Environmental Health Perspectives
Vol. 76, pp. 89-95, 1987

Role of Protein Kinase C in Regulation of Gene Expression and Relevance to Tumor Promotion

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The tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate (TPA) has highly pleiotropic effects on cells in culture and on tissues *in vivo*, including effects on protein kinase C (PKC) activation and gene expression. In order to determine the mechanism of activation of gene transcription by TPA, DNA sequences whose transcription is modulated in cells undergoing a mitogenic response to TPA were isolated by differential screening of a cDNA library from TPA-treated cells. TPA-S1 corresponds to an mRNA species whose abundance is increased within 1 hr of exposure of quiescent C3H 10T1/2 mouse embryo fibroblasts. TPA-R1 corresponds to an mRNA species whose abundance is decreased in TPA-treated cells. The induction of TPA-S1 is blocked by actinomycin D and is specific for phorbol esters with tumor-promoting activity. The transcription of this sequence is not induced by cycloheximide, nor is there an enhancement of the TPA response. Several lines of evidence demonstrate that PKC activation plays a critical role in the regulation of TPA-S1 expression. The nucleotide and predicted amino acid sequence of TPA-S1 exhibits homology with sequences representing a peptide with erythroid-potentiating activity, a metalloproteinase inhibitor protein, and a murine protein with β-interferon-like activity. The role of TPA-S1 in tumor promotion is suggested by the expression of this sequence in mouse skin carcinomas induced by dimethylbenzanthracene-TPA treatment, but not in papillomas or in control tissue. The consideration of signal transduction pathways may be useful in the design of short-term risk assessment assays for agents that act as tumor promoters.

Introduction

Any scientific approach to the problem of carcinogen risk assessment must consider that carcinogenesis is usually a multistage process, resulting in profound alterations in the control of cell proliferation and differentiation. The elucidation of the basis of these alterations is limited by our lack of understanding of the molecular mechanisms involved in regulation of proliferation and differentiation in normal cells. Recent studies of the responses of cells to specific growth factors and modulators of differentiation have identified several pathways of receptor-mediated signal transduction, involving specific second messengers (Fig. 1). The binding of growth factors to their membrane-associated receptors initiates a cascade of events, leading eventually to nuclear events and alterations in gene expression. It is of interest that several of the characterized oncogenes are homologous to various components in signal transduction pathways and the mitogenic response, including those related to growth factors (*sis*), growth factor receptors (*erb*-A, *erb*-B, *neu*, *fms*), and G proteins that are involved in signal transduction (*ras*). In addition, the expression of several oncogenes (*myc*, *fos*) is inducible in response to mitogenic stimulation (*1–4*). Therefore, the perturbation of pathways of responses of cells to growth factors may be a common mechanism in the transformation process.

Protein phosphorylation plays a central role in the signal transduction process (Fig. 1). Membrane receptors for platelet-derived growth factor (PDGF), epidermal growth factor (EGF), insulin, and colony-stimulating factor (CSF) have tyrosine kinase activity (*5*). The kinase activity of these receptors is activated by binding of the respective growth factor to the receptor. In addition, the response of cells to several mitogens is mediated, at least in part, by the stimulation of phospholipid turnover, through the activation of the membrane-associated enzyme phospholipase C. This results in the hydrolysis of phosphatidylinositol 4,5-diphosphate to generate diacylglycerol (DAG) and inositol triphosphate (IP_3) (*6*). DAG is an activator of an important serine and threonine protein kinase, which is Ca^{2+}- and

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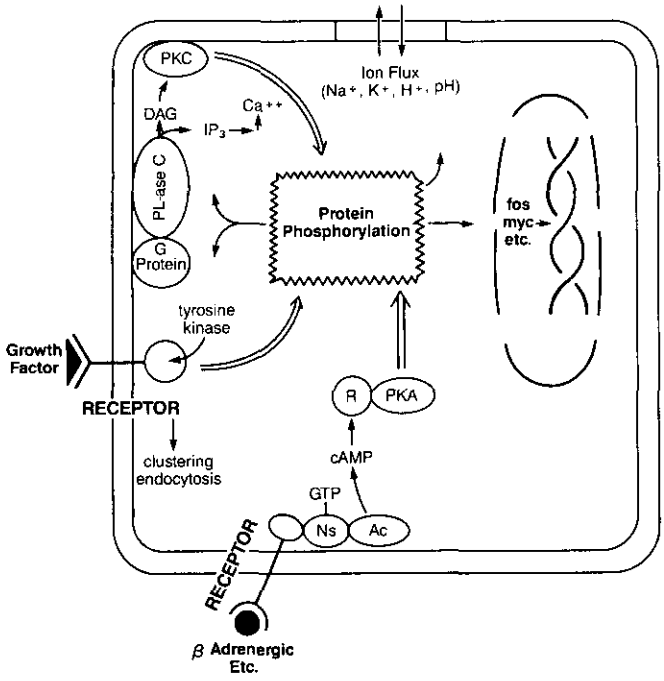

FIGURE 1. A schematic diagram showing various pathways of signal transduction that are mediated by protein phosphorylation. The growth factor receptors include those for EGF, PDGF, insulin, and colony-stimulating factor, which have tyrosine kinase activity. The β-adrenergic receptor pathway involves the coupling to adenyl cyclase (AC) through a G regulatory protein (Ns). The stimulation of AC activity results in the synthesis of cAMP, which binds to the regulatory subunit (R) or protein kinase A (PKA) and releases the catalytic activity. The activation of phospholipase C (PL-ase C), through an associated G protein, results in the generation of diacylglycerol (DAG), which activates protein kinase C (PKC), and inositol 1,4,5-triphosphate, which stimulates the release of calcium from the endoplasmic reticulum. Activation of these pathways has been associated with the induction of expression of specific genes, including c-*fos* and c-*myc*.

phospholipid-dependent, protein kinase C (PKC). PKC plays a central role in numerous cellular processes, including neurotransmitter release, hormone secretion, the action of several growth factors, and tumor promotion. PKC also has a wide range of substrates, including growth factor receptors, ion channel proteins, and cytoskeletal proteins (*7*). Thus, the activation of PKC can produce a highly pleiotropic response on cells.

Studies in our laboratory, and others, have demonstrated that PKC represents a complex gene family of related kinases (isozymes), based on the isolation of distinct cDNA clones and the purification of distinct forms of PKC proteins (*8*). The enzyme has two functional domains, a carboxy terminal region with consensus sequences for ATP binding and for kinase activity, and an amino terminal regulatory domain, which contains the putative sites for lipid, Ca^{2+}, and phorbol ester binding. The deduced amino acid sequences from PKC cDNA clones reveal considerable homology to other serine and threonine protein kinases, especially the catalytic regions of the cyclic AMP-dependent protein kinase (protein kinase A) and the cyclic GMP-dependent protein kinase (protein kinase G) (*8*).

The essential role of the PKC pathway in tumor promotion and cellular transformation was suggested by the observation that PKC is the major receptor for the potent tumor promoter 12-*O*-tetradecanoyl phorbol-13-acetate (TPA) (*9*), which binds to PKC and mimics the effect of DAG in the activation of the kinase activity of PKC (*10*). The treatment of cells with TPA and related tumor promoters induces a highly pleiotropic set of responses *in vitro*, as summarized in Table 1 (*11,12*). An important question is whether the activation of PKC mediates all of these complex effects of TPA on membrane function, mitogenesis, differentiation, and gene expression. Activators of PKC, such as synthetic diacylglycerols, can mimic many of the effects of TPA *in vitro* (*13,14*). Also, inhibitors of PKC have been shown to block specific effects of TPA (*15*). The observation that H-*ras*-transformed fibroblasts have elevated levels of diacylglycerol suggests that perturbations of the PKC pathway may also be involved in maintenance of the transformed phenotype in tumor cells (*16*).

Treatment of cells with TPA can induce or inhibit the expression of several mitogen-responsive genes (Table 2) (*1,11,17–21*). We have recently described the isolation and characterization of a cDNA clone (TPA-S1), which corresponds to a gene whose expression is markedly increased in murine fibroblasts exposed to TPA. We have also isolated a cDNA (TPA-R1) that corresponds to a gene whose RNA abundance is markedly decreased in cells treated with TPA (*21*). In this paper we describe these results and provide evidence that the induction of TPA-S1 RNA by TPA is mediated by PKC. We also briefly discuss the relevance of studies of PKC and signal transduction to the development of short-term assays for tumor promoters and the science of risk assessment.

Materials and Methods

Cell Culture. C3H 10T1/2 mouse embryo fibroblasts (*22*) were cultured within passage 12 in DMEM medium (Gibco) with 10% fetal bovine serum (FBS, Flow Laboratories) and 25 U/mL penicillin-25 μg/mL streptomycin (Gibco).

Reagents. The chemicals used in these studies were obtained from the following sources: DMSO (Aldrich

Table 1. Summary of TPA effects.

Tumor promotion
Cell proliferation
Cell morphology
Cell differentiation
Intercellular communication
Membrane transport
Growth factor-receptor binding
Phospholipid metabolism
Protein phosphorylation
Sister chromatid exchange
Gene transcription

Table 2. Summary of TPA-induced genes.

Protooncogenes	Growth modulation	Protease modulation	Cytoskeleton	Other proteins
c-*myc*	Ornithine decarboxylase	Plasminogen activator	Actin	Metallothionein
c-*fos*	Prolactin	MEP-Lysosomal protein	Vimentin	Transglutaminase
c-*sis*	MRP-Proliferin	Stromolysin		Calcitonin
c-*fms*	PDGF	Collagenase		
	Interleukin-1	Metalloproteinase inhibitor		
	T-Cell growth factor			
	BSF-Interferon B_2			
	Interferon-erythroid potentiating activator			

Chemicals); TPA, mezerein and 4α-phorbol didecanoate (4α-PDD) (LC Services); A23187 (Sigma).

RNA Isolation. Cells in culture plates were rinsed in cold phosphate-buffered saline (PBS), then lysed in a 4 M guanidinium thiocyanate (GTC). The RNA was isolated by centrifugation through a 5.7 M CsCl–0.1 M EDTA gradient. The poly A^+ fraction was isolated following two rounds of selection through an oligo dT cellulose column (*21*). RNA from mouse tissues was prepared by homogenization of frozen samples and isolated by a lithium chloride extraction method (*23*).

RNA Analysis. The RNA samples were elctrophoresed through 1% agarose-formaldehyde gels and blotted onto nylon membranes in 10× SSC. The blots were hybridized to nick-translated probes (TPA-S1 and TPA-R1) in 50% formamide, 5× SSPE, 5× Denhardt's, 10% dextran sulfate, and 20 μg/mL salmon sperm DNA, then washed with 0.1× SSC at 68°C and autoradiographed.

Results

A λGT10 cDNA library was synthesized from poly A^+ RNA obtained from quiescent C3H 10T1/2 cells 4 hr after treatment with 100 ng/mL TPA. Several clones were detected by differential hybridization analysis, two of which were isolated and characterized in detail. One of these corresponded to an RNA species that was induced by TPA treatment (TPA-S1) and the other to an RNA species inhibited by TPA treatment (TPA-R1). Figure 2 shows a Northern blot of poly A^+ RNA isolated from control cells and from cells treated with TPA for 4 hr, then hybridized to TPA-S1 and to TPA-R1 probes. TPA-S1 hybridized to a 0.8 kb transcript that was induced about 20-fold in response to TPA treatment (as determined by densitometric scan). TPA-R1 hybridized to a 4 kb transcript whose abundance was markedly decreased in response to TPA.

The induction of TPA-S1 RNA levels by TPA was transient, showing an increase after 1 hr of treatment, a maximum increase by 9 hr, then a return to basal levels by 24 hr (*21*). TPA-S1 was also induced to an equivalent extent after 4 hr of treatment with mezerein, a second-stage tumor promoter (Fig. 3). However, 4α-PDD, a nonpromoting phorbol ester, had no effect on TPA-S1 RNA levels, indicating the specificity of TPA-S1 induction for tumor-promoting phorbols. Also, treatment with the calcium ionophore A23187 showed no change in TPA-S1 RNA levels, although we have observed an increase in the abundance of ornithine decarboxylase RNA (data not presented). Replacing the culture medium with fresh medium containing 10% FBS resulted in an increased level of TPA-S1 mRNA, but to a lesser degree of induction than that observed with TPA. Addition of fresh bovine calf or fetal bovine serum to serum-depleted cultures resulted in a strong induction of TPA-S1 expression (data not presented). The role of PKC in the regulation of TPA-S1 is based on the concordance of agents that activate PKC (TPA, PDGF, serum, diacylglycerol, and EGF) with their ability to induce TPA-S1, the ability of PKC inhibitors to block the induction of TPA-S1 by TPA, and the inhibition of TPA-S1 inducibility in cells with down-regulated PKC activity.

TPA treatment of the 10T1/2 cells led to a reduction of TPA-R1 RNA levels with a time course that was similar to the TPA induction of TPA-S1 RNA levels. Mezerein and A23187 also caused a decrease in the levels of TPA-R1 RNA, while 4α-PDD had no appreciable effect (*21*). Although PDGF, serum, EGF, and diacylglycerol induced an increase in TPA-S1 RNA, the RNA level of TPA-R1 was not altered by treatment with these agents (data not presented). The mechanism of regulation is unclear, but the modulation of TPA-R1 RNA levels may occur by a PKC-independent process.

Northern blot analysis of RNAs from tissues isolated after treatment of mouse skin with DMBA, followed by repeated TPA doses [as described in (*23*)], revealed that TPA-S1 mRNA was not detectable in spleen or liver RNA samples. TPA-S1 was also not detected in papillomas induced by DMBA alone or by DMBA and TPA treatment. However, TPA-S1 was detected in two skin carcinomas induced by DMBA and TPA treatment (Fig. 4). Interestingly, the mRNA was not observed in tumors induced by DMBA alone. The transcript size was the same as that observed in mouse fibroblasts (0.8 kb). Hybridization of the TPA-S1 probe to RNA from other mouse tissues revealed extremely low basal RNA levels, with highest expression in the testes and lung, and lowest expression in liver, kidney, and brain (data not presented).

We have determined the complete nucleotide sequence of the TPA-S1 cDNA clone. The predicted amino acid sequence indicates a peptide of 205 amino acids with a molecular weight of 22.6 kd. The amino terminus of

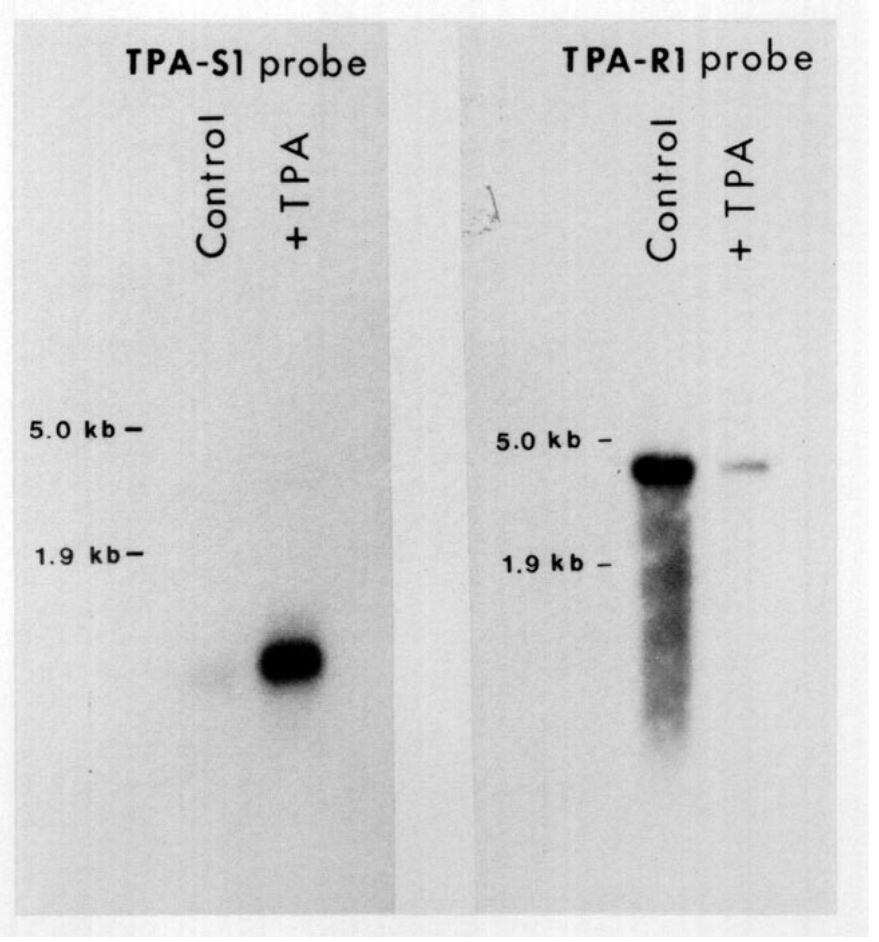

FIGURE 2. Effect of TPA on the expression of TPA-S1 and TPA-R1 transcripts. Poly A^+ RNA was isolated from postconfluent C3H 10T1/2 cells, 4 hr after treatment with either 0.1% DMSO (control) or 100 ng/mL TPA (in 0.1% DMSO). Each lane contains 10 μg of poly A^+ RNA, electrophoresed through 1.0% agarose-formaldehyde gel, blotted onto nylon membrane, hybridized to ^{32}P-labeled probes of TPA-S1 or TPA-R1, and then autoradigraphed. Location of ribosomal RNA bands (kb) are indicated.

this sequence contains a hydrophobic region with homology to signal peptides found in secreted proteins (*21*). Comparison of TPA-S1 sequence to the National Biomedical Research Foundation (NBRF) data base shows strongest homology (76%-DNA and protein) to a human cDNA sequence that corresponds to a protein that has erythroid potentiating activity (EPA) (*24*) and also to a human cDNA that corresponds to an inhibitor of metalloproteinases (*25*). These two human sequences are identical to each other. The alignment of these sequences with TPA-S1 is shown in Figure 5. TPA-S1 also shows 98% homology to a cDNA sequence that corresponds to a protein that has β-interferonlike activity (*26*). TPA-S1 is identical to another murine sequence (16C8), isolated independently from a cDNA library from serum-stimulated cells (*27*).

Discussion

The responses of cells to mitogens and tumor promoters reveal several common features, which we refer to as the "mitogenic program" (*4*). Included in these responses are effects on the expression of a specific set of genes, which demonstrate multiple levels of regulation. The induction of TPA-S1 by TPA appears to be a primary affect on gene transcription that does not require the synthesis of a *trans*-acting protein, unlike the induction of c-*myc* in PC12 cells, which requires protein synthesis for the activation of transcription (*28*). No change in the TPA-S1 RNA levels was observed in response to inhibition of protein synthesis; however, the induction of other mitogen-responsive genes, for example, c-*fos*, c-*myc*, and actin in fibroblasts, apparently involves labile repressor proteins that control transcription (*28*). Therefore, the control of gene expression in the mitogenic program can involve multiple levels of regulation. Studies on the pattern of expression of these genes must consider this complexity.

There are at least two possible mechanisms by which PKC might regulate the transcription of specific genes. One is that the activation of the kinase activity leads to the phosphorylation of proteins that translocate to the nucleus to activate transcription of a specific set of genes. An alternative hypothesis is that PKC or a fragment of the enzyme actually enters the nucleus where it affects transcription. It is of interest that the cystine-

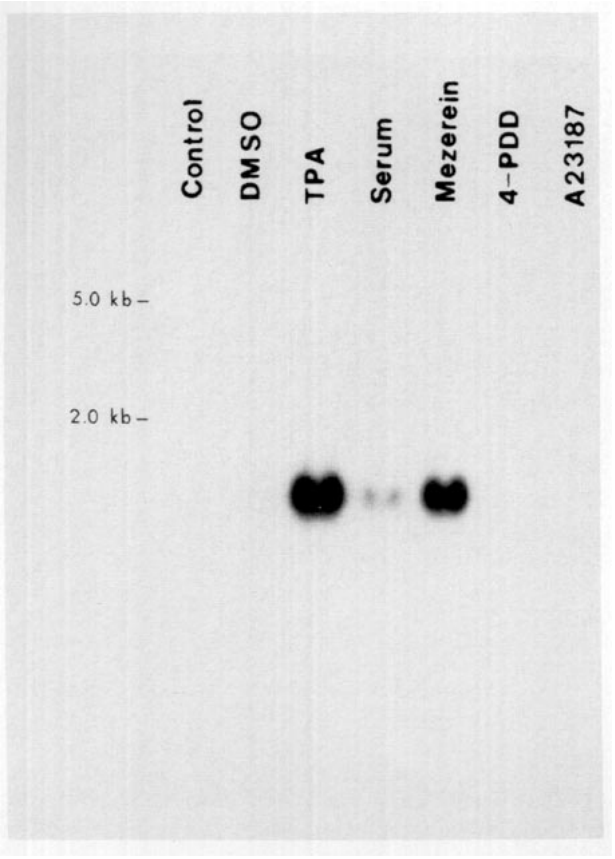

FIGURE 3. Specificity of TPA-S1 induction by mitogens and tumor promoters. Postconfluent C3H 10T1/2 cultures were treated with 0.1% DMSO, 100 ng/mL TPA, 100 ng/mL mezerin, 100 ng/mL 4α-PDD or 1.0 μM A23187, in 0.1% DMSO or a medium change with DMEM-10% fetal bovine serum. Total RNA was isolated after a 4-hr treatment with the various agents, then 10 μg of each sample was electrophoresed, blotted, hybridized to a ^{32}P-labeled TPA-S1 probe, and autoradiographed.

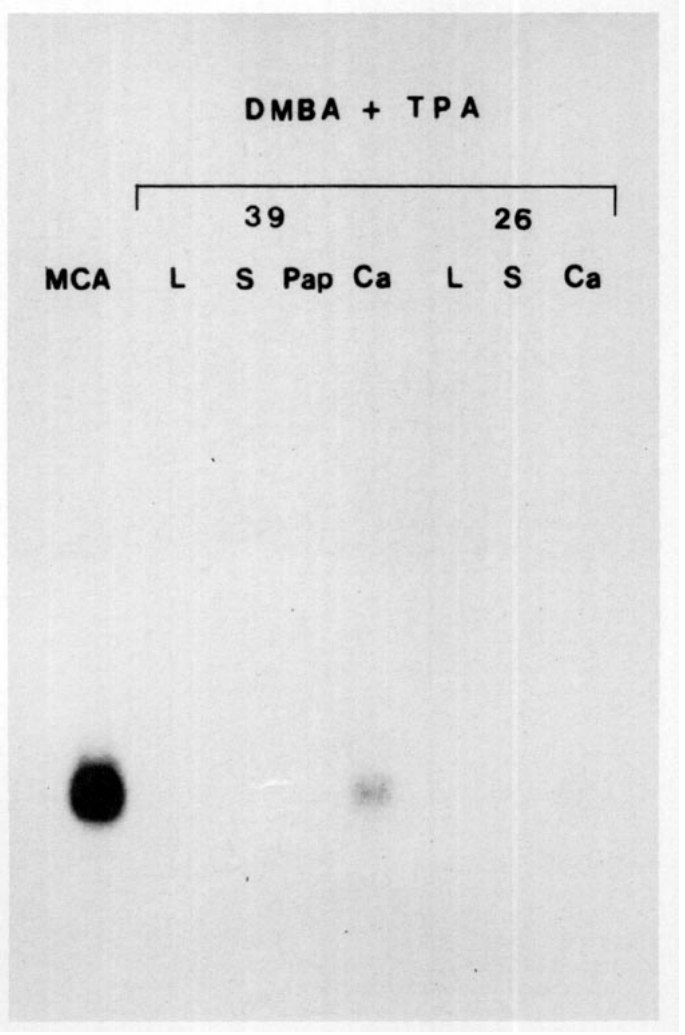

FIGURE 4. Expression of TPA-S1 in mouse skin tissues and tumors. HA/ICR mice were treated with dimethylbenz[a]anthracene and TPA, as described (*23*). Poly A$^+$ RNA was isolated from liver (L), spleen (S), papillomas (Pap) and carcinomas (Ca). The RNA from two animals (nos. 39 and 26) was gel electrophoresed (10 μg/sample), blotted, and hybridized to ^{32}P-labeled TPA-S1 probe and autoradiographed.

rich repeat elements in the amino terminal domain of PKC (*29*) display homology to sequences in several DNA-binding proteins, including the estrogen and glucocorticoid receptors (*30*), and may play a regulatory function in gene transcription. Although there is no direct evidence for either mechanism, the evidence that neither TPA (*31*) nor PKC (*7*) are present in appreciable amounts in the nucleus favors the former mechanism.

Several findings suggest that activation of PKC may not be sufficient to explain all of the effects of TPA. The induction of multinucleated cell formation in HTLV-I-infected cells by TPA is not affected by PKC inhibitors (*32*). The compound bryostatin is a potent activator of PKC, yet it lacks tumor-promoting activity on mouse skin and does not mimic the effects of TPA on cell differentiation (*33*). The activation of PKC by diacylglycerols is insufficient to induce the differentiation of HL60 cells (*34*). We have observed that the reduction in TPA-R1 levels by TPA was not mimicked by PDGF, EGF, serum, or diacylglycerol, nor was the effect of TPA blocked by PKC inhibitors (unpublished data). Therefore, it appears that PKC activation may be a necessary but insufficient step in mediating many of the biological effects of TPA. The existence of multiple forms of PKC that are differentially expressed in different tissues suggests that the regulation and physiological effects of each form may differ. This could explain some of the above discrepancies. Alternatively, there may be pathways of responses to TPA that are not mediated via PKC.

The significance of the homology of TPA-S1 to EPA (*24*), to a tissue inhibitor of metalloproteinases (TIMP) (*25*), and to a β-interferon (*26*) is unclear at present. Although it has been demonstrated that the EPA and TIMP activities are TPA-inducible, the role of these proteins in the biologic effects of TPA and other mitogens has not yet been determined. It is of interest that TPA treatment can result in the induction of proteases,

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                                                                                  *   *
TPA-S1      M M A P F A S L A S G I L L L L S L I A S S K A | C S C A P
EPA/TIMP    -         E P                   W       P   R   |   T   V

                              *
TPA-S1      P H P Q T A F C N S D L V I R A K F M G S P E I N E T T L Y
EPA/TIMP                                          V   T     V   Q

TPA-S1      Q R Y K I K M T K M L K G F K A V G N A A D I R Y A Y T P V
EPA/TIMP          E             Y       Q   L   D           F V       A

                    *
TPA-S1      M E S L C G Y A H K S Q N R S E E F L I T G R L R N G N L H
EPA/TIMP          V       F   R   H                 A   K   Q D   L

                    *                                                 *
TPA-S1      I S A C S F L V P W R T L S P A Q Q R A F S K T Y S A G C G
EPA/TIMP      T T       V A     N S     L     R   G   T       T V     E

              *         *         *               *
TPA-S1      V C T V F P C L S I P C K L E S D T H C L W T D Q V L V G S
EPA/TIMP    E                           Q   G                 L   Q

                                      *               *
TPA-S1      E D - Y Q R S R H F A C L P R N P G L C T W R S L G A R *
EPA/TIMP      K G F           L           E             Q     R S Q I A *
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FIGURE 5. Homology of TPA-S1 amino acid sequence to human EPA and TIMP. The predicted amino acid sequence of TPA-S1 is displayed, based on complete nucleotide sequence analysis (*21*). The alignment of this sequence with the predicted protein sequence for EPA (*24*) and TIMP (*25*) is shown. The first 24 amino acids contain the putative signal sequence of the nascent protein. The mark (|) indicates the predicted cleavage site for the signal peptide. Cysteine (C) residues are designated (*). The underlined regions represent the potential glycosylation sites.

Table 3. Potential assays for tumor promoters based on signal transduction pathways.

Direct activation of protein kinase C *in vitro*
Direct activation of phospholipase C *in vitro*
Stimulation of DAG production and PI turnover
Induction of translocation of PKC to membrane
Stimulation of phosphorylation of PKC substrates
Enhancement of T24-induced transformation in culture

such as plasminogen activator (*17*) and collagenase (*35*), as well as protease inhibitors, such as TIMP (*35*). These dual responses could provide a physiological mechanism by which cells could perform limited extracellular proteolysis. Therefore, alterations in the balance between these activities could be an important step in tumor development, invasion, and metastases. Studies are now underway to determine the biologic activity of TPA-S1 protein, using a retroviral expression vector system.

As the role of signal transduction pathways in the carcinogenic process is being uncovered, it may be useful to consider how these advances could lead to the development of short-term assays for agents that might act as tumor promoters. Possible assays which could be used to detect potential tumor promoters and related compounds are summarized in Table 3. These assays include the direct or indirect activation of PKC, which could be detected by specific substrate phosphorylation; the release of endogenous PKC activators; alterations in PKC-mediated gene transcription; and the enhancement of oncogene-induced transformation (*36*). At the present time, the major short-term tests for carcinogens detect only genotoxic agents, which seriously limits the process of risk assessment. It is hoped that assays of the type described in Table 3 will broaden the detection of agents that can enhance the carcinogenic process and therefore improve the science of risk assessment.

We thank Catherine Reznikoff for providing C3H 10T1/2 cells and James Murphy for technical support. This research was supported by NCI Grant CA 02656 (to I.B.W.), NIH Post-doctoral Fellowship Award CA 07870 (to M.D.J.), and the Medical Scientist Training Program (to G.M.H.).

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