Protein Kinase C and Its Substrates in Tumor Promoter-sensitive and -resistant Cells*

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Calcium- and phospholipid-dependent protein kinase C activity and substrates were characterized in cell lysates of preneoplastic JB6 cells, a model system of genetic variants for sensitivity to tumor promoter-induced neoplastic transformation. Protein kinase C activity was similar for sensitive and resistant variants, as measured by calcium- and phospholipid-dependent phosphorylation of an exogenous substrate (histone HIII). Of 13 endogenous protein kinase C substrates, identified by labeling proteins with [γ-32P]ATP, at least two (80 and 23 kDa) are potential candidates for mediating events on the pathway for promotion of transformation. 32P incorporation into the 80-kDa protein kinase C substrate was stimulated by tetradecanoylphorbol acetate and correlated with phenotype: the highest incorporation was found in promotion-insensitive cells, an intermediate level in promotion-sensitive cells and the lowest in the transformed cells. The phosphorylation of an 80-kDa protein, found by labeling intact cells in monolayer growth with [32P]orthophosphate, was also stimulated by tetradecanoylphorbol acetate and correlated inversely with phenotype. The 80 kDa protein kinase C substrate from cell lysates and the 80-kDa phosphoprotein from intact cells appear to be identical, as indicated by peptide mapping with protease V8 from Staphylococcus aureus. This finding suggests that the 80-kDa substrate is relevant to promoter-induced signal transduction in the intact cell. The 23-kDa protein kinase C substrate exhibited a band shift in sodium dodecyl sulfate gels in response to another transformation promoter in JB6 cells, the calcium analog, lanthanum (Smith, B. M., Gindhart, T. D., and Colburn, N. H. (1986) Carcinogenesis 7, 1949–1956). In summary, there are no unique substrates that distinguish the variants. Quantitative differences in certain substrates or their phosphorylation may, however, account for the difference in promotion sensitivity among the variants.

The receptor for the tumor promoting phorbol esters appears to be identical to calcium- and phospholipid-dependent protein kinase C (PKC)1 (1–3). This finding indicates that protein kinase C activation and substrate phosphorylation should be early signal transduction events in phorbol ester-induced neoplastic transformation. Phosphorylation of specific protein substrates may be on the pathway for promoter-induced preneoplastic progression (4–7).

The JB6 cell system, originally derived from primary mouse epidermal cells, is analogous to second stage tumor promotion in mouse skin (8, 9), and is a useful in vitro model for studying the promotion of neoplastic transformation. Promotion sensitive (P*) JB6 cells undergo irreversible transformation when treated with agents that are tumor promoting in mouse skin, including phorbol esters. The promotion-insensitive (P−) cells are thought to represent an earlier stage of preneoplastic development and are resistant (showing about 1% of the P* response) to promotion induction of anchorage independence and tumorigenicity (8, 9). Tumorigenic JB6 cell lines, established by selective cloning of TPA-induced transformants (10), exhibit anchorage independence in the absence of tumor promoters. Thus, cell lines bearing the P*, P−, and transformed (Tx) phenotypes represent cell populations at successive stages of neoplastic progression.

Unlike the P− cell lines, cells bearing the P* phenotype harbor activated promotion sensitivity genes (termed pro-1 and pro-2) (9, 11). Transfection of either of the activated pro genes from the P* cells to the P− cells confers promotion sensitivity, indicating that the capacity to be transformed by TPA, at least in this model, can be specified by one gene (9, 11). Thus, this unique system of genetic variants allows us to compare among phenotypes components of the phorbol ester signal transduction system and to identify phosphoprotein changes that may be specifically relevant to promoter-induced transformation. In fact, we have previously identified a phosphoprotein of 80 kDa stimulated by TPA treatment that correlates inversely with degree of preneoplastic progression (12).

In the present article, we present evidence that this transformation-linked 80-kDa protein is a PKC substrate in JB6 cells. We also characterize PKC activity and identify a total of 13 PKC substrates in JB6 cell lysates, several of which appear to be substrates in the intact cell. Thus, an altered signal transduction event, namely phosphorylation of a PKC substrate, may account for the sensitivity of the JB6 cells to tumor promoter-induced neoplastic transformation.

MATERIALS AND METHODS

Chemicals

Acrylamide, bisacrylamide, molecular weight standards, Coumassie Brilliant Blue G-250 and R-250 stain, ammonium per sulfate, Temed, and glycine were all purchased from Bio-Rad. Phenylmethylsulfonyl fluoride, phosphatidyserine, 1,2-diolein, and histones were from Sigma. Tris was from Boehringer Mannheim. Amersham was the

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1 The abbreviations used are: PKC, protein kinase C (Ca2+/phospholipid-dependent enzyme); TPA, 12-0-tetradecanoylphorbol-13-acetate; Temed, N,N,N′,N′-tetramethylethylenediamine; EGT, (ethylenebis(oxyethylenenitrilo))tetraacetic acid; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PL, phospholipid.
Phosphorylation in Intact Cells Identified by [32P]Orthophosphate Labeling

JB6 cells in monolayer growth were treated with TPA (10 ng/ml) for the indicated times and labeled with 0.5 μCi/ml [32P]orthophosphate for the last hour of treatment. Cells were trypsinized, washed with phosphate-buffered saline, and resuspended in sucrose buffer (0.25 M sucrose, 10 mM Tris-HCl, 10 mM NaCl) and 10 μg/ml leupeptin. Cells were lysed with 20% Nonidet P-40 on ice and centrifuged for 4.5 min at 0 °C. DNase (1 mg/ml) and RNase (10 mg/ml) were added to supernatants to degrade nucleic acids and chelators EDTA and EGTA were added to decrease protease activity as described (12). Samples were standardized by adjusting for cell number or by trichloroacetic acid-precipitable counts and were subjected to SDS-PAGE analysis.

Peptide Mapping

The standard reaction mixture described under “PKC Phosphorylation in Cell Lysates” was used to activate PKC and obtain the phosphorylated 80-kDa PKC substrate. Cells growing in monolayer were TPA-treated and labeled with [32P]orthophosphate (as described in the previous section) to obtain the 80-kDa phosphoprotein. After cells were fractionated, samples were boiled (the 80-kDa protein stays in solution; 16, 17) and were subjected to SDS-PAGE. Gels were washed overnight in isopropyl alcohol to remove SDS. Bands corresponding to the 80-kDa protein from both of the above treatments were sliced from the wet 0.75-mm gel and inserted into a well of a stacking gel of 1.5-mm thickness. Gel bands in the well were treated with staphylococcal V8 protease (endoproteinase Glu-C) obtained from Boehringer Mannheim at a concentration of 1 or 10 μg/ml for 1.5 h and electrophoresed at 17 mA/gel. Gels were stained, dried, and subjected to autoradiography.

RESULTS

The first step in the phorbol ester-induced signal for promotion of transformation should involve activation of the phorbol ester receptor PKC and resultant phosphorylation of PKC protein substrates, a subset of which may be necessary for the transformation response. To determine whether a difference in protein kinase C activity or substrates could account for the lack of promotion sensitivity of JB6 P+ cells, calcium- and phospholipid-dependent phosphorylation of exogenous (histone) and endogenous substrates was measured and compared in clonal lines of each phenotype.

Total cellular PKC was quantitated and compared among the three JB6 cell phenotypes (Table I). Total cellular PKC activity was assayed by: 1) measuring cytosolic PKC after chelator-extracting membrane-bound PKC into the cytosolic fraction, and 2) measuring residual PKC activity of the chelator-extracted, detergent-solubilized membrane fraction. (Methods of quantitation are described under “Materials and Methods.”) The chelator extraction yielded 95–97% of PKC activity in the cytosolic fraction indicating that chelator extraction is very efficient and appropriately measures PKC activity in JB6 cells (Table I). It should be noted that under-estimation of residual PKC due to possible inhibition by Nonidet P-40 seems unlikely since the 0.02% Nonidet P-40 employed in our experiments decreased PKC activity in the cytosolic fraction by less than 8% in all cell lines. The results in Table I show that enzyme activity detected in the cytosolic fraction was similar in clones of all phenotypes, ranging from 152 to 360 pmol of 32P incorporated/min/mg protein. It appears clear that the promotion resistance of the P+ cells cannot be attributed to a deficiency of protein kinase C activity.

In measuring PKC activity, saturating concentrations of phosphatidylserine (24 μg/reaction mixture) and 1.2-diolein (0.8 μg/reaction mixture) were used. Samples lacking phospholipids, or lacking calcium, had less than 10% of the incorporated radioactivity present in samples containing both of these agents. It should also be noted that for clonal lines of
phospholipids were detected. Protein kinase C substrates phosphorylation of endogenous substrates. When phosphatidyserine and calcium were added to the reaction mixture (lane 4, Fig. 1), calcium- and phospholipid-dependent phosphorylation was detected. Protein kinase C substrates were found predominantly in the molecular weight region from 14 to 45 kDa (as indicated by the arrows). Neither phospholipids (lane 2) nor calcium (lane 3) when added alone could support significant phosphorylation of these substrates. As indicated by asterisks, phosphorylation of two proteins at 60 and 63 kDa was independent of calcium and phospholipid, suggesting the activity of another kinase(s) in the cytosolic fraction. Also, a phosphoprotein dependent only upon calcium was detected at 107 kDa (asterisk). Calcium- and phospholipid-dependent activity was greatest when cells were homogenized with high concentrations of chelators (total of 7.0 mM EGTA and EDTA) and reactions performed at 5.0 mM calcium and 75.0 mM magnesium.

Thirteen PKC substrates were identified in JB6 cells. Fig. 2 is a representative experiment showing substrates present in JB6 Cl 25 with apparent molecular masses of 14, 17, 20, 21, 23, 25, 26, 30, 32, 34, 37, 57, and 80 kDa. The apparent molecular weights of these substrates have been calculated by comparison with known standards. Combining the cytosolic and membrane fractions resulted in an increase in detectable phosphorylation of substrates already found in the cytosolic fraction, but did not result in the detection of new substrates that might be specifically membrane associated (compare lanes 2 and 6). When cells were treated 1 h prior to harvest with TPA, an increase in the phosphorylation of the 80-kDa substrate was observed. The phosphorylation of other substrates (such as the 37 kDa) appear to be decreased. This is likely due to differences in the kinetics of substrate phosphorylation and dephosphorylation.

Table II presents a quantitative summary of the substrates identified in JB6 Cl 25 cell lysates. Radioactivity incorporated into each band and the fold increase over minus phospholipid background is presented. It is known that there is a variation in magnesium requirement for PKC depending upon substrates phosphorylated (15). We observed that decreasing magnesium concentrations 10-fold (from 75 to 7.5 mM) increased phosphorylation of histone substrates of 34 and 32 kDa (data not shown). This raised the possibility that decreasing magnesium concentrations might allow detection of other endogenous PKC substrates. Under conditions of dialyzed cytosol (to remove chelators used during fractionation), 7.5 mM magnesium and 1.0 μM calcium, all 13 of the substrates found under higher cationic conditions were detected, but no new substrates were detected.

To determine whether unique or lacking PKC substrates might explain promotion sensitivity, we simultaneously tested P+ and P− cell lines (under cation conditions of 5.0 mM calcium and 75 mM magnesium), and examined them for the 13 substrates previously identified or for additional substrates. The PKC substrates found in the promotion-resistant Cl 25
transformed cells. No other quantitative difference in a PKC substrate in P' and P- cells, and the lack of phosphorylation in the Tx cells. Fig. 2.

### FIG. 2. Thirteen endogenous PKC substrates identified in JB6 C125 cells. Lanes 3, 4, 7, 8, intact C125 JB6 cells in monolayer growth were incubated with solvent control dimethyl sulfoxide or TPA (10 ng/ml or 16 nM) for 1 h prior to cell fractionation by sonication in homogenization buffer. All lanes, cells were fractionated in high chelator buffer (5.0 mM EGTA and 2.0 mM EDTA) to bring PKC into the cytosolic fraction. Cell suspensions were then centrifuged at 100,000 x g for 1 h. The supernatant recovered was the cytosolic fraction (CS) and the pellet was the membrane fraction (MB). The reaction mixture contained 5.0 mM CaCl₂, 75 mM MgAc, 50 μg of cytosolic protein or 50 μg of cytosolic plus 50 μg of membrane protein, 10 μg/ml leupeptin, and 100 μM ATP with 100 μCi of [³²P]ATP in the absence or presence of phospholipids. Lanes 1-4 contain only the cytosolic fraction; lanes 5-8 contain both cytosolic and membrane fraction. Arrowheads mark bands which increased with addition of phospholipids (+PL lanes). Molecular weight standards were run on each gel and were used for determining the molecular weight of PKC substrates.

(32P)orthophospho-

(Fig. 2) were detected in JB6 cells of all three phenotypes (P⁺, P⁻, and Tx). Fig. 3 presents the 13 JB6 cell substrates in two cell lines each of the P⁺ and P⁻ phenotypes. The exposure of the autoradiogram presented in Fig. 3 best visualizes substrates from 17 to 34 kDa. Analysis of several autoradiograms of the same clonal line was performed to confirm the presence of every calcium- and phospholipid-dependent PKC substrate. For ease in comparison of substrates, only the phospholipid containing lanes are shown in Fig. 3. In summary, it appears that sensitivity to promotion of transformation among JB6 cell variants does not involve substrates unique to either sensitive or resistant phenotypes.

Although no qualitative difference was observed in PKC substrates, a quantitative difference among the phenotypes was consistently observed in phosphorylation of the 80-kDa PKC substrate (Fig. 4, A and B). Fig. 4A shows the phospholipid-dependent phosphorylation of the 80-kDa PKC substrate in P⁺ and P⁻ cells, and the lack of phosphorylation in the Tx cells. Fig. 4B shows that the phosphorylated 80-kDa PKC substrate was highest in the P⁻ cells, of an intermediate level in P⁺ cells, and very low or non-detectable in the transformed cells. No other quantitative difference in a PKC substrate was observed between phenotypes. These findings regarding the 80-kDa PKC substrate were particularly interesting because they directly paralleled our previous finding that TPA treatment of JB6 cells growing in monolayer increases the level of phosphorylation (using [³²P]orthophospho-

### TABLE II

**Summary of substrates for protein kinase C in JB6 cell lines**

JB6 cell homogenates were centrifuged at 100,000 x g for 1 h. The reaction mixture and protocol is described in the legend to Fig. 1. Dried gel bands corresponding to the phosphate-labeled bands (substrates) in the autoradiograph were excised and the counts/min determined by liquid scintillation counting. Each substrate is represented by the amount of counts/min incorporated into the band. The counts/min in the phospholipid containing lanes divided by the counts/min in lanes lacking phospholipids (+PL/-PL ratio) presents the fold increase of the counts/min of each substrate over background. (All lanes contained calcium so that any calcium-dependent kinase phosphorylation would be calculated as background and would not be attributed to PKC activity.

| Gel band M × 10⁻³ | Radioactivity incorporated | +PL/-PL ratio |
|-------------------|---------------------------|---------------|
| 8                 | 2692                      | 1.6           |
| 57                | 1010                      | 2.2           |
| 37                | 1060                      | 2.9           |
| 34                | 1120                      | 2.7           |
| 32                | 654                       | 1.8           |
| 30                | 446                       | 1.5           |
| 26                | 580                       | 1.9           |
| 25                | 424                       | 1.4           |
| 23                | 1066                      | 2.9           |
| 21                | 830                       | 3.0           |
| 20                | 1422                      | 2.8           |
| 17                | 618                       | 1.6           |
| 14                | 1264                      | 2.9           |

**FIG. 3. Identical 13 substrates detected in promotion-sensitive and promotion-resistant JB6 cells.** Cytosolic fractions were harvested from two promotion-sensitive (C1 41 and C1 22) and two promotion-resistant (C1 30 and C1 25) cell lines by sonication and centrifugation at 100,000 x g for 60 min. The standard reaction mixture contained 5.0 mM CaCl₂, 75 mM MgAc, and 100 μM ATP with 100 μCi of [³²P]ATP in the presence or absence of phospholipids (as described in the legend to Fig. 1). Samples have 100 μg of cytosolic protein. Molecular weight standards are indicated.
The phosphorylation of 80-kDa phosphoprotein (12). Like the phosphorylated 80-kDa PKC substrate, the level of the 80-kDa phosphoprotein from intact cells also correlates inversely with stage of preneoplastic progression: the promotion-resistant cells show the highest levels of phosphorylated pp80 in both unstimulated and TPA-stimulated cells, the promotion-sensitive cells show intermediate levels, and the transformed cells show marginally detectable levels. In this report, we have extended our observations on the 80-kDa protein from intact cells by showing that the greatest degree of TPA stimulation of phosphorylation of the 80-kDa protein occurred during 1 h of treatment of intact cells (Fig. 5). The observation of TPA-induced phosphorylation at 1 and 4 but not 20 h is compatible with our observation that approximately 25% of PKC activity for phosphorylation of endogenous substrates remains after 4 h of TPA exposure, while the level at 20 h is not measurable.

To test the alternative possibility that TPA treatment had altered the uptake of \(^{32}P\) in cells and thus altered the specific radioactivity of the \(^{32}P\) pool available for phosphorylation of the 80-kDa protein, we quantitated the amount of \(^{32}P\) incorporated into protein by using trichloroacetic acid precipitation. Cells that were TPA-treated for 1, 4, or 20 h showed protein specific radioactivities of 0.8- to 2.2-fold those of untreated controls. They did not show an increase in incorporation of \(^{32}P\) into protein that paralleled the increase in phosphorylation of the 80-kDa protein (Table III). For example, Cl 25 shows a 1.5- to 1.6-fold increase at all time points, including the 20 h when stimulation of phosphorylation of the 80-kDa protein is not seen. Also, Cl 30 is not increased at 4 h when stimulation of phosphorylation of the 80-kDa protein is observed. Also, the radioactivity in the soluble (cytosolic) fraction was unaffected by TPA treatment for 1 h, the time at which the 80-kDa protein was maximally stimulated (Fig. 5). These data indicate that the observed increase (Fig. 5) in phosphorylation of the 80-kDa protein is not due to increased \(^{32}P\) uptake into TPA-treated cells, but reflects a true increase in pp80 phosphorylation.

The phosphorylation of 80-kDa PKC substrate and the 80-

\(^{32}P\) incorporation into protein after TPA treatment

| Clone | Phenotype | Control | TPA treatment (h) |
|-------|-----------|---------|-------------------|
|       |           | 1       | 4                 | 20                |
| Cl 41 | P*        | 1.0     | 1.1               | 1.8               | 2.2               |
| Cl 22 | P*        | 1.0     | 0.8               | 0.9               | 1.0               |
| Cl 30 | P-        | 1.0     | 1.1               | 1.0               | 0.9               |
| Cl 25 | P*        | 1.0     | 1.2               | 1.1               | 1.6               |

B. M. Smith et al., unpublished data.
promoter-induced signal transduction that might account for protein, which stays in solution after boiling. protein labeled by [\(^{32}P\)]orthophosphate treatment of intact cells polyacrylamide gels of 0.75-mm thickness. Gel slices were inserted into the stacking gel of a standard reaction mixture (lane 1); and the TPA-stimulated 80-kDa protein labeled by [\(^{32}P\)]orthophosphate treatment of intact cells (lane 2). Samples were boiled after harvest to partially purify the 80-kDa protein, which stays in solution after boiling. B, gel slices corresponding to the 80-kDa bands were excised from wet, freshly run SDS-polyacrylamide gels of 0.75-mm thickness. Gel slices were inserted into the stacking gel of a 1.5 mM gel and treated with staphylococcal V8 protease (1 or 10 g/ml). The resulting peptide maps are indicated.

lysates is the same as the 80-kDa protein from TPA-treated intact cells. This finding further emphasizes that substrates identified in cell lysates may also be substrates in the intact cell, and in this case, may be a marker for a transformation essential event.

**DISCUSSION**

This investigation has considered two components of tumor promoter-induced signal transduction that might account for the difference in sensitivity to phorbol ester-promoted transformation in JB6 cell variants: a difference in activity and a difference in substrates phosphorylated by protein kinase C, the phorbol ester receptor (5-7, 18). We have measured total PKC activity in JB6 cells by chelator extracting PKC into the cytosolic fraction, a procedure that results in 95% or more of PKC activity in the cytosol. The lack of any consistent phenotypic difference in PKC among the variants (Table I) indicates that a deficiency in protein kinase C activity is not the cause of the resistance of the P- cells. Others have also observed no PKC deficiency in variants resistant to phorbol ester induced effects. Herschman (19) reported that 3T3 cell variants resistant to TPA-induced mitogenesis exhibit PKC activity comparable to that in mitogen-sensitive cell lines; while Garte et al. (20) found that protein kinase C activity was similar in several mouse strains sensitive or resistant to tumor promotion by TPA (20). Whether P+ and P- cells differ in PKC translocation was not addressed by these experiments.

To compare substrates among cells of different phenotypes, we first characterized calcium- and phospholipid-dependent phosphorylation of endogenous proteins in JB6 cell lysates (Figs. 1 and 2). We identified 13 protein kinase C substrates in JB6 cell lysates with molecular masses ranging from 14 to 80 kDa. There was no qualitative difference in substrate phosphorylation among the phenotypes, indicating that lack of a substrate does not explain the resistance of the P- variants. It is noteworthy, however, that the level of the phosphorylated 80-kDa PKC substrate was quantitatively different and was inversely correlated with degree of neoplastic progression.

Others of the 13 PKC substrates identified in cell lysates may be candidates for mediating events on the promotion pathway. For example, the 23-kDa PKC substrate is sensitive to lanthanides, pharmacological analogs of calcium that are strong transformation promoters in JB6 cells (21). Increasing concentrations of lanthanum (50-300 \(\mu M\)) increased migration of the 23-kDa PKC substrate to an apparent molecular mass of 21 kDa in SDS gels, an effect not dependent on activation of PKC (21, 22). Calcium-binding proteins, some known to be PKC substrates, respond to lanthanum with such a band shift (23-25). It is noteworthy that we have found that extracellular calcium plays a regulatory role in TPA-promoted transformation (26).

Also, two-dimensional PAGE analysis has shown that TPA-treated intact JB6 cells exhibit TPA-sensitive protein phosphorylation of 23-, 25-, 37-, and 80-kDa phosphoproteins (12). On the basis of molecular weight, abundance, PI, and changes in response to TPA or phospholipids, these phosphoproteins appear to be the same in vivo as well as in vitro. It is possible that any of these phosphoproteins may transduce the TPA signal that ultimately results in neoplastic transformation.

Other investigators have also begun to characterize proteins in intact cells and in cell lysates that are PKC substrates (27-29). Purified proteins already identified as PKC substrates include proteins important in signal transduction and gene expression, such as receptors, other kinases or enzymes, proteins that regulate DNA metabolism, and cytoskeletal proteins. Substrates include receptors for insulin (30) and epidermal growth factor (31, 32), myosin light chain kinase (33), glycogen synthase (34) and tyrosine hydroxylase (35), phospholamban (36), topoisomerase II (37), and vinculin (38, 39). PKC substrate differences have been detected in genetic variants of EL4 thymoma cells sensitive or resistant to TPA-induced interleukin-2 production. In contrast to the present results with JB6 cells, EL4 thymoma cell variants resistant to phorbol ester effects lacked three substrates found in sensitive cells of 45, 40, and 36 kDa. One or more of these substrates could be essential in eliciting the interleukin-2 response in EL4 thymoma cells (40).

The 80-kDa substrate for PKC appears to be ubiquitous to...
and to be a major specific substrate for PKC (41). The protein apparently is found in a variety of species and tissue types and exhibits slightly different molecular weights from species to species and significant differences in antibody cross-reactivity (16, 17, 41). The ubiquitous nature of the 80-kDa PKC substrate suggests that it has some fundamental function in PKC-mediated signal transduction, that is perhaps usurped by phorbol esters during the promotion process. The isoelectric point of the 80-kDa protein as identified by orthophosphate labeling of intact cells was characterized in JB6 cells earlier (12) and appears similar to the pI of pp80 found in other studies (16, 17). The kinetics of dephosphorylation of the 80-kDa PKC substrate in 3T3 cells has been correlated with removal of PKC-activating agents, including phorbol esters, vasopressin, and bombesin (42, 43). These studies describe the half-life of the phosphorylated 80-kDa protein and also show that 80-kDa phosphorylation is uniquely dependent on protein kinase C activation.

It is assumed that the observed phosphorylation of the 80-kDa protein in TPA-treated cells (Fig. 5) reflects both new phosphorylation and dephosphorylation. The transient increase in net phosphorylation observed at 0-1 and 3-4 h may occur because PKC is first activated and subsequently down-modulated with accompanying loss of new substrate phosphorylation. Our measurements of PKC down-modulation after TPA indicate sufficient PKC activity in JB6 cells to produce stimulated phosphorylation of endogenous substrates at 0-1 and 3-4 h but not at 19-20 h, consistent with the observed results. Such an observation is in agreement with that of Wolfman and Macara (44). The kinetics of phosphorylation and dephosphorylation probably vary for different PKC substrates.

As discussed above, the P-, P*, and transformed JB6 cell variants can be distinguished by their level of phosphorylated 80-kDa PKC substrate. This quantitative PKC substrate difference represents the only detectable substrate variation among the three JB6 cell phenotypes. Using cell lysates, phosphorylation of the 80-kDa PKC substrate was greatest in the promotion-resistant cells, less in promotion-sensitive cells, and least in the transformed cells (Fig. 3). These data paralleled earlier findings that TPA-enhanced phosphorylation of an 80-kDa protein in intact cells was inversely correlated with phenotypic progression (12). In the present article we have shown by peptide mapping (Fig. 6) that the 80-kDa PKC substrate identified in cell lysates (Figs. 2 and 3), and the 80-kDa phosphoprotein from intact cells (Figs. 5 and 6) appear to be identical. This suggests that the 80-kDa PKC substrate is relevant to tumor promoter-induced signal transduction in the living cell.

Bishop et al. (45) have found that variants of 3T3 cells that are not sensitive to mitogenic stimulation by phorbol esters exhibit similar levels of phosphorylation of the 80-kDa protein. It may be the case that the 80-kDa protein is on a promotion pathway, and not on a mitogenic pathway. In fact, mitogenesis has been dissociated from promotion of transformation to JB6 cells (46).

The results presented here are compatible with the possibility that TPA may regulate the 80-kDa protein at the level of synthesis, at the level of phosphorylation, or at both levels. Experiments to distinguish among these possibilities are in progress, and are the focus of another article.

The 80-kDa phosphoprotein is a candidate for a negative regulator or suppressor of transformation in JB6 cells. Further studies will focus on approaches to elevate the phosphorylated 80-kDa phosphoprotein in transformed cells and to determine whether these cells consequently acquire nontransformed phenotype. If the 80-kDa phosphoprotein has a transformation suppression function, then elevation of it may cause phenotypic reversion to an earlier stage of progression.

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