Phorbol Diester and Epidermal Growth Factor Receptors in 12-0-Tetradecanoylphorbol-13-acetate-resistant and -sensitive Mouse Epidermal Cells


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ABSTRACT

Several cell variants have been isolated from promotable mouse JB6 epidermal cells which are resistant either to mitogenic stimulation at quiescence or to promotion of anchorage independence by 12-O-tetradecanoylphorbol-13-acetate (TPA). Such resistant variants would be expected to lack one or more steps in the TPA response pathway leading to mitogenesis or promotion of tumor cell phenotype. This report is concerned with determining whether resistance is attributable to lack of receptors for phorbol diesters or epidermal growth factor (EGF, a potential mediator) or to absence of receptor down modulation following ligand binding. The results show that neither lack of phorbol diester receptors nor absence of EGF receptors can account for TPA promotion of anchorage independence in JB6 cells but may mediate mitogenic stimulation of these cells by TPA.

INTRODUCTION

Although many biochemical and biological responses to tumor-promoting phorbol esters have been described (3) and although virtually all mammalian cells except erythrocytes have specific receptors for phorbol esters (12, 15, 16, 18), it remains to be demonstrated what role phorbol ester receptor binding or any of these other events play in the process of tumour promotion. Similarly, the possible causal relationship to phorbol ester mitogenesis of any of these responses has not been clarified.

We have described previously the use of the JB6 mouse epidermal model system for studying the mechanism of promotion (2–4, 6, 8, 9). JB6 cells respond to phorbol esters and other tumor promoters with irreversible induction of anchorage independence and tumorigenicity (4, 6). They also show a plateau density mitogenic response to phorbol esters and other promoters (5, 10). The approach we have used in recent studies (11, 13, 14, 30, 31) is to determine whether variants of promotable mitogen-sensitive mouse epidermal JB6 cells (6), which are resistant to mitogenesis or to promotion of anchorage independence by TPA, lacked one or more responses to TPA postulated to be required events in the process of mitogenesis or promotion. Among the responses to TPA currently being investigated for their significance in promotion of anchorage independence or mitogenesis in JB6 cells are inhibition of collagen synthesis (13, 14), inhibition of trisialoganglioside synthesis (29, 30), and changes in hexose uptake (11). Absence of a given response to TPA in promotion or mitogen-resistant variants would suggest but not prove a requirement for the response in promotion or mitogenesis, while absence of the response in sensitive variants would rule out the response as an absolute requirement.

Hormone resistance has been found to be associated with a receptor deficiency in a number of instances including such clinical disorders as insulin resistance, testicular feminization, and Laron dwarfism (25). Pruss and Herschman (26) have found that selection for EGF resistance yielded EGF-receptorless variants.

The studies reported here are aimed at determining whether phorbol ester resistance in JB6 cells is attributable to a receptor deficiency. In particular, we hope to obtain evidence concerning the role in phorbol ester biological activity of both phorbol diester and EGF receptors. The results indicate a possible requirement for phorbol diester but not EGF receptors in promotion of tumor cell phenotype by TPA and a possible requirement for EGF receptors as well as TPA receptors in TPA mitogenesis.

MATERIALS AND METHODS

Materials. [3H]PDBU (7.2 Ci/mmol) was obtained from Chemical Carcinogenesis, Eden Prairie, Minn., and [3H]PDBU (5.0 Ci/mmol) was obtained from Dr. Mohammed Shoyab, Laboratory of Viral Carcinogenesis, National Cancer Institute, as a gift. [125I]-EGF was prepared as described (22).

Derivation of TPA-resistant Variants. The derivation of TPA mitogen-resistant variants of JB6 mouse epidermal cells has been described elsewhere (5, 10, 11). The promotable JB6 CI 41 was exposed

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after reaching plateau density to TPA and colchicine in a selection procedure analogous to that described by Pruss and Herschman (26) for producing EGF-resistant variants of 3T3 cells. The cells which showed a mitogenic response to TPA at plateau density were trapped in mitosis by colchicine, detached, and washed off. The resistant cells remaining were carried through a total of 2 to 6 selection cycles and cloned. The TPA promotion-resistant variants of JB6, CI 25 and CI 30, were derived by nonselective cloning of the JB6 parent line which was promotable to anchorage independence by phorbol diesters and other tumor promoters (8).

Assay for Mitogenic Response. Clonal derivatives of JB6 cells were allowed to reach plateau density in 5% serum. The cells were then exposed to TPA (1 to 100 ng/ml; 1.6 × 10^{-7} M), conditions which lead to an approximate doubling by JB6 CI 41 cells (5, 10, 11), and were enumerated by hemocytometer or Royco counter.

Assay for Promotion of Anchorage Independence. JB6 cell lines were exposed to TPA in 0.33% agar medium, and TPA-dependent colony induction was determined as described (6, 9).

Assay of [3H]PDBU Binding to Membrane Fractions. Cell homogenates in Tris buffer, pH 7.4, were prepared, 100,000 × g pellets were obtained, and assay of [3H]PDBU binding to membrane fractions was carried out as described elsewhere (15).

Assay for Specific [3H]PDBU Binding to Cells in Monolayer Culture. Twenty-four to 48 hr after plating in 6-well plates, cells at a density >600,000 cells per 35-mm well were washed twice with binding medium containing bovine serum albumin (1.0 mg/ml; Sigma Chemical Co.) and then incubated for 30 min at 37° in a 5% CO2 incubator with [3H]PDBU in binding medium at concentrations ranging from 1 to 100 × 10^{-9} M. After removing aliquots of supernatants to determine the concentration of bound [3H]PDBU, plates were washed twice with cold binding medium, and the cell layer was lysed with 1.0 ml 0.5% NaOH. Radioactivity was determined in a Beckman LS-350 scintillation counter with an efficiency of 40%. Nonspecific binding in the presence of 25 μM unlabeled PDBU was measured in parallel plates. Binding affinities and number of binding sites per cell were determined by Scatchard analysis. All points are the means of duplicates. [3H]PDBU binding is expressed as amount specifically bound per 10^6 cells.

Down Modulation of [3H]PDBU Binding. Cells cultured as above were exposed to 25 μM unlabeled PDBU for 1, 3, 6, and 12 hr. After 3 washes at 37° for 10 min each (which removes >98% of bound [3H]PDBU), specific binding of 30 nM [3H]PDBU was determined.

Assay of 125I-EGF Binding to Cells in Culture. EGF was isolated from designated sources and iodinated as described previously (22). 125I-EGF binding assays and Scatchard analysis were performed as described (22). Binding assays were carried out using cells in monolayer in 35-mm dishes in the presence of 125I-EGF and 1% bovine serum albumin for 4 hr at 4°. Specific 125I-EGF binding was determined by subtracting the amount of radioactivity bound nonspecifically in the presence of 1000-fold excess of unlabeled EGF. Pretreatments with TPA were carried out at 37° for periods up to 24 hr in the presence of TPA (200 ng/ml).

RESULTS

Recently, we have described a selection of promotable JB6 CI 41 cells for resistance to plateau density mitogenic stimulation by TPA (5, 10, 11). Using the approach of Pruss and Herschman (26), cells at plateau density were exposed to TPA plus colchicine. This traps in mitosis and permits washing off of the mitogenically responsive cells, leaving behind resistant cells which are collected and recycled through the selection.

Chart 1A shows the TPA mitogen resistance of 2 cell lines obtained by cloning the selected cell population, R219 and R23. Additional mitogen-resistant lines (10) included R6141 and R28. Qualitative resistance was indicated by the lack of response to TPA (1 to 100 ng/ml), the concentration range effective in promotion of anchorage independence in JB6 cells. Of these mitogen-resistant lines, R219, R23, and R6141 were sensitive to promotion of anchorage independence by TPA (M+P-) but R28 was not (M−P−) (Chart 1B). In addition, the responses are shown for 2 cell lines derived from JB6 by nonselective cloning. JB6 CI 41 (the parent line for the mitogen-resistant variants) was mitogen sensitive and promotion sensitive (M+P+) and JB6 CI 25 was mitogen sensitive and promotion resistant (M+P−) to TPA.

These resistant variants have been investigated for missing responses to TPA, since resistance would be expected to arise from lack of one or more responses which are required steps leading to mitogenesis or promotion. Because binding of phorbol esters to specific receptors occurs as an initial event which precedes biological responses in a variety of cells, the TPA-resistant variants were examined for possible aberrations in receptor binding. Table 1 shows that, whether binding was determined on membrane fractions or on intact cells, all of the resistant variants had phorbol ester binding sites. Furthermore,
neither the mitogen- nor promotion-resistant variants showed a decrease in receptor number. The median values for number of binding sites per cell (3 to 6 cell lines of each phenotype) were for P+, P−, M+, and M− variants, respectively, 394, 453, 392, and 424 × 10^3. As indicated in Table 1 and Ref. 10, the selection for M− variants yielded not only P− and P+ clones but also anchorage-independent transformants, a not-surprising result in view of the 2 to 6 periods of TPA exposure to which the cells were subjected. These anchorage-independent transformants derived by cloning after the selection for TPA mitogen resistance (R24, R21) and other transformants derived by cloning from agar after induction from JB6 cells by TPA (T3 6272) showed levels of phorbol ester binding similar to non-transformants. The apparent lack of quantitative agreement between binding to membrane preparations and binding to intact cells for JB6 CI 30 may arise from loss or masking of binding sites in membrane preparations to nonmembrane binding sites in intact cells (19) or to large cells and consequent high levels of membrane protein per cell.

Since down modulation of receptor-ligand complexes following binding has been postulated to be a means of reaching intracellular targets for biological activity, the possibility that phorbol ester-resistant variants might lack receptor down modulation was investigated. Chart 3 shows the time-dependent decrease in [3H]PDBU binding for 4 different cell lines after preincubation with 25 nM unlabeled PDBU for varying periods of time. In each case, the onset of phorbol diester receptor down modulation occurred within 3 hr after the start of phorbol ester incubation, and by 24 hr, binding was 30 to 50% of the initial value.

Although phorbol ester binding to its receptor may directly trigger a second messenger for mitogenesis or promotion, an alternative possibility is that phorbol ester binding stimulates the binding of an endogenous growth factor which then functions as a mediator required for the biological activity of the phorbol ester. Such a growth factor might be EGF or a related factor which utilizes EGF receptors as described by Todaro et al. (31). In this case, lack of EGF receptors would produce resistance to phorbol ester activity. The results of testing this possibility are shown in Table 2. Of the 3 TPA-nonpromotable variants tested, R28 was without available EGF receptors. However, 2 of the TPA promotable variants, R219 and R23, also lacked available EGF receptors, thus ruling out EGF or EGF receptor as an obligatory mediator of TPA promotion of

### Table 1

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Phenotype</th>
<th>[3H]PDBU bound (pmol/mg protein)</th>
<th>No. of binding sites/cell (x10^-3)</th>
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<tr>
<td>JB6</td>
<td>M+P+</td>
<td>2.52</td>
<td>210</td>
</tr>
<tr>
<td>JB6 CI 41</td>
<td>M+P+</td>
<td>1.88</td>
<td>392</td>
</tr>
<tr>
<td>JB6 CI 22</td>
<td>M+P+</td>
<td>1.71</td>
<td>729</td>
</tr>
<tr>
<td>JB6 CI 25</td>
<td>M+P−</td>
<td>2.10</td>
<td>347</td>
</tr>
<tr>
<td>JB6 CI 30</td>
<td>M+P−</td>
<td>1.71</td>
<td>1333</td>
</tr>
<tr>
<td>R23</td>
<td>M+P−</td>
<td>2.26</td>
<td>603</td>
</tr>
<tr>
<td>R219</td>
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<td>1.73</td>
<td>253</td>
</tr>
<tr>
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<td>2.77</td>
<td>396</td>
</tr>
<tr>
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<tr>
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<td>M−Tx</td>
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<tr>
<td>T³ 6272</td>
<td>M+Tx</td>
<td>2.53</td>
<td>296</td>
</tr>
</tbody>
</table>

⁸ Tx, anchorage-independent and tumorigenic phenotype.

Chart 2. TPA-sensitive and -resistant variants show similar phorbol diester binding affinities. Scatchard analysis of specific [3H]PDBU binding to intact cells was carried out as described in “Materials and Methods.” Correlation coefficients were greater than 0.8. Bₜ, bound specific dPM; F, free dPM; B.S., number of binding sites.

Chart 3. TPA resistance not attributable to a defect in receptor down modulation. Determination of specific [3H]PDBU binding was carried out after preincubation with 25 nM unlabeled PDBU for 1 to 24 hr as described in “Materials and Methods.” Each point is the mean of duplicate values, and each cell line was tested twice. The results are expressed as cPM specifically bound per cell. t, time; Bₜ, bound specific.
tumor cell phenotype in JB6 cells. Since the 4 TPA mitogen-resistant lines, R219, R23, R28, and R6141, show little or no detectable EGF binding, while all 5 of the 5 M+ lines tested showed moderate to high levels of EGF binding, the possibility exists that EGF could be involved in at least one pathway of TPA-induced mitogenesis.

Finally, if the inhibition of EGF binding produced by TPA has biological significance in TPA responses, defects in this parameter would be expected to produce resistance to TPA. The data in Table 2 indicate that TPA exposure produces substantial inhibition of EGF binding in both sensitive and resistant variants by 2 hr after the start of TPA exposure. The P-resistant variants JB6 CI 25 and CI 30 showed a slower and less extensive recovery of EGF binding than did their sensitive counterparts JB6 CI 41 and JB6 CI 22.

**DISCUSSION**

Since all of the TPA-sensitive variants had phorbol ester receptors, the possibility remains that phorbol ester binding may be required for its biological activity. Because the selection for resistance did not yield any phorbol ester-receptorless variants, the possibility also exists that these binding sites may function in maintaining cell viability. A similar observation has been reported by Fisher et al. (17), who showed similar phorbol ester binding for Friend erythroleukemia cells which were sensitive or resistant to TPA inhibition of differentiation, and by Solanki et al. (28), who found similar binding by human leukemic HL-60 cells which were sensitive or resistant to TPA induction of differentiation.

Absence of phorbol ester receptor down modulation has been found by Solanki et al. (28) in a TPA-resistant variant of the human leukemic line HL-60. These authors have suggested that this lack of down modulation may indicate lack of receptor-ligand internalization which may be needed for biological activity. The results which we report here show that phorbol ester receptor down modulation occurs in all of the resistant variants of JB6 cells and therefore leave unanswered the question as to the role of down modulation in mitogenic and promotion responses to TPA in JB6 cells. The mechanism and functional significance of phorbol ester receptor down modulation is unknown. Whether down modulation involves receptor internalization as occurs with polypeptide hormones or some other mechanism is not clear.

EGF mediation can be ruled out as an obligatory requirement for promotion of anchorage independence in JB6 cells, since the TPA-promotable R219 and R23 lines lack EGF receptors and show promotion resistance to EGF (Table 2 and ref. 7). The possible role of EGF as a mediator of one pathway of TPA mitogenesis (23) is suggested by the TPA mitogen resistance of 3 EGF-receptorless variants and by the presence of EGF receptors on all the TPA mitogen-sensitive variants. It is noteworthy that selection of 3T3 cells for TPA mitogen resistance as reported by Butler-Gralla and Herschman (1) also yielded an EGF-receptorless variant (TNR2) as well as a variant with reduced EGF binding (TNR9). The presence of EGF receptors on TNR9 and the presence of TPA mitogenic response by the EGF-receptorless NR6 variant selected for EGF resistance by Pruss and Herschman (26) indicate, however, a dissociation between EGF receptors and TPA mitogenesis in 3T3 cells. Perhaps there exist EGF receptor-dependent and EGF receptor-independent pathways of TPA mitogenesis.

Phorbol ester-induced inhibition of EGF binding as described by Lee and Weinstein (20, 21), Shoyab et al. (27), and Murray and Fussenig (24) occurs in TPA-promotable and -nonpromotable JB6 cell lines. Whether this decrease or its recovery plays a role in TPA mitogenic activity as suggested by Matrisian et al. (23) remains an unanswered question. However, the possible importance of EGF receptor recovery in promotion sensitivity is suggested by the observation that both of the nonpromotable lines tested appeared defective in their EGF-binding recovery following phorbol ester exposure.

The correlation between resistance to TPA-induced mitogenesis and the EGF-receptorless state may or may not have occurred repeatedly in the case of the JB6 variants. As dis-
cussed elsewhere (10), the clonal lines of the same phenotype obtained from the selection for TPA resistance may be isolates of the same preexisting variant, since all of these lines were obtained from the same parent JB6 Cl 41. To ascertain whether the M—EGF-receptorless correlation occurs repeatedly requires independent selections from other JB6 clonal lines. This is currently under way.

The molecular basis for resistance to promotion of anchorage independence by TPA is under continuing investigation in our laboratory. Recent results indicate that the nonpropagated variants fail to undergo specific changes in ganglioside biosynthesis which occur in their promatable counterparts (29, 30).

REFERENCES


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