Partial Purification and Characterization of a Binding Protein for Biologically Active Phorbol and Ingenol Esters from Murine Sera*

Mohammed Shoyab and George J. Todaro
From the Laboratory of Viral Carcinogenesis, National Cancer Institute, Frederick, Maryland 21701

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We have purified a protein (Mr ~ 71,000) from murine sera 104-fold which directly binds biologically active phorbol esters, ingenol esters, and mezerein in a specific, reversible, and saturable manner. The binding of labeled phorbol-12,13-dibutyrate (PDBu) to the purified protein is rapid and dose-dependent. Those phorbol and ingenol esters which stimulate cell growth in culture and have tumor-promoting activity in vitro inhibit the binding of labeled PDBu, while the biologically inactive derivatives fail to do so. Other nontidepther tumor promoters, retinoids, steroids, and prostaglandins do not interfere with PDBu-protein interaction. Epidermal growth factor, insulin, bovine serum albumin, hemoglobin, ovalbumin, ferritin, myoglobin, fettin, and lipase do not interact directly with PDBu. The purified binding protein competitively inhibits the binding of PDBu to its specific receptors. It is non-glycosylated and slightly hydrophobic. The protein is heat- and acid-labile and is present in sera of various mammalian species. Its concentration in murine sera is age-, sex-, and strain-independent.

Tumor promoters are compounds which are themselves noncarcinogenic but which can induce tumors in animals previously treated with a suboptimal dose of certain chemical carcinogens (1-3). Most of the experimental work on tumor promotion has been carried out with phorbol esters, especially 12-O-tetradecanoylphorbol-13-acetate, initially isolated from croton oil derived from the seed of the plant Croton tiglium (4, 6). TPA and other biologically active phorbol esters elicit and modulate a variety of biochemical and biological responses in mouse skin, including stimulation of macromolecular synthesis, histone phosphorylation, synthesis of phospholipids, and modulation of the metabolism of polyamines and cyclic nucleotides (1-5, 7-13). In addition, these compounds induce ultrastructural changes in and affect the differentiation of murine epidermis (7, 14). Tumor-promoting phorbol esters also evoke pleiotrophic responses in cultured cells, including the stimulation of macromolecular synthesis and cell proliferation, induction of plasminogen activator and ornithine decarboxylase, loss of surface-associated fibronectin, alterations in the metabolism of cyclic nucleotides and polyamines, stimulation of prostaglandin synthesis, either the inhibition or stimulation of differentiation, and alterations in cell morphology and cell permeability, and elevation in the level of (Na+, K+)-ATPase activity (1-5, 15-23).

Several biochemical and biological studies provide evidence that the initial site of action of tumor-promoting phorbol esters may be the membrane of target cells (3-5, 21, 23-26). The tumor-promoting phorbol esters have been found to modulate the interaction between epidermal growth factor and its membrane receptors in a variety of cells in culture (27-31). The pleiotopic effects of TPA and related tumor promoters in vivo as well as in vitro seem to mimic the several actions of growth-stimulating polypeptide hormones such as EGF (32) and sarcoma growth factor (33). However, the effect, although rapid in modulating the EGF receptors, is indirect as it cannot be shown using low temperatures (28) and/or fixed cells or in isolated cell membranes. This would suggest that TPA produces its membrane effects through an interaction distinct from the EGF-receptor interaction.

Recently, we and others have reported the presence of specific high affinity receptors for biologically active phorbol and ingenol esters in a variety of cells and tissues using [3H]phorbol dibutyrate as a ligand (34, 35). The discovery of specific receptors for biologically active phorbol and ingenol esters, compounds of plant origin, led us to propose that TPA and certain analogues may have some structural resemblance to the endogenous growth promoting and/or differentiation modulating substance(s) (agonists or antagonists) that have specific membrane receptors. These compounds recognize and interact with the receptors, mimicking the action of the endogenous putative ligand(s) (34).

During the extensive search for the putative endogenous ligand(s) for the receptor, we have found that sera from a variety of mammalian species contain a protein which competitively inhibits the binding of PDBu to its receptors. Further investigation has revealed that this protein is a binding protein for biologically active phorbol and ingenol esters. We report here the partial purification and characterization of the binding protein from murine sera.

**MATERIALS AND METHODS**

**Chemicals**—Phorbol and its congeners were purchased from CMC Cancer Research Chemical Inc., Brewster, NY. Mezerein was obtained from Dr. P. Borchert, Eden Prairie, MN. Ingenol and its esters were generously supplied by Dr. E. Hecker, Heidelberg, Germany. Sephadex G-200 and phenyl-Sepharose CL-4B were bought from Pharmacia Fine Chemicals. Bio-Gel P-10 was from Bio-Rad Laboratories, Richmond, CA. [3H]PDBu was labeled at position 20 as described by Kreibich and Hecker (36) to a specific activity of 4.85 Ci/mmole.

**Preparation of Murine Brain Membranous Fraction**—Pooled murine brains (from approximately 2-month-old mice, NFS strain) were minced with scissors, suspended in 1 nmtriethanolamine/HCl, pH 7.4, and disrupted with 1 Polytron P-10 (Brinkmann). This and all

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1 The abbreviations used are: TPA, 12-O-tetradecanoylphorbol-13-acetate; PDBu, phorbol-12,13-dibutyrate; EGF, epidermal growth factor; BMP, brain membranous fraction; BB, binding buffer; PEG, polyethylene glycol; PBS, phosphate-buffered saline; PBA, PDBu binding inhibiting activity; BP, binding protein.

2 M. Shoyab and G. J. Todaro, unpublished results.
subsequent steps were done at 0-4 °C. The homogenate was centrifuged for 10 min at 1700 X g, the supernatant was removed and centrifuged for 60 min at 105,000 X g. The resulting pellet was suspended in one-fourth of the initial volume of PBS. The suspension was aliquoted into small volumes and stored at -70 °C.

**PDBu-binding Assays**—The binding of [³H]PDBu to cells was performed as previously described (34). The binding of [³H]PDBu to BMF or soluble receptors was performed in duplicate in disposable glass tubes (12 X 75 mm) (Kimax) either in the absence or presence of 20 μg/ml of unlabeled PDBu. The binding mixture contained 5 ng of [³H]PDBu (~4 X 10⁶ cpm), 0.2% final concentration of dimethyl sulfoxide and BMF (~50 μg of protein) or soluble receptors in a total volume of 0.25 ml of binding buffer consisting of Dulbecco’s minimum essential medium (DME medium) containing serum albumin (1 mg/ml) and N,N-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid (5 mM), adjusted to pH 6.8. After incubation for 30 min at 23 °C, the tubes were chilled, 0.25 ml of cold 4% calf serum (Colorado Serum Co., Denver, CO) and 0.7 ml of cold 25% polyethylene glycol 6000 in 1 mM Tris-HCl, pH 7.4, was added to each tube, the contents vortexed, the tubes allowed to stand for 15 min at 4 °C, and centrifuged for 10 min at 1500 X g at 4 °C. The supernatant solution was carefully drained off, the pellet was suspended in 2.5 ml of cold 10% PEG in 0.1 M Tris-HCl, pH 7.4, again centrifuged as earlier, the supernatant solution was removed, and pellets were solubilized in 0.8 ml of lysing buffer (0.01 M Tris-HCl, pH 7.4, containing 0.5% sodium dodecyl sulfate and 1 mM EDTA). The mixture was transferred to counting vials and 10 ml of Aquasure (NEN) was added to each vial. The vials were vigorously shaken and radioactivity determined using a Beckman liquid scintillation counter. The radioactivity bound in the presence of 20 μg/ml of [³H]PDBu was considered to be nonspecific binding.

**Assays for PDBu-binding Inhibiting Activity**—The competition assays either with cells or with BMF were performed as described above except the test material was added to the reaction mixture along with other components.

**Cell Filtration Assay for PDBu Binding**—The reaction mixture consisting of 2.5 ng of [³H]PDBu (~20,000 cpm), 0.5% final concentration of Me₂SO, 0 or 5 μg of unlabeled PDBu and soluble receptors or binding protein in a total volume of 0.125 ml was incubated for the desired time and temperature. The mixture was applied to a column of Bio-Gel P-10 (0.9 x 7.9 cm) previously equilibrated with phosphate-buffered saline (pH 7.1) at 4 °C. The flow rate was maintained at approximately 10 ml/h, 10-drop fractions were collected and radioactivity determined as described above.

**Purification of PDBu-binding Protein**—Forty ml of murine sera were applied to a column of Sephadex G-200 (5 x 90 cm) previously equilibrated with PBS at 4 °C. The flow rate was approximately 30 ml/h and 300-drop fractions (20 ml) were collected. Fractions 20 to 67 were pooled (Fig. 1) and applied to a column of phenyl-Sepharose CL-4B (1.5 x 7 cm) previously equilibrated with PBS at room temperature. The column was washed with approximately 100 ml of PBS. The bound proteins were eluted with distilled water. Five-ml fractions were collected. An aliquot of each fraction was assayed for PDBu binding inhibiting activity. Most of the activity appeared in fraction 3 (Fig. 2) which was divided into 0.5-ml aliquots, lyophilized, and stored at -70 °C. Each aliquot was dissolved in 0.5 ml of cold PBS for use in these studies.

**RESULTS**

**Purification of the Protein**—The elution profile of the PDBu binding inhibiting activity of murine sera from a column of Sephadex G-200 is shown in Fig. 1. The inhibiting activity emerged from the column as a single peak with a median size slightly larger than bovine serum albumin (M₀ ~ 71,000). Fig. 2 depicts the results of chromatography of pooled Sephadex G-200 fractions containing the inhibiting activity on phenyl-Sepharose. Most of the activity was eluted by water in fraction 3. The purification is summarized in Table I. A 104-fold purification with a 57.7% yield has been achieved in a two-step process. Attempts to purify the protein further by ion exchange chromatography or by affinity chromatography on various kinds of lectin-Sepharose have not been successful.

The competition of binding of labeled PDBu to BMF by various fractions (Table I) is presented in Fig. 3. The degrees of inhibition increased with increasing concentrations of protein. However, the maximum inhibition by unfractonated sera was never found to be more than 58% even at very high concentrations of serum (20 mg of protein in 0.25 ml of total reaction mixture).

The partially purified PDBu binding inhibiting activity is heat- and acid-labile, as well as protease sensitive. However, PBIA is resistant to DNase, RNase, neuraminidase, or galactosidase. Furthermore, protein is not retained on various lectin-Sepharose columns indicating the absence of glycosyl residues. The activity is stable to extensive dialysis against water (4 °C), lyophilization, and storage at -70 °C (~6 months with no appreciable loss of activity).

**Distribution of Protein (PBIA) in Sera from Various Species**—We tested mouse, rat, hamster, guinea pig, rabbit, goat, fetal calf, calf, monkey, and human sera for the presence of PBIA in order to determine the richest source for purification purposes (Table II). The activity was found to be highest in...
TABLE I
Purification of murine serum PDBu-binding protein

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume</th>
<th>Protein</th>
<th>Units*</th>
<th>Specific activity</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum</td>
<td>40</td>
<td>3610</td>
<td>1155</td>
<td>0.32</td>
<td>100</td>
</tr>
<tr>
<td>Sephadex G-200</td>
<td>145</td>
<td>858</td>
<td>1142</td>
<td>1.29</td>
<td>98.9</td>
</tr>
<tr>
<td>Phenyl-Sepharose</td>
<td>6</td>
<td>20</td>
<td>667</td>
<td>33.35</td>
<td>57.7</td>
</tr>
</tbody>
</table>

*Protein needed for 40% inhibition of [3H]PDBu binding to BMF.

Fig. 3. Inhibition of [3H]PDBu binding to BMF by different concentrations of protein at various stages of purification. The binding and competition assays were performed as described in the text. ○, murine serum; ●, Sephadex G-200 fraction; □, phenyl-Sepharose fraction.

TABLE II
Distribution of PDBu binding inhibiting activity of serum from various species

The binding assays in the absence or presence of serum (containing 15 mg of protein) in a total volume of 0.25 ml were carried out as described in the text.

<table>
<thead>
<tr>
<th>Species</th>
<th>[3H]PDBu bound</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>4798</td>
<td>0</td>
</tr>
<tr>
<td>Mouse</td>
<td>2162</td>
<td>56.2</td>
</tr>
<tr>
<td>Rat</td>
<td>4686</td>
<td>2.3</td>
</tr>
<tr>
<td>Hamster</td>
<td>3048</td>
<td>36.5</td>
</tr>
<tr>
<td>Guinea pig</td>
<td>4444</td>
<td>7.4</td>
</tr>
<tr>
<td>Rabbit</td>
<td>4560</td>
<td>5.0</td>
</tr>
<tr>
<td>Goat</td>
<td>4292</td>
<td>10.5</td>
</tr>
<tr>
<td>Fetal calf</td>
<td>4844</td>
<td>1.0</td>
</tr>
<tr>
<td>Calf</td>
<td>4332</td>
<td>9.3</td>
</tr>
<tr>
<td>Monkey</td>
<td>4238</td>
<td>13.8</td>
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<td>Human-A</td>
<td>3762</td>
<td>21.6</td>
</tr>
<tr>
<td>Human-B</td>
<td>3460</td>
<td>27.9</td>
</tr>
<tr>
<td>Human-C</td>
<td>2718</td>
<td>42.5</td>
</tr>
</tbody>
</table>

mouse serum followed by hamster, human, monkey, goat, and calf sera. Sera from other species contained negligible amounts of PBIA. Sera from various strains of male and female mice (NFS, NIH, Balb/c, DBA, AKR, C57/Bl, and C3H) at different ages were found to exhibit almost similar degrees of inhibition (data not shown).

Nature of Inhibition by PBIA—The effect of PDBu concentration on the binding of PDBu to BMF in the absence and presence of 80 μg/ml or 200 μg/ml of partially purified protein is shown in Fig. 4. The inhibitory effects of the protein were much greater at lower concentrations of PDBu. As the concentration of PDBu was increased, decreasing the ratio of receptors to PDBu molecules, the protein-elicited inhibitory effects lessened until they vanished at a PDBu concentration of 96 ng/ml. At this concentration, the same quantity of PDBu bound in the absence or presence of protein. The double reciprocal plots of binding in the absence and presence of protein are shown in the inset of Fig. 4. All three curves intersect the ordinate at the same point indicating that the protein competitively inhibited PDBu binding to its receptor. Thus, Vmax remained the same in the absence or presence of protein.

Fig. 4. PDBu concentration and PDBu-BMF interaction. A, effect of PDBu concentration on the binding of PDBu to BMF. Binding assays were performed as detailed in the text using various concentrations of [3H]PDBu either in the absence or presence of binding protein. ○, 0 μg/ml of BP; ●, 80 μg/ml of BP; □, 200 μg/ml of BP. The double reciprocal plots of data are presented in the inset. 1, 0 μg/ml of BP; 2, 80 μg/ml of BP; 3, 200 μg/ml of BP. B, Scatchard analysis of data in A. Symbols are the same.
protein. $K_m$ values for PDBu binding to its receptors were $1.32 \times 10^{-8}$ M, $2.83 \times 10^{-8}$ M, and $3.96 \times 10^{-8}$ M in the presence of 0, 80, or $100 \mu$g/ml of protein, respectively. Thus, the protein reduced the affinity of PDBu for its receptors. The analysis of data in Fig. 4 using a Scatchard plot revealed the decrease of receptor affinity in the presence of the protein (Fig. 4B).

Fig. 5 shows the effects of protein concentration on the extent of PDBu binding to BMF in the absence and presence of protein at two different concentrations. The inhibition was not overcome by increasing the receptor concentration, again indicating the competitive nature of inhibition.

Direct Binding of PDBu to Protein—PBlA could act to inhibit the binding of PDBu to receptors using one of the following mechanisms: 1) directly or indirectly masking or destroying the receptors, 2) transforming PDBu to a form incapable of binding to receptors, or 3) directly binding to PDBu or masking PDBu. We performed experiments to test these possibilities and found that PBlA binds directly to biologically active phorbol and ingenol esters.

Fig. 6 shows the direct binding of [$^3$H]PDBu to the partially purified protein. Free [$^3$H]PDBu was not found to be excluded from Bio-Gel P-10 and most of the radioactivity appeared in fractions 4 to 8 (panel A). However, when [$^3$H]PDBu was incubated with protein and then subjected to gel filtration, most of the label appeared in the void volume with protein in fractions 3 to 5 (panel B). The binding of [$^3$H]PDBu to protein was competed for by an excess of unlabeled PDBu (panel C) but not by phorbol (not shown in figure). Even 500 pg of bovine serum albumin did not bind any significant amount of [$^3$H]PDBu (panel D). Ovalbumin, DNase, RNase, actin, myosin, tubulin, calmodulin, phospholipase A or D, lipase, ferritin, insulin, EGF, hemoglobin, rabbit immunoglobulin G, Protein A (Staphylococcus aureus), and myoglobin all failed to bind [$^3$H]PDBu. Similarly, the binding of [$^3$H]PDBu to unfractionated sera and its competition with unlabeled PDBu were also observed.

The effect of protein concentration on the binding of [$^3$H]PDBu to the partially purified protein is shown in Fig. 7a. As expected, the protein always eluted in the void volume. However, the distribution of label in different fractions at the lower concentration of protein was unexpected and intriguing. As the concentration of protein was increased, the radioactivity started to shift from the unbound position (A) toward void volume fractions and finally co-eluted with the protein (F). We expected that [$^3$H]PDBu would either appear in fractions 3 to 5 (bound position) or fractions 7 to 9 (unbound position), and that the amount of label in fractions 3 to 5 would be directly proportional to protein concentration similar to the binding of [$^3$H]PDBu to soluble brain receptors (as shown in Fig. 7b). We feel that the on and off rates of PDBu binding to protein depend on protein concentration and at lower concentrations of protein, PDBu is rapidly dissociated and during filtration does not have an opportunity to recombine with protein and co-elute with the protein.

Protein bound very rapidly to PDBu. The binding was reversible. When an aliquot of fraction 4 ([$^3$H]PDBu-BP complex) was incubated for 10 min at 4 or 23 °C and chromatographed, almost all the radioactivity appeared at the position of free [$^3$H]PDBu.

Fig. 8 summarizes the effect of increasing concentrations of unlabeled PDBu on the binding of [$^3$H]PDBu to protein. As the concentration of unlabeled PDBu was increased, the amount of label in fractions 3 to 5 decreased and started to appear toward the free position. Most of the [$^3$H]PDBu was found to elute in fractions 7 to 9 at 1 or 10 $\mu$g/ml of unlabeled PDBu (F and G).

**Relationship between the Structures of Phorbol and Ingenol Derivatives and Modulation of [$^3$H]PDBu Binding to PBlA—**There are now several natural and synthetic derivatives of phorbol and ingenol with various degrees of promoting activity in the two-stage tumorigenesis model available (1-5).
We studied their inhibition of the binding of [3H]PDBu to the binding protein (Fig. 9). The biologically active derivatives of phorbol and ingenol, such as TPA, PDBu, phorbol-12,13-didecanoate, phorbol-12,13-dibezoate, 12-deoxy-phorbol-13-tetradecanoate, ingenol-3-hexadecanoate, and mezerein inhibited the [3H]PDBu-binding protein interaction, while inactive derivatives such as phorbol-12,13-diacetate, 4α-phorbol-12,13-didecanoate, 4-O-methyl-TPA, phorbol-12-acetate, phorbol-13-acetate, phorbol, ingenol-3,5,20-triacetate, or ingenol did not affect the interaction of [3H]PDBu to binding protein. Thus, only biologically active and tumor-promoting derivatives interact with binding protein exactly as previously reported by us for EGF modulation and PDBu binding to its receptors (28, 34).

![Image of Figure 7](http://www.jbc.org/)

**Fig. 7.** Binding of [3H]PDBu to BP or BSR. *A*, effect of BP concentration on PDBu binding. Two and one-half ng of [3H]PDBu and the indicated concentration of BP in 0.125 ml of BB were incubated for 30 min at 23 °C and analyzed as described in Fig. 6. *A*, 0 μg of BP; *B*, 9 μg of BP; *C*, 22.5 μg of BP; *D*, 45 μg of BP; *E*, 90 μg of BP; *F*, 180 μg of BP; *G*, 270 μg of BP; *H*, no binding protein but 500 μg of bovine serum albumin. *B*, effect of crude brain soluble receptors (BSR) on PDBu binding. 2.5 ng of [3H]PDBu and the indicated concentration of BSR in BB were incubated for 30 min at 23 °C and analyzed as described in the legend to Fig. 6. *A*, 0.1 mg of BSR; *B*, 0.25 mg of BSR; *C*, 1 mg of BSR; *D*, 2.5 mg of BSR.

![Image of Figure 8](http://www.jbc.org/)

**Fig. 8.** Effect of various concentrations of unlabeled PDBu on [3H]PDBu-BP interaction. Two and one-half ng of [3H]PDBu, 180 μg of BP, and the indicated concentration of unlabeled PDBu in 0.125 ml of BB were incubated for 30 min at 23 °C and assayed as in Fig. 6. *A*, 0 μg/ml; *B*, 0.01 μg/ml; *C*, 0.03 μg/ml; *D*, 0.1 μg/ml; *E*, 0.3 μg/ml; *F*, 1 μg/ml; *G*, 10 μg/ml.
Phorbol Ester-binding Protein

Phorbol ester tumor-promoting agents such as bile acids, barbiturate, laurate, limonene, saccharin, oleate, catharin, cyclamate, or anthralin (1-5) did not affect the [3H]PDBu binding to its receptors in cells or to BMF, it is not an endogenous ligand (either agonist or antagonist) (34) of PDBu receptors. It neither modulates EGF receptor interaction nor affects TPA-elicited modulation of EGF binding to its receptor (28). This protein neither induces nor modulates TPA-induced adhesion of human promyelogenous leukemia cells HL60 (38). We routinely use these two tests in our laboratory to determine the biological responses of tumor-promoting diterpene esters. Although the temptation is great, a competitive inhibitor of animal origin of PDBu binding to its receptor should not be christened an endogenous ligand unless it elicits biological responses akin to TPA in vivo and in vitro or it modulates TPA-induced biological responses.

Thus, why should mammalian sera contain specific binding protein for biologically active phorbol and ingenol esters, compounds of plant origin? The discovery of binding protein strengthens our belief in the proposal we presented in the report on specific receptors for these compounds (34). We now propose that TPA and certain analogues may have a structural resemblance to the endogenous growth-promoting and/or differentiation-modulating substance(s) (agonists and/or antagonists) that have specific binding proteins and specific receptors. These compounds recognize and interact with binding proteins and receptors, mimicking the action of the putative substances. Azaserine, cordycepin, curare, opiate, physostigmine, plant lectins, puromycin, and tubercidin appear to exert their action by such biological mimicry (39-41). The isolation and characterization of putative endogenous ligand(s) should help in understanding the mechanism of tumor-promotion.

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