 5% of the H2B histones are phosphorylated, although 30 to 40% of the H4 histones contain bound phosphate (25). Relatively low amounts of H2B and H4 phosphorylation have been reported in rat liver (12), mouse uterus (41), and the protozoan Tetrahymena thermophila (1). These results agree with our finding that the extent of H2B or H4 phosphorylation is in general low in untreated cells. Neither partial hepatectomy (12) nor induction of the uterine decidual cell reaction (41), treatments which stimulate cell division, were reported to cause an increase in H2B or H4 phosphorylation. Consistent with these observations, we have found that treatment of splenocyte cultures with pokeweed mitogen did not increase the phosphorylation of H2B or H4. The stimulation of H2B and H4 phosphorylation by TPA occurred in nondividing cells, moreover, indicating that the phosphorylation of H2B and H4 was not involved in cell division-related events. In contrast, the low level of H2B and H4 phosphorylation in Tetrahymena was associated with the mitotically active but transcriptionally inactive micronuclei, suggesting a role in cell division-related events (1) in this simple eukaryote. In vitro studies of histone-histone interactions have demonstrated that the phosphorylation of H2B decreases its binding affinity for H4 and H2A (45). It is possible that H2B phosphorylation may therefore alter chromatin structure and, like that of H2A, affect gene expression.

Active phorbol esters elicit a wide range of biochemical and biological responses reflecting modulation of gene expression in lymphocyte and other cell types, and many of these appear related to changes induced by the same agents following topical application to mouse skin. The specific histone modifications described herein appear not merely to be involved in events related to cell division. Their further study may therefore be...
valuable in elucidating the mechanism of phorbol diester effects on gene expression in susceptible cells in vivo and in vitro.

REFERENCES


Fig. 1. Fractionation of histones using 2-dimensional polyacrylamide gel electrophoresis. Spleen cell suspensions (2 × 10^7 lymphocytes/ml) were incubated for 2 hr with ^32P, and either TPA or solvent alone. Equal amounts of protein from control and TPA-treated cells were added to the gels.
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