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

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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 vivo inhibit the binding of labeled PDBu, while the biologically inactive derivatives fail to do so. Other nontumor-promoting tumor promoters, retinoids, steroids, and prostanoids do not interfere with PDBu-protein interaction.

Epidermal growth factor, insulin, bovine serum albumin, hemoglobin, ovalbumin, ferritin, myoglobin, fetuin, and lipase do not interact directly with PDBu. The purified binding protein competitively inhibits the binding of PDBu to its specific receptors. It is nonglycosylated 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 (1-5). Most of the experimental work on tumor promotion has been carried out with phorbol esters, especially 12-O-tetradecanoylphorbo1-13-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 pleiotypic 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/ mmol.

Preparation of Murine Brain Membranous Fraction—Pooled murine brains (from approximately 2-month-old mice, NFS strain) were minced with scissors, suspended in 1 mM triethanolamine/HCl, pH 7.4, and disrupted with a 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; PBLA, 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 cen-
trifuged for 10 min at 1700 X g, the supernatant was removed and cen-
trifuged for 60 min at 105,000 X g. The resulting pellet was sus-
pended 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 per-
curred essentially as 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 10^6 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 bovine 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 removed, and pellets were
suspended in 0.8 ml of lysis 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 Aquassure (NEN) was added to each vial. The vials
were vigorously shaken and radioactivity determined using a Beck-
man counter. The radioactivity bound in the presence of 20 μg/ml
(50 to 200 cpm) of unlabeled PDBu was considered to be nonspecific
and all data were corrected accordingly.

**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.

**Gel Filtration Assay for PDBu Binding**—The reaction mixture
consisting of 2.5 ml of cold 10% PEG in 0.1 M Tris-HCl, pH 7.4, tubes were again centrifuged as earlier, supernatant
solution was removed, and pellets were solubilized in 0.8 ml of
lysis 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 Aquassure (NEN) was added to each vial. The vials
were vigorously shaken and radioactivity determined using a Beck-
man counter. The radioactivity bound in the presence of 20 μg/ml
(50 to 200 cpm) of unlabeled PDBu was considered to be nonspecific
and all data were corrected accordingly.

**Results**

**Phorbol Ester-binding Protein**

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.

The partially purified PDBu binding inhibiting activity is heat- and acid-labile, as well as protease sensitive. However, PBlA is resistant to DNase, RNase, neuraminidase, or galac-
tosidase. 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

![Fig. 1. Sephadex G-200 chromatography of murine sera.](http://www.jbc.org/)

**Fig. 2. Phenyl-Sepharose CL-4B chromatography of the pooled Sephadex G-200 fractions.** See "Materials and Methods" for details.
TABLE I

Purification of murine serum PDBu-binding protein

| Fraction          | Volume (ml) | Protein (mg) | Units* | Specific activity (units/mg protein) | Yield (%) |
|-------------------|-------------|--------------|--------|-------------------------------------|-----------|
| Serum             | 40          | 3610         | 1155   | 0.32                                | 100       |
| Sephadex G-200    | 145         | 858          | 1142   | 1.29                                | 98.9      |
| Phenyl-Sepharose  | 6           | 23           | 667    | 33.35                               | 57.7      |
| CL-4B             |             |              |        |                                     |           |

*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.

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.

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/B1, 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.
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 BMP 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—PDBu 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 PDBu binds directly to biologically active phorbol and ingenol esters.

Fig. 6 shows the direct binding of $[^3H]$PDBu to the partially purified protein. Free $[^3H]$PDBu was not found to be excluded from Bio-Gel P-10 and most of the radioactivity appeared in fractions 6 to 8 (panel A). However, when $[^3H]$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 $[^3H]$PDBu to protein was competed for by an excess of unlabeled PDBu (panel C) but not by phorbol (not shown in figure). Even 500 ng of bovine serum albumin did not bind any significant amount of $[^3H]$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 $[^3H]$PDBu. Similarly, the binding of $[^3H]$PDBu to unfractionated sera and its competition with unlabeled PDBu were also observed.

The effect of protein concentration on the binding of $[^3H]$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. The reaction mixture was chromatographed on a Bio-Gel P-10 column as described in the text, A, no BP; B, 180 ng of BP; C, 180 ng of BP and 1.25 ng of unlabeled PDBu; D, 500 ng of bovine serum albumin. Blue dextran was used for the void volume.

FIG. 6. Direct binding of $[^3H]$PDBu to BP. The reaction mixture in 0.125 ml of BB contained 2.5 ng of $[^3H]$PDBu and other constituents. After incubation for 30 min at 23 °C, the mixture was chromatographed on a Bio-Gel P-10 column as described in the text, A, no BP; B, 180 ng of BP; C, 180 ng of BP and 1.25 ng of unlabeled PDBu; D, 500 ng of bovine serum albumin. Blue dextran was used for the void volume.
We studied their inhibition of the binding of [\textsuperscript{3}H]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-dibenzoate, 12-deoxy-phorbol-13-tetradecanoate, ingenol-3-hexadecanoate, and mezerein inhibited the [\textsuperscript{3}H]PDBu-binding protein interaction, while inactive derivatives such as phorbol-12,13-diacetate, 4\alpha-phorbol-12,13-didecanoate, 4\alpha-O-methyl-TPA, phorbol-12-acetate, phorbol-13-acetate, phorbol, ingenol-3,5,20-triacetate, or ingenol did not affect the interaction of [\textsuperscript{3}H]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).

**Fig. 7.** Binding of [\textsuperscript{3}H]PDBu to BP or BSR. A, effect of BP concentration on PDBu binding. Two and one-half ng of [\textsuperscript{3}H]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 [\textsuperscript{3}H]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.

**Fig. 8.** Effect of various concentrations of unlabeled PDBu on [\textsuperscript{3}H]PDBu-BP interaction. Two and one-half ng of [\textsuperscript{3}H]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

specifically interacts with biologically active phorbol and ingenol esters, and mezerein but not with biologically inactive derivatives of these diterpenes. The binding protein has been partially purified from murine sera. PDBu interaction with this protein is specific, reversible, and saturable. The nonditerpene ester tumor-promoting agents apparently do not interact with this protein, consequently, they do not modulate PDBu-BP interaction. Also, potent inhibitors of tumor promotion such as retinoids, anti-inflammatory steroids, prostaglandins, and cyclic nucleotides fail to affect the binding of PDBu to the protein. The above characteristics of the binding proteins are quite similar to the interaction between PDBu and its receptors in cells and tissues (34). However, BP appears to be different from PDBu receptors in many respects. The binding of PDBu to protein appears to be time- and temperature-independent and very much protein concentration-dependent (Fig. 7). In contrast, PDBu binding to its specific receptors is a concentration-, time-, and temperature-dependent phenomenon (34). The on and off rates of FDBu-BP interactions are apparently very different from PDBu-receptor interaction. Soluble brain receptors for PDBu resolve into three distinct peaks ($M_r \sim 440,000, 230,000, and 94,000$) during gel filtration (Bio-Gel A-0.5) while binding protein appears as a single peak ($M_r \sim 71,000$) (Fig. 1). Finally, binding protein inhibits the binding of labeled PDBu to cells or BMF, whereas soluble brain receptors show synergetic effects on the binding of PDBu to BMF. However, the final answer to the question of chemical relatedness and differences between the binding protein and receptors would come after purification to homogeneity of these proteins and their physical and chemical analyses.

Although the binding protein competitively inhibits the binding of PDBu 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-elicted 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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