Identification of the primary growth response gene, ST2/T1, as a gene whose expression is differentially regulated by different protein kinase C isozymes

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Abstract Individual protein kinase C isozymes have been shown to play different roles in mediating proliferation, differentiation and transformation, but it is not known to what extent these effects involve induction of expression of particular genes. To explore the differential gene expression that might be induced by activation of different PKC isozymes, we stably transfected NIH 3T3 cells with expression vectors that encode the isozymes PKC-α, -βII, -γ, -δ, -ε, -ζ and -η. Using differential display-reverse transcription-polymerase chain reaction we isolated a small cDNA that encodes a portion of the primary response gene, ST2 (also referred to as T1 or DER4), and we confirmed by RNA blot studies that ST2/T1 expression is differentially regulated by PKC isozymes. ST2/T1 mRNA is undetectable in the unstimulated parental NIH 3T3 cells that express only the α isozyme of PKC, but it can be induced by phorbol ester treatment. Clones that overexpress PKC-α, -δ or -ε similarly do not express ST2/T1 until they are stimulated with phorbol esters, which induces expression of ST2/T1 with kinetics similar to wild-type NIH 3T3 but to different extents. In contrast, ST2/T1 mRNA is already present in unstimulated cells that overexpress PKC-βII, -γ, -ζ and -η, but phorbol ester greatly enhances ST2/T1 expression in these cells. These results suggest a differential role for PKC isozymes in mediating the ST2/T1 expression that is induced by growth stimuli.

Key words: Protein kinase C (mouse); Isozyme; Differential display; ST2; T1; Expression

1. Introduction

The protein kinase C (PKC) isozymes comprise a family of at least eleven different serine/threonine kinases that are implicated in a variety of cellular responses including proliferation, differentiation, gene expression, membrane transport as well as secretion of hormones and neurotransmitters (see [1–3] for reviews). Initially, PKC was described as a Ca^{2+}- and phospholipid-dependent serine/threonine kinase [4,5], and later it was shown to be the major intracellular receptor for the tumor-promoting phorbol esters [6,7]. cDNA cloning and careful examination of the encoded proteins has revealed that the members of the PKC family can be classified into three major groups, namely, classical, cPKCs-α, -βI, -βII and -γ, [8]; novel, nPKCs-δ, -ε, -η, -θ, and -μ [9–15]; and atypical, aPKCs -ζ and -ι (the human counterpart of mouse aPKC-λ) [10, 16–18]. Only the cPKCs require Ca^{2+} for activity.

Most PKC isozymes appear to be quite similar in basic structural features and in means of activation, since they translocate from the cytosol to different intracellular sites upon activation with diacylglycerol (DAG) and phospholipids [2]. Phorbol esters, such as 12-*O*-tetradecanoylphorbol-13-acetate (TPA) mimic DAG in that they can bind and activate all PKCs, except the aPKCs, by substituting for endogenous DAG [6,7]. However, the different isozymes exhibit distinct tissue and cellular localization [19–22]. Furthermore, only certain isozymes are involved in particular physiological functions. For example, PKC-α and -δ, but not PKC-βII, -ε, -η or -ζ, mediate differentiation of myeloid 32D cells to mature macrophages [23]. What is more, overexpression of PKC-ε in NIH/3T3 cells leads to oncogenic transformation [24,25], whereas overexpression of PKC-δ inhibits proliferation in different cell systems [24,26]. The highly diverse biological actions of PKC clearly call for a determination of the specific biochemical functions of each PKC isoform.

In an early attempt to dissect the transcriptional responses to activation of the different PKC isozymes, Hata et al. [27] showed that overexpression of PKC-α, -βII, or -ε in 3Y1 cells enhanced transcription of c-jun, [which contains an upstream TPA-responsive element (TRE)], while overexpressed PKC-γ did not. PKC-γ did, however, induce transcriptional activation of genes that contain upstream serum response elements (SRE), as did all the other PKC isozymes tested. These experiments suggested that activation of the different PKC isozymes results in distinct and isozyme-specific transcriptional activities. To extend these studies, we used the method of differential display reverse-transcription polymerase chain reaction (DDRT-PCR, [28,29]) on NIH 3T3 cells that stably overexpress PKC-α, -βII, -γ, -δ, -ε, -ζ and -η [22,24]. To our knowledge, the data presented in this paper are the first comparisons of gene induction by 7 PKC isozymes within the same cellular environment.

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Abbreviations: DAG, diacylglycerol; DDRT-PCR, differential display, reverse transcription polymerase chain reaction; GAPDH, glyceraldehyde phosphate dehydrogenase; PKC, protein kinase C; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; TRE, TPA-responsive element; SRE, serum-responsive element.

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2. Materials and methods

2.1. Expression vectors

Overexpression of the 7 PKC isozymes [23,24] was achieved using pMTH and pLTR expression vectors. pMTH-βII,-δ, and -ζ were constructed by inserting the corresponding blunt-ended cDNAs into the blunt-ended *Bam*HI site of pMTH (kindly provided by Dr. G. Shen-Ong) which contains the mouse metallothionine promoter and the neomycin resistance gene as a selectable marker [30]. pLTR-α, which overexpresses PKC-α in the expression vector pLTR, and pLTR were obtained from Dr. N. Mazurek. pLTR contains the Rous sarcoma virus LTR enhancer and promoter and the mycophenolic acid resistance gene as a selectable marker [32]. pLTR-ε and -η were constructed by inserting the PKC-ε and -η cDNA *Eco*RI fragments into pLTR.

2.2. Generation of overexpressing cell lines

Our clone of NIH 3T3 cells expressed only PKC-α until it was transfected with each PKC expression vector, as described elsewhere [22–24]. Transfected cells were selected in the appropriate medium, and 12 resistant clones from each transfection were randomly screened for PKC protein expression. The clone that displayed the highest level of protein expression for each isoform was used in the following experiments. The PKC-γ-overexpressing NIH 3T3 cells (3T3-SG-g10) were a kind gift from Dr. J. Silvio Gutkind [33].

2.3. PKC kinase assays

PKC activity was determined on cell lysates that were partially purified over a DEAE column as described [23]. 5×10^6 cells were lysed in 500 μl lysis buffer that contained 30 mM Tris pH 7.5, 2 mM EDTA, 2 mM EGTA, 2 mM dithiothreitol, 0.5 mM PMSF and 5 mM benzamidine and sonicated on ice for 10 s. The insoluble fraction was removed by centrifugation at $14,000 \times g$ for 10 min. 10 μl of lysate was added on ice to 40 μl of assay mix that contained 20 mM Tris-HCl pH 7.5, 1 mM $CaCl_2$, 10 mM MgAc, 10 nM TPA, 80 μg/ml phosphatidylcholine, 20 μg/ml phosphatidylserine, 10 μM ATP/[γ-^{32}P]ATP (specific activity 20 Ci/mmol) and 0.2 mg/ml PKC-α substrate peptide (BRL). The reactions were incubated at 30°C for 10 min, and 25 ml of each reaction were spotted onto a Whatman DE-52 paper. The paper was washed three times with 0.1 M H_3PO_4 and once with acetone, air dried and counted. Background (phospholipid-independent) kinase activity was assayed under identical conditions without TPA and phospholipid and subtracted from the total kinase activity measured in the presence of TPA and phospholipid.

2.4. RNA isolation and Northern blot analysis

Poly(A)$^+$ RNA from clones of NIH 3T3 cells that overexpressed the different PKC isozymes was isolated following overnight starvation and stimulation with TPA for different time points as previously described [19]. 5 μg of each RNA were fractionated on 1% agarose gels containing formaldehyde. The RNA was transferred to Hybond-N nylon membranes (Amersham) by capillary blotting and hybridized overnight with 3×10^6 cpm/ml of random-primed cDNA probes, washed at high stringency (0.2× SSC, 0.1% SDS at 65°C) and exposed to Kodak XAR-2 film overnight or for 4 h in the case of glyceraldehyde-3-phosphate-dehydrogenase (GAPDH). For sequential hybridization of the same blots with different DDRT-PCR-generated probes, membranes were stripped with boiling water. After hybridization with these short cDNA probes, the blots were probed with a full-length cDNA of T1 [36] and with a probe specific for the 'housekeeping' gene GAPDH [34] to confirm the identity of the DDRT-PCR probe and to normalize for the amount of mRNA loaded in each lane, respectively.

2.5. Differential display

1 μg of mRNA from each of the different NIH 3T3 lines was reverse transcribed with the Superscript kit (BRL) following the manufacturer's protocol, using the oligonucleotide 5′ T_{11}-CA 3′ as a primer. DDRT-PCR was performed with 10 ng of single-stranded cDNA in each 50 μl reaction using Taq Polymerase (BRL) and the 5′ T_{11}-CA 3′ oligonucleotide as an anchor primer combined with an 10-mer arbitrary primer, taken from a primer set (primers OPA-01 to OPA-20, Operon Biotechnology Inc. Alameda, CA). dNTP concentration was 20 μM each, supplemented with 5 μCi of [α-^{33}P]dATP (3000 Ci/mmol; ICN Biomedicals, Inc.). Each of the 35 cycles of PCR included 1 min annealing at 40°C, 30 s extension at 72°C and 30 s denaturing at 94°C. The initial denaturating step lasted 2 min, the final extension 5 min. The reaction products were resolved on a 6% polyacrylamide sequencing gel. After electrophoresis the gel was dried on 3MM Whatman chromatography paper and exposed to a Kodak XAR-2 film overnight. Bands of interest were cut out of the gel, and the DNA was recovered by boiling the gel slice in H_2O for 15 min and subsequent ethanol precipitation in the presence of 0.3 M sodium acetate. DNA fragments were reamplified by PCR with the same primers used for the display PCR.

2.6. Cloning and sequencing

20 ng of each reamplified DNA fragment were phosphorylated using T4-kinase, ligated into 10 ng of *Sma*I-digested pBS (Stratagene), and the resulting DNA was transfected into electrocompetent DH10B cells (BRL) using electroporation. Positive colonies were identified by colony hybridization using the reamplified DNA fragment as a probe and confirmed by restriction digestion of DNA isolated from positive clones. Restriction fragments were purified on a 1% low melting point agarose gel and used as probes on Northern blots. DNAs that detected differentially expressed mRNAs were sequenced with the Sequenase sequencing kit (United States Biochemical Corporation) according to the manufacturer's protocol.

3. Results

Western blots of the overexpressing cell lines have been published elsewhere [22,24]. To ensure that the immunologically detected PKCs in each stably transfected, overexpressing clone of NIH 3T3 cells showed kinase activity, kinase assays were performed on cell lysates that had been partially purified as described [23]. The results of these assays, presented in Table 1, show that all the overexpressed PKC isozymes are active kinases.

To study TPA-induced genes in the different overexpressers, the cell lines were transferred to serum-free medium overnight and then treated with 100 nM TPA for 0, 15, 90, or 300 min. At each time point the cells were harvested, and poly(A)$^+$ RNA was isolated as described previously [19]. 1 μg poly(A)$^+$ RNA was reverse transcribed and used for the subsequent display PCR, using the oligonucleotide 5′ T11-CA 3′ as an anchor primer combined with the oligonucleotide 5′ TGCCGAGCTG 3′ (OPA-02) as an arbitrary primer.

As shown in Fig. 1, several amplified bands appeared to be differentially expressed in the different PKC overexpressers. Eight bands that were of potential interest (indicated by downward arrows) were excised, eluted and reamplified. The prod-

Table 1
PKC kinase assays of NIH 3T3 wild type and lines that overexpress PKC isozymes

Cell line	PKC activity cpm/mg protein
NIH 3T3	32,000
3T3-LTR-α2	58,000
3T3-MTH-β10	72,000
3T3-SG-γ10	64,000
3T3-MTH-δ9	58,000
3T3-LTR-ε2	78,000
3T3-MTH-ζ4	43,000
3T3-LTR-η1	74,000

The kinase activity was measured in cell lysates partially purified by stepwise elution of DEAE-52 columns as described [23] and is expressed as cpm ^{32}P incorporation into the PKC-α substrate peptide per μg lysate protein.

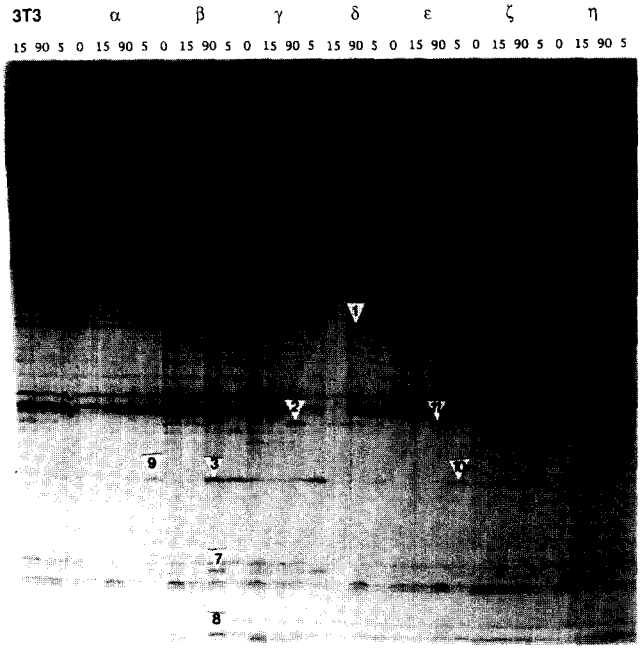

Fig. 1. Autoradiograph of PCR products resolved on a 6% polyacrylamide gel under denaturing conditions. cDNAs of either wild-type NIH 3T3 (3T3) or the PKC overexpressers (as indicated by the Greek letters on the top of the figure) which were stimulated with 100 nM TPA for 0, 15, and 90 min or 5 h (as indicated on the top) were used for the different reactions. The fragments that appeared to be differentially expressed and that were subsequently excised are indicated by downward-pointing arrows.

ucts of the reamplification were electrophoresed on an agarose gel (not shown). While most of the reamplifications resulted in a single DNA fragment that appeared to be of the same size as the original display PCR product, some reactions yielded fragments that did not amplify well (e.g. fragment 7) and some yielded fragments that were multiple (e.g. fragment 1) or inconsistent in size with the original DNA. These were not used for further experiments.

To evaluate which of the isolated DNAs represent differentially expressed RNA, the remaining six reamplified DNA fragments were labeled by random priming and used to probe selected Northern blots of RNA. Our experience indicated that it is desirable to perform this screening test early, since only one (fragment 9) of the six DNAs isolated from the display gel was, in fact, differentially expressed Two (fragments 3 and 10) did not hybridize on Northern blots and therefore were discarded as artifacts (probably due to contamination with genomic DNA); one (fragment 8) encoded a portion of the 18S ribosomal RNA, and two (fragments 2 and 4) were expressed essentially equally in all samples.

The single differentially expressed fragment was cloned and sequenced. The sequence of the 427-bp fragment showed 100% identity to the murine ST2 gene [35], also referred to as T1 [36] or DER4 [37]. Northern blots of RNA from overexpressing clones of NIH 3T3 that had been stimulated with TPA for 0, 15, 90, or 300 min were probed with the cloned and sequenced PCR fragment and subsequently, to avoid any misleading artifacts due to a PCR-derived probe, with a full-length T1 cDNA [36]. Both hybridizations yielded virtually identical results. As shown in Fig. 2, fragment 9 or ST2/T1 is expressed predominantly as a 3.2-kb mRNA, with an additional mRNA of ca. 6 kb visible in the samples that show high expression. Expression can be induced in wild-type NIH 3T3 cells by TPA treatment, reaching a maximum after 5 h. Similar results can be seen in the PKC-α, -δ and -ε overexpressers.

In the clones that overexpressed PKC-βII, -γ, -η, and -ζ, ST2/T1 is expressed even in the absence of TPA, but expression is induced to even higher levels with TPA. Interestingly, overexpression of the cPKCs (βII and γ) results in a peak of ST2/T1 expression at 90 min and a clear decline after 5 h, while the nPKCs (ε and η) as well as aPKC-ζ show either similar levels at 90 and 300 min (ζ and η) or highest levels of induction at 300 min (ε), similar in respect to the time course, but not to signal intensity, to wild-type NIH 3T3. We also probed bulk cultures of PKC overexpressers with the T1 probe and obtained results that were similar to those from the cloned cell lines shown in Fig. 2 (data not shown).

4. Discussion

ST2/T1 has been described as a primary response gene to growth stimuli in BALB/c 3T3 cells and as a $p21^{H\text{-}ras}$-inducible gene in NIH 3T3 fibroblasts [35,36]. Its expression can be induced by serum, growth factors and TPA and peaks after 6–10 h [35,38]. ST2/T1 belongs to the carcinoembryonic antigen family of tumor markers and shows high homology to the IL-1 receptor type 1 and 2. It is expressed predominantly as a 2.7-kb mRNA [37,38], and its gene product, a protein with an apparent molecular weight of 50–70 kDa (due to *N*-glycosylation heterogeneity) is secreted into the medium [38]. Another ca. 5.5-kb transcript [38,39], termed ST2L, seems to be an integral membrane protein. Its ligand, however, is still unknown. As shown in Fig. 2, we find bands of the expected size for both transcripts on the Northern blots. The difference from the sizes reported in the literature (we find them to be ca. 3.2-kb and 6-kb transcripts) is most likely due to the use of different RNA size markers. Our results confirm and extend the published findings that ST2/T1 is a PKC-inducible gene. It can be induced by TPA treatment of cells, but it is expressed at significantly higher levels if specific members of the PKC family are overexpressed. The fact that NIH 3T3 cells that overexpress PKC-ε, -ζ, and -η, the three isoforms that seem to induce transformation of NIH 3T3 ([24,40] and Goodnight and Mischak, unpublished), show a strong and prolonged ST2/T1 induction after PKC activation by TPA, makes it tempting to speculate that ST2/T1 might contribute to PKC-mediated transformation of NIH 3T3. This hypothesis correlates with reports of Werenskiold et al. [36] that ST2/T1 is an inducible gene and that ST2/T1 might be involved in the process of tumorgenicity. Experiments to test this notion involving the overexpression of ST2/T1 in NIH 3T3 cells are underway and will be reported elsewhere.

Our results also show that PCR display can be a powerful tool to pinpoint genes that are induced by only certain PKC isozymes. When comparing Figs. 1 and 2, the patterns of ST2/T1 induction can be seen both in the DDRT-PCR (in the bands indicated by arrows 3, 9 and 10), and, with similar kinetics, on the Northern blot. It is apparent that bands 3, 9 and 10 on the differential display (Fig. 1) are likely to represent the same gene

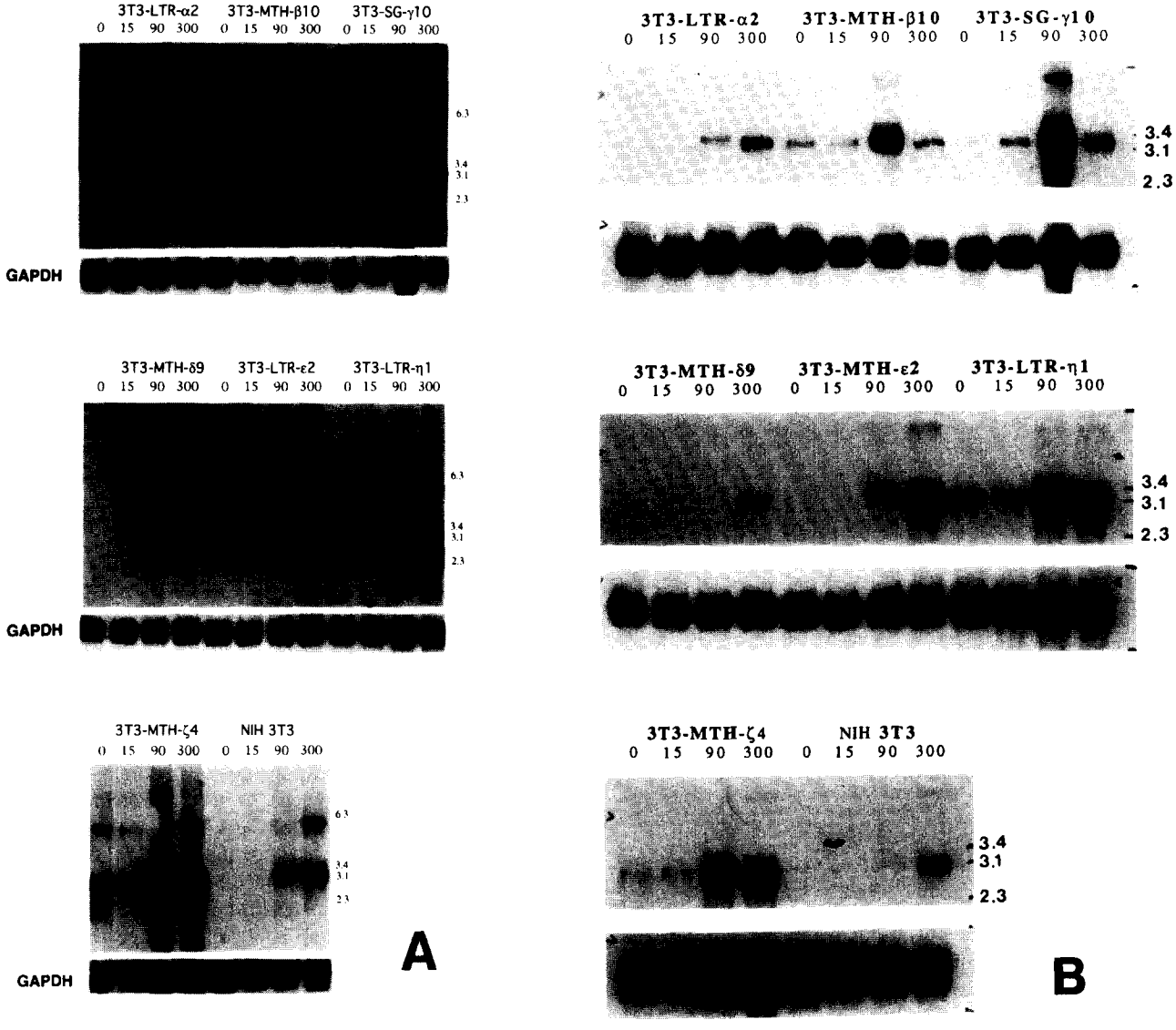

Fig. 2. Expression of ST2/T1 in NIH 3T3 cells. Northern blots of either wild-type NIH 3T3 or the different overexpressers that were stimulated with 100 nM TPA for the time (min) indicated on the top. 5 μg mRNA were loaded per lane. The blots were probed with cloned lane 9 product (panel A) and then with full-length T1 cDNA [36] (panel B). The size and position of RNA size standards are indicated on the right. The hybridization of these Northerns blots with GAPDH (to normalize for the amount of mRNA loaded in each lane) is shown below the lane 9 and T1 hybridization results.

transcript (ST2/T1), and bands 2 and 11 may also represent identical transcripts of a different gene. Variability in cloning of the different isolates may have contributed to the fact that only band 9 yielded usable data that permitted identification of the differentially expressed transcript as ST2/T1. It should be pointed out that the quantitative differences in ST2/T1 expression seen in several PKC overexpressers do not necessarily reflect direct activation of this gene's expression by PKC itself. Instead, they could be due to modulators downstream of PKC.

At first glance, the data on PKC-ζ in Fig. 2 suggest that PKC-ζ can be activated by TPA, which is contrary to the usual experience. It is more likely that overexpression of PKC-ζ leads to a constitutive expression of ST2/T1, which can be further enhanced upon stimulation of the endogenous PKC-α. It cannot be ruled out as yet that PKC-ζ can be activated by still unknown pathways (e.g. via phosphorylation by other kinases that are stimulated by TPA in vivo).

So far, only one report in the literature [27] showed clear differences in transcriptional activation of a reporter gene under the influence of SRE. The use of additional primers or other cell lines in PCR display should enable us and others to identify other genes that are differentially regulated by the different PKC isozymes. Cloning of their promoters should reveal the elements involved in PKC isozyme-specific regulation of transcription.

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