Specific High-Affinity Binding of the Phorbol Ester Tumor Promoter 12-O-Tetradecanoylphorbol-13-acetate to Isolated Nuclei and Nuclear Macromolecules in Mouse Epidermis

Frank W. Perrella, Curtis L. Ashendel, and R. K. Boutwell

ABSTRACT

The tumor promoter 12-O-tetradecanoylphorbol-13-acetate (TPA) binds reversibly and with high affinity and specificity to nuclear macromolecules in mouse epidermis. The dissociation constants determined from Scatchard analysis of epidermal nuclei and nuclear macromolecules are 3.58 ± 0.66 (S.E.) and 2.18 ± 0.54 nm, respectively. The solubilization of TPA receptors from epidermal nuclei by DNase I was examined. Following a 20-min digestion at 22°C, more than a 2-fold increase in specific TPA binding was observed in the supernatant relative to non-nuclease-treated nuclei (0.71 versus 0.32 pmol/mg protein, respectively). Our data indicate that epidermal nuclei contain saturable and specific TPA-binding macromolecules and that these binding components may be associated with regions of chromatin that are preferentially susceptible to nuclease cleavage. These data suggest the existence of nuclear receptors for the phorbol ester tumor promoters. These observations may necessitate a more critical assessment of plasma membrane binding as the sole binding site responsible for triggering the multistep process of tumor promotion in mouse epidermis.

INTRODUCTION

It is established that the carcinogenic process in experimental animals is composed of multiple stages (45). The 2-stage mouse skin carcinogenesis system demonstrates that at least 2 separate phases of carcinogenesis exist, initiation and promotion (7, 43, 45). Fundamental to the prevention of cancer, therefore, must be an understanding of the biochemical mechanisms which underlie the promotion process.

Tumor promoters produce changes in mouse epidermis and cell culture that alter differentiation and development (1, 5, 7, 13, 22, 34–37). The identification of specific phorbol ester receptors in target tissues is necessary, therefore, to understand the biochemical mechanisms which link cellular differentiation with tumor promotion. It is believed that phorbol esters exert part of their promoting activities by interacting with receptors in the cell membrane (5, 7, 13, 40, 46). The most active phorbol ester is TPA. TPA is highly lipophilic and tends to penetrate the plasma membrane, making it a likely candidate for interactions with intracellular binding proteins (5, 13, 20, 42). Since tumor promoters appear to be involved in gene transcription, it is conceivable that nuclear receptors may exist for TPA. The subcellular fractionation of mouse epidermis following in vivo exposure to radioactive phorbol esters revealed that a significant portion of the recovered activity was associated with the nuclear fraction (5, 20, 42). We report here that epidermal nuclei contain specific high-affinity binding sites for TPA, and we propose a nuclear site of action for the phorbol ester tumor promoters in addition to the plasma membrane (3–5, 12, 40, 46) and sera sites (41).

MATERIALS AND METHODS

Materials. Female CD-1 mice from Charles River Breeding Laboratories, Wilmington, Mass., were used throughout. [3H]TPA (specific activity, 17.2 Ci/mmol) was purchased from New England Nuclear, Boston, Mass. TPA was purchased from Lifesystems, Newton, Mass., and Chemical Carcinogenesis, Eden Prairie, Minn. PMSF and cetylpyridinium chloride were purchased from Calbiochem-Behring Corp., La Jolla, Calif. Hexylene glycol was purchased from Eastman Kodak Co., Rochester, N. Y. Nudit cream hair remover was purchased from Helena Rubinstein, Inc., New York, N. Y. DNase I (electrophoretically pure) was purchased from Sigma Chemical Co., St. Louis, Mo.

Isolation of Mouse Epidermal Nuclei. Epidermal preparations were prepared from the skins of shaved female CD-1 mice. The mice were killed by cervical dislocation, and the hair was removed from their backs with Nudit cream hair remover. The epidermis was prepared by scraping the skins at 0°C with a razor blade. All subsequent procedures were performed at 0–4°C. Epidermal tissue was homogenized in volumes of 1 mM N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid:1 mM CaCl2:0.5 mM hexylene glycol:0.025% cetylpyridinium chloride:0.2 mM PMSF (pH 7.0) by a 5-sec burst of a Polytron homogenizer (Setting 6) followed by 20 strokes in a Dounce homogenizer. The homogenate was filtered through 8 layers of surgical gauge and centrifuged at 1000 x g for 10 min. The crude nuclear pellet was washed twice by gentle homogenization in a Dounce homogenizer in 0.1 mM N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid:1 mM CaCl2:0.5 mM hexylene glycol (pH 7.0) and centrifuging at 1000 x g for 10 min. Following resuspension by homogenizing in 1 mM sucrose:0.5 mM CaCl2:0.5 mM hexylene glycol, the nuclear suspension was sedimented by centrifugation at 1500 x g for 20 min. The nuclear pellet was then resuspended by homogenization in a Dounce homogenizer in 1.5 mM sucrose:0.75 mM CaCl2 and centrifuged for 60 min in a Beckman SW41 rotor at 15,000 rpm. The supernatant was decanted, and the purified nuclear preparation was washed once by centrifugation through 1.0 M sucrose:0.5 mM MgCl2:0.2 mM PMSF (pH 7.4) for 60 min at 0°C. The recovered pellet following centrifugation at 12,000 x g for 10 min was referred to as residual chromatin.

TPA Binding Assay. [3H]TPA was dissolved in dimethyl sulfoxide with or without a 500-fold excess of unlabeled TPA just before use. The binding studies were carried out in dichlorodimethylsilane-treated glass tubes using the cold-acetone filter assay of Ashendel and Bou-
Enzyme Assays. All preparations to be assayed for enzyme activity were added to the assay medium in 0.1 ml of homogenizing buffer to give the final volumes indicated. All phosphohydrolases were assayed at 37° in a total volume of 250 μL. The reactions were stopped after 15 min by the addition of perchloric acid (0.5 M final concentration), centrifuged at 8000 × g for 5 min, and neutralized with potassium hydroxide (0.5 M final concentration). After centrifugation (8000 × g, 5 min), an aliquot of the supernatant was assayed for Pi by the method of Hess and Derr (21). The glucose-6-phosphatase assay medium contained 50 mM imidazole (pH 6.6), 10 mM 2-mercaptoethanol, and 10 mM glucose-6-phosphate. The ADPase assay medium contained 30 mM Tris-HCl (pH 7.5), 9 mM MgCl₂, 1.2 mM EDTA, 2.4 mM 2-mercaptoethanol, and 6 mM ADP. The alkaline phosphomonoesterase assay medium contained 30 mM Tris-HCl (pH 8.5), 9 mM MgCl₂, 1.2 mM EDTA, 2.4 mM 2-mercaptoethanol, and 6 mM AMP. Leucine aminopeptidase was assayed at 37° in a total volume of 0.5 ml. The reaction was stopped after 15 min by the addition of HClO₄ (0.5 M final concentration). After centrifugation (8000 × g, 5 min), 0.5 ml of 2 M Tris-HCl (pH 9.0) was added followed by 3 ml of toluene. The reaction tubes were vigorously vortexed for 1 min in order to extract the p-nitroaniline. An aliquot of the extract was then assayed for p-nitroaniline by the method of Nakagawa et al. (31). The assay medium contained 70 mM phosphate buffer (pH 7.2), 4.2 mM 2-mercaptoethanol, and 10 mM leucyl-nitroanilide.

Assay for Protein, DNA, and RNA. Protein, DNA, and RNA were quantitated by the methods of Lowry et al. (27), Burton (8), and Schneider (38), respectively.

RESULTS

The characterization of specific TPA binding to nuclear components was investigated using nuclei isolated by a modification of the aqueous hexylene glycol technique of Wray and Stubblefield (53, 54). Hexylene glycol stabilizes the nuclear membrane allowing the removal of adhering cytoplasmic material by shearing forces during homogenization. Furthermore, the inclusion of the cationic detergent cetylpyridinium chloride facilitates both the disruption of plasma membranes and the removal of adherent cytoplasm (32). The RNA:DNA ratio, a criterion often used for testing the purity of nuclear preparations, was 0.31 in the nuclei prepared from epidermis. This value is slightly higher than that observed in nuclei prepared from other tissues (9, 53). This is suggestive of possible cytoplasmic contamination. However, since the nucleus contains only about 6 to 10% of the RNA of the cell, even a relatively small cytoplasmic contamination will cause a significant increase in the RNA:DNA ratio of the nuclear preparation. It must be borne in mind, however, that cellular RNA is not a constant component and may vary from tissue to tissue.

Mouse epidermal nuclei were further characterized by measuring the activities of intracellular enzyme markers. The activity of several marker enzymes in total tissue homogenates and in nuclei, together with the percentage of the activity in the homogenate found in the nuclear fraction, is shown in Table 1. The low level of glucose-6-phosphatase activity in the nuclear fraction suggests that there was very little contamination of isolated nuclei by microsomal material. The activities of 3 other enzymes that have been proposed as plasma membrane markers were determined, anticipating that they would be useful in determining the extent of plasma membrane contamination in the nuclear fraction (Table 2). The marker enzymes used for the identification of plasma membranes were ADPase (33, 51), leucine aminopeptidase (17, 47), and alkaline phosphomonoesterase (16, 47, 50). The percentage of membrane marker activity identifiable in the nuclear fraction was between 3 and 7% of that found in the homogenate. However, ADPase and glucose-6-phosphatase are also localized in the nuclear envelope (19). Therefore, the level of membrane contamination in the nuclear preparation would appear to be at a minimum. Accepting these functional criteria as a means of assessing the purity of the nuclear preparation, the role of the nucleus in the specific binding of phorbol ester tumor promoters was studied.

Isolated epidermal nuclei were extracted with 0.35 M NaCl:10 mM Tris-0.2 mM MgCl₂:0.2 mM PMSF (pH 7.4), and the extract was assayed for [³H]TPA binding (Chart 1). The competition of radioactive TPA with a 500-fold excess of unlabeled TPA at each [³H]TPA concentration allowed an estimate of the nonspecific binding. The nonspecific binding was subtracted from the total binding to give the level of specifically bound TPA. The term "specific binding" was defined by having the properties of high-affinity and low-capacity TPA binding of a saturable nature. The specific binding of [³H]TPA to the nuclear extract gave a linear response for the soluble nuclear proteins (Chart 1, inset).

The binding of [³H]TPA (10 to 15 nM) to the nuclear protein extract was very rapid at 0°, giving a t₁/₂ of association of approximately 5 min (Chart 2A). The displacement of specifically bound [³H]TPA from the nuclear protein extract by the addition of a 500-fold excess of unlabeled TPA at equilibrium gave an approximate dissociation t₁/₂ of 9 min at 0° (Chart 2B). The reversibility of TPA binding at 0°was observed following a 1-hr incubation with 10 nM [³H]TPA. When a 500-fold excess of unlabeled TPA was added and the incubation was continued

### Table 1

<table>
<thead>
<tr>
<th>Component</th>
<th>Glucose-6-phosphatase</th>
<th>ADPase</th>
<th>Leucine aminopeptidase</th>
<th>Alkaline phosphomonoesterase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homogenate</td>
<td>15.2 (100)</td>
<td>174.9 (100)</td>
<td>192.1 (100)</td>
<td>7.3 (100)</td>
</tr>
<tr>
<td>Nuclei</td>
<td>0.43 (2.8)</td>
<td>11.5 (6.6)</td>
<td>11.3 (5.9)</td>
<td>0.22 (3.0)</td>
</tr>
</tbody>
</table>

*Enzyme activities were measured in filtered epidermal homogenates and in suspensions of purified nuclei from the same homogenates (μmol/15 min/100 mouse epidermal homogenate).

**Numbers in parentheses, percentage.

Enzyme activities were adjusted for 100% yield.

### Table 2

<table>
<thead>
<tr>
<th>Component</th>
<th>pmol/mg protein</th>
<th>pmol/100 mouse epidermis</th>
<th>Molecules x 10⁻³</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homogenate</td>
<td>2.07</td>
<td>252</td>
<td>194/cell</td>
</tr>
<tr>
<td>Nuclei</td>
<td>0.53</td>
<td>23</td>
<td>18/nucleus</td>
</tr>
</tbody>
</table>

*Adjusted through gauze.

**Adjusted for 100% yield.
Chart 1. Effect of [3H]TPA concentration on binding to epidermal nuclear extract and [3H]TPA binding to increasing amounts of nuclear extract (inset). Nuclear extract (100 μg/ml) was incubated with [3H]TPA ± 500-fold excess of unlabeled TPA for 2 hr at 0° in 20 mM Tris, pH 7.4, containing 2 mM 2-mercaptoethanol to determine specific binding activity. Specific TPA binding to different amounts of nuclear extract (inset) containing a fixed amount of [3H]TPA (10 nM) was determined similarly. Details of the TPA-binding assay are described in “Materials and Methods.” Data are presented as independent determinations of duplicate experiments using 100 mice. •, total TPA binding; ■, specific TPA binding; ▲, nonspecific TPA binding.

Chart 2. Time course of association and reversibility of TPA binding to nuclear extract. The nuclear extract was prepared, and TPA binding was assayed as described in “Materials and Methods.” The nuclear extract (80 μg/ml) was incubated with 15 nM [3H]TPA at 0° for different periods of time to determine the association time of total [3H]TPA binding (A). The addition of 7.5 μM unlabeled TPA at the time of equilibrium allowed an estimate of the dissociation of specific [3H]TPA binding for a further 60 min (B).

for 1 hr longer, the displacement of bound [3H]TPA gave an approximate value for the nonspecific binding of 20% (data not shown).

Scatchard analyses of the affinity and capacity of specific [3H]TPA binding to epidermal nuclei, nuclear protein extract, and extracted nuclei (residual chromatin) are presented in Chart 3. When the binding of [3H]TPA was measured for all 3 nuclear fractions, it was found that there exist 2 major classes of binding sites, those that have a high affinity and low capacity (nuclei and nuclear protein extract) and those that have very low affinity and are difficult to saturate (residual chromatin). The data show that most of the high-affinity binding components are extractable from nuclei.

Scatchard analysis gave values for the equilibrium dissociation constant (Kd) of 3.58 ± 0.66 (S.E.) nM for nuclei and 2.18 ± 0.54 nM for the nuclear extract. The TPA-binding capacity for the same 2 fractions was 0.53 ± 0.17 and 1.21 ± 0.24 pmol/mg protein, respectively. The nuclear extract showed an enrichment in the specific activity of TPA binding relative to nuclei, a result compatible with the selective release of TPA-binding components into the extract. Moreover, we estimated that the number of TPA-binding sites per epidermal nucleus was of the order of 18,000 (Table 2). The nucleus, therefore, represents approximately 9% of the specific TPA binding of the cell.

The specificity of the nuclear extract for [3H]TPA binding was demonstrated by the competition of unlabeled TPA and PDBU, a very weak tumor promoter in mouse epidermis, for [3H]TPA-binding sites (Chart 4). Although the slopes of the dose-response curves for the 2 phorbol esters were similar, the molar concentration of PDBU that produced a 50% inhibition of binding was approximately 67-fold higher. This relationship is comparable to data showing that TPA is 72- and 194-fold more active as a tumor promoter in mouse epidermis than is PDBU (39, 48).

The use of nucleolytic digestion of isolated nuclei was used to assess the nuclear origin of TPA-binding activity (Table 3). Epidermal nuclei were incubated in the presence or absence of DNase I (960 units/3.6 x 10⁷ nuclei) for 20 min at 22°.
Following centrifugation, the supernatants (S₁) of the control and DNase I-treated nuclei were assayed for specific TPA binding. The nuclear pellets were extracted with 0.35 M NaCl:10 mM Tris (pH 7.4):0.2 mM MgCl₂:0.5 mM PMSF for 20 min at 0°C and assayed for specific TPA-binding activity following centrifugation at 1000 x g for 10 min (4°). The data represent the average of 2 independent experiments using 90 mice each.

### Table 3

<table>
<thead>
<tr>
<th></th>
<th>S₁</th>
<th>S₂</th>
<th>% of control specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control nuclei</td>
<td>0.32</td>
<td>0.57</td>
<td>100</td>
</tr>
<tr>
<td>DNase I-treated nuclei</td>
<td>0.71</td>
<td>0.74</td>
<td>163</td>
</tr>
</tbody>
</table>

Following centrifugation, the supernatants (S₁) of the control nuclei sample following termination served as the control. The pellets from control and treated nuclei were extracted with 0.35 M NaCl:10 mM Tris (pH 7.4):4 mM MgCl₂:2 mM PMSF for 20 min at 22°C. Following termination by centrifugation at 1000 x g for 10 min (4°), the supernatants (S₂) were assayed by exchange at 0°C for 2 hr with 10 nM [³H]TPA ±5 nM TPA to determine specific binding activity. Addition of an equivalent amount of DNase I to the supernatant by exchange at 0°C for 2 hr with 10 nM [³H]TPA ±5 nM TPA to determine specific TPA-binding activity following centrifugation at 1000 x g for 10 min (4°).

The mechanism of action of the phorbol ester tumor promoters is of considerable interest in view of their diversity in eliciting particular biological responses. Previous studies have demonstrated the existence of receptors for TPA in plasma membranes of mouse epidermis (3, 5), skin (5, 12), brain (5, 15, 30), and cells in culture (5, 14, 18, 40). Although these studies emphasized the importance of membrane binding as a means of eliciting a particular TPA response, the possibility of alternative sites of action for TPA should not be overlooked. Murine serum, for instance, contains a protein that binds phorbol esters in a specific, reversible, and saturable manner (41). It is known that phorbol esters affect the growth and differentiation of mouse epidermis (2, 28, 34–36, 45) and cells in culture in different ways (1, 37, 45, 52, 55, 56), which leads to a consideration of possible multiple sites of action as well as multiple receptors. The nuclear binding of TPA observed in present study may therefore represent a third class of phorbol ester receptors.

The evidence that supports but does not necessarily prove the concept that nuclear receptors exist for the phorbol ester tumor promoters will be summarized. TPA is highly lipophilic and tends to penetrate membranes readily (23). Uptake studies using radioactive TPA applied to mouse skin or added to 3T3 cells revealed a subcellular distribution that indicated that 16 to 24% of the bound TPA was localized in the nucleus (20, 42). Our data indicate that isolated epidermal nuclei contain 2 apparent binding components, one of high affinity and extractable from the nucleus and another of low affinity, not easily saturable, and retained in the extracted nuclei. The latter binding component may represent unspecific binding to the nuclear membrane or perhaps to the nuclear protein matrix.
resulting from down regulation of membrane-binding components onto nuclear sites.

The evidence that phorbol ester tumor promoters function at the level of gene regulation are indirect; however, accumulating evidence suggests that gene derepression may be involved (4, 7, 13, 36). Two independent aspects of tumor promotion have been discussed in a review by Berenblum and Armuth (4). One involves the interaction at the cell membrane while the other site of action occurs at the gene-regulatory level in the nucleus, consistent with our data. A speculation that could be considered is whether these 2 aspects of tumor promotion are involved in the 2 stages of promotion originally reported by Boutwell (6, 7) and extended by Slaga et al. (43, 44).

It is reasonable to believe that TPA interacts in vivo with nuclear components naturally involved in developmental stimuli. Our data indicate that epidermal cell nuclei contain high-affinity binding macromolecules that bind phorbol esters in a saturable yet reversible manner and show specificity for the potent tumor promoter TPA.

REFERENCES

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