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Differential Regulation of Protein Kinase C Isoforms by v-Src

by

Qun Zang

A dissertation submitted to the Graduate Faculty in Biology in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

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Abstract

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by

Qun Zang

Advisor: Dr. David A. Foster

The protein kinase C (PKC) family consists of at least 11 distinct isoforms. In both murine and rat fibroblasts, we detected expression of the conventional PKC α, the novel PKCs δ and ε, and the atypical PKC ζ. Except for the atypical PKCs, membrane association has been used to determine PKC activation. In cells transformed by v-Src, there was a Ca^{2+}-dependent increase in membrane association of the α isoform relative to the non-transformed parental cells. Of the novel PKC isoforms δ and ε, the δ isoform was preferentially associated with the membrane in v-Src-transformed cells. Since it is not clear whether the subcellular distribution of aPKCs correlates with their activation, we could not determine whether ζ isoform is activated. PKC δ and PKC ε are both activated by exogenous diacylglecerol and phorbol ester. Thus, the differential activation of the δ and ε isoforms by v-Src suggests that the regulation of the novel PKC isoforms involves more complex mechanism.

We found that PKC δ co-immunoprecipitates with v-Src and is phosphorylated on tyrosine. The tyrosine phosphorylated PKC δ was primarily localized in membrane

fraction. However, tyrosine-phosphorylated PKC δ has reduced enzymatic activity relative to non-tyrosine-phosphorylated PKC δ. c-Src did not co-immunoprecipitate with PKC δ and an activated c-Src mutant (c-Src 527F) did, suggesting that the association between Src and PKC δ requires active Src kinase. An additional mutation at the N-terminal of c-Src 527F, abolishing membrane association of Src, prevented its association with and tyrosine phosphorylation of PKC δ. A deletion within the SH2 domain of Src did not prevent the Src/PKC δ interaction. Interestingly, both the association between c-Src-527F and PKC δ and the tyrosine phosphorylation of PKC δ were substantially enhanced by mutating the PKC phosphorylation site at Ser 12 in Src, suggesting that phosphorylation of Src by PKC destabilizes the interaction in a negative feedback loop. These data suggest a complex regulation of PKC δ isoform in which direct interaction between Src and PKC δ may result in PKC δ tyrosine phosphorylation and down-regulation of its kinase activity.

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Also, I want to extend my thanks to all my colleagues and collaborators: Drs. Jianguo Song and Ruchika Gupta for their great help when I joined the lab as a newcomer; Dr. Marcello Curto, Dr. Youwei Jiang, Paul Frankel and Zhimin Lu for their collaborations; Jingqing Luo, Sergey Bychenok and Armond Hornial for their helpful advises and discussions; Nancy Barile for technical assistants; all the other members in Dr. Foster's lab for providing a friendly environment. Dr. Irvin Gelman (Mount Sinai Medical School) and Dr. David Shalloway (Cornell University) generously provided cell lines and plasmids.

Finally, I would like to thank all the other members in my thesis committee, Drs. Laurel Eckhardt, Joseph S. Krakow, Marius Sudel and Lu-Hai Wang, for their advice and help; and Dr. Thomas Schmidt-Glenewinkel who let me in the program and gave me a lot of help, especially during the early time of my graduate study.

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List of Abbreviations

PKC: protein kinase C

cPKC: conventional PKC

nPKC: novel PKC

aPKC: atypical PKC

PLD: phospholipase D

PLC: phospholipase C

TPA: 12-*O*-tetradecanoylphorbol 13-acetate

EGTA: (ethylenebis(oxyethylenenitrilo))tetraacetic acid

DG: diacylglycerol

PS: phosphatidylserine

PC: phosphatidylcholine

PI: phosphotidylinositide

BAPTA/AM: bis-(O-aminophenoxy)-ethane-N,N,N',N'-tetraacetic acid tetra-(acetoxymethyl)-ester

DIC_8: 1,2-dioctanoyl-*sn*-glycerol

TRE: TPA response element

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Chapter I. Introduction

Since its discovery in 1977, protein kinase C (PKC) has been implicated as a critical component in number of signaling mechanisms because it is activated by a variety of extracellular agents, such as hormones, growth factors, neurotransmitters (Nishizuka, 1992a; 1988). These external stimuli cause the elevated levels of a second messenger, *sn*-1,2-diacylglycerols (DG), which binds to and activates PKC. PKC activation elicits cellular responses by phosphorylating target proteins on serine and threonine residues. It is now clear that PKC is a serine/threonine kinase family consisting of at least 11 distinct isoforms. Much more study remains to be done to define the specific activation, regulation and the biological roles of these PKC isoforms.

1. PKC family

11 members of PKC family have been identified to date by molecular cloning. These isoforms are derived both from multiple genes and from alternative splicing of a single RNA transcript (Coussens et al., 1986; Ono et al., 1987; Ono et al., 1988). They possess a primary structure containing conserved structure motifs with a high degree of sequence homology. Based on their activation requirements, this family has been divided into three major categories (Nishizuka, 1992a) (Figure 1). The schematic structures of PKC isoforms are illustrated in figure 1.

All of the PKC isoforms require phosphatidylserine for activation. The conventional PKCs (cPKCs) consist of four members: α, βI, βII and γ. Their activation requires both Ca^{2+} and DG. A second class of PKCs lack a conserved Ca^{2+}-binding

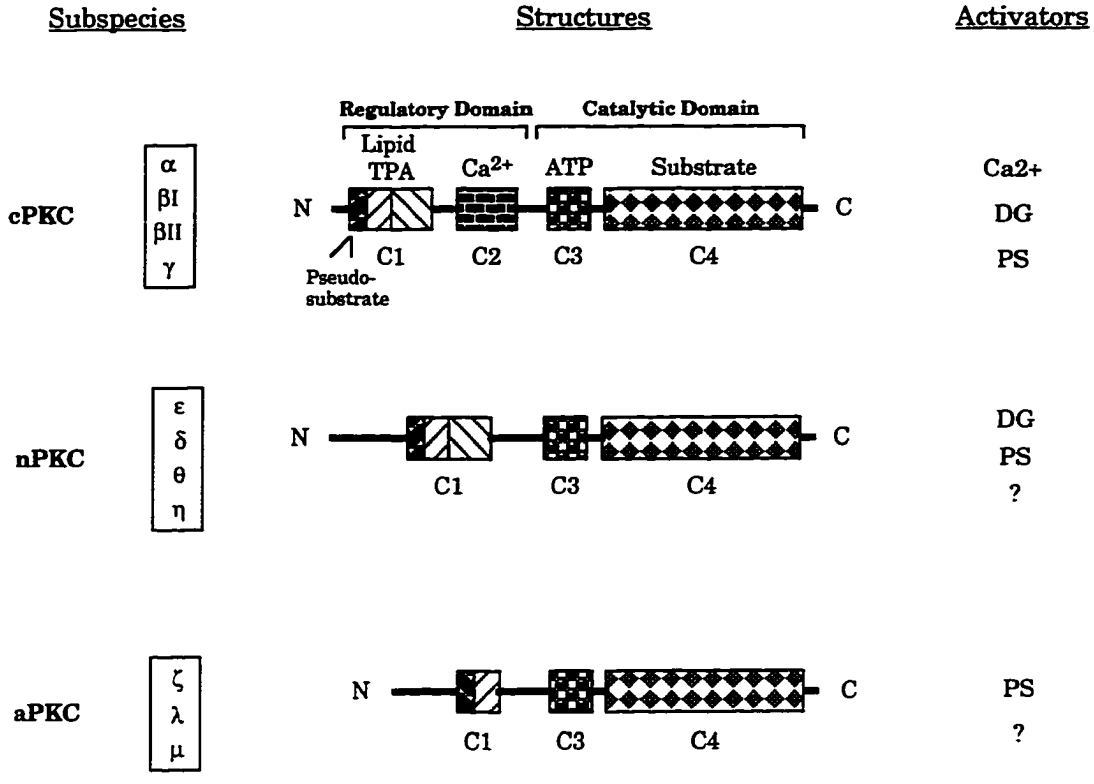


Figure 1. **Schematic of the structures of PKC isoforms.**

Showing here are the domains of PKC isoforms involved in binding lipid, phorbol ester and zinc (C1), Ca^{2+} (C2), ATP (C3) and substrates (C4). Also indicated at the N-terminus of C1 is the psudosubstrate domain. Ca^{2+}-independent PKC isoforms (nPKC and aPKC) lack the Ca^{2+} binding region. aPKC isoforms only have a truncated lipid binding domain (C1).

domain (C_2) and have been designated novel PKCs (nPKCs). This group includes the δ, ε, η, and θ isoforms. Their activation is independent of Ca^{2+}. The atypical PKCs (aPKCs), ζ , λ and μ, lack the C_2 Ca^{2+}-binding domain and possess only a single DG binding site, which apparently renders them insensitive to both Ca^{2+} and DG/phorbol esters. There is a pseudosubstrate domain for every PKC isoform. This domain has been proposed to bind to the active site of PKC and maintains the kinase in an inactive state by steric inhibition. Binding of activators to PKC molecules induces a conformational change that displaces the pseudosubstrate domain and thus allows access to substrates (Nelsestuen and Bazzi, 1991; Quest and Bell, 1994).

The reasons for PKC heterogeneity are not yet understood. Northern and western blot analysis have demonstrated that most cell types contain multiple PKC isoforms and the expression patterns of PKC isoforms are tissue and cell type specific (Nishizuka, 1988). For example, PKC α, β, δ and ζ are widely distributed. However, PKC γ is exclusively localized in brain and spinal cord (Nishizuka, 1988), and PKC η is expressed mainly in epithelial tissues (Bacher et al., 1991; Osada et al., 1993) . The distinct expression patterns and activation requirements suggest that the PKC family is not mere a family of isoforms with identical function, but rather different enzymes likely to have distinguishable functions.

2. Activation of PKC by diacylglycerols and reporter systems for PKC activation

Second messenger DG activates most PKC isoforms except the aPKCs. Tumor promoter, phorbol esters, mimics DG and activates PKC. In *in vitro* studies, over 50 DG analogues have been tested and the results showed that PKC activation by DG is stereo-

specific and requires *sn*-1,2-diacylglycerols (Boni and Rando, 1985; Ganong et al., 1986). Figure 2 illustrates the model of PKC activation by membrane bound lipids. However, it should be pointed out that since cPKCs were the first isoform group to be characterized in detail with the respect to their activation by lipids, the model described here relies heavily on the data obtained for this group of PKCs.

As shown in figure 2, elevated Ca^{2+} levels promote the localization of cPKCs at the membrane. Binding of DG increases the affinity of PKC to phosphotidylserine (PS) and strengthens PKC association with membrane (Mosior and Epand, 1993; Orr and Newton, 1992a; 1992b). DG together with PS induces a conformational change that exposes the pseudosubstrate domain (Orr et al., 1992) and makes a transition of PKC from a membrane associated to a membrane inserted state (Bazzi and Nelsestuen, 1988; Brumfeld and Lester, 1990; Lester and Brumfeld, 1990; Lester et al., 1990)

In most cells and tissues, inactive PKC resides within cytosol. Activation of PKC results in the translocation of PKC to membrane fraction (Kraft and Anderson, 1983; May et al., 1985; Wolf et al., 1985). Therefore, the level of membrane-associated PKC in stimulated cells is used as a measurement for its intracellular activation. The distribution of PKC isoforms can be detected by either immunoblotting or immumocytochemistry. The development of isoform specific PKC antibodies has greatly facilitated the study of distinct activation status of individual PKC isoforms. However, the activation of aPKCs, which are DG independent, can not be determined by their membrane association.

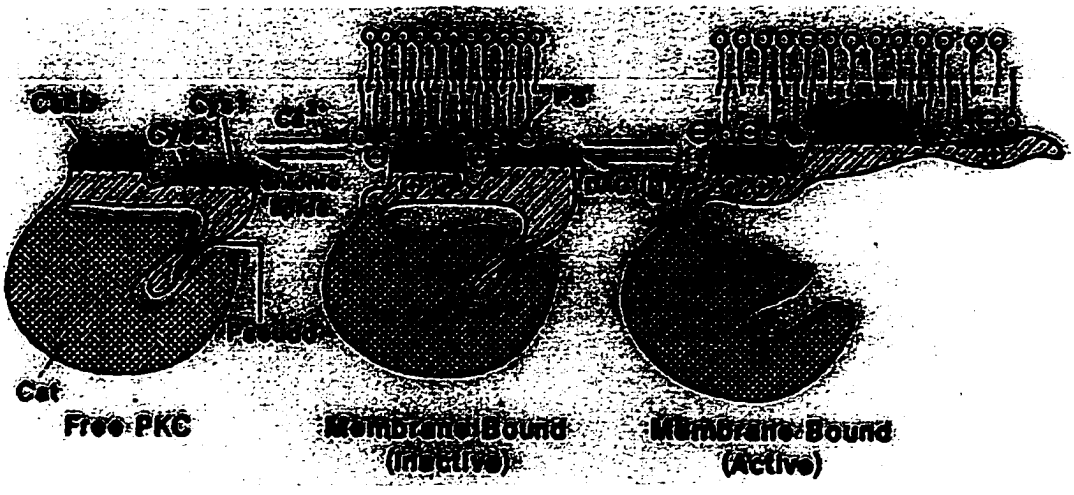


Figure 2. **Mechanism of cPKC activation by membrane bound lipids.**

Lipid interaction sites in regulatory domain of PKC are indicated as dark areas. Cys1 and Cys 2, cysteine-rich region 1 and 2 in lipid binding domain (C1) (see figure 1). CalB, calcium dependent lipid binding site in C2 region (see figure 1). Cat, catalytic domain. Figure shows three steps in the sequence of events leading to PKC activation. (1) PKC resides in cytosol in an inactive state with the pseudosubstrate motif buried within the catalytic domain. Initial PKC association with membrane mediated through CalB motif depends on the presence of calcium (left). (2) Upon association, in particular with PS (black circles), a conformational change could bring C1 region closer to the membrane to allow subsequent interaction with DG. In this stage, the pseudosubstrate domain has not yet been exposed and the kinase is not activated (middle). (3) DG binding within C1 region promotes insertion of the protein into the hydrophobic core of the membrane, which releases the pseudosubstrate domain from catalytic domain and activates the kinase (right).

Another widely used method for studying PKC activation is to detect the phosphorylation state of PKC substrates, such as myristoylated alanine-rich C kinase substrate (MARCKS). However, it is now clear that MARCKS is a substrate of multiple PKC isoforms (Fujise et al., 1994). So far, little is known about the *in vivo* substrates for individual PKC isoforms.

3. Is the activation of PKC isoforms differentially regulated ?

DG can be generated from various phospholipids by phospholipases, such as phospholipase C (PLC) and phospholipase D (PLD). PKC activation by DG generated from agonist stimulated phosphoinositide (PI) metabolism via PLC is well established (Leach et al., 1991). However, hydrolysis of other phospholipids, particularly PC by PLD or PLC in cell signaling has been suggested (Diaz-Laviada et al., 1990; Song et al., 1991).

Upon cell stimulation, PI hydrolysis by PLC results in a rapid and transient elevation of DG, frequently followed by a sustained DG increase (Nishizuka, 1992a). It was reported that the second phase DG formation is from PC hydrolysis in response to some mitogens, growth factors (Leach et al., 1991) and oncogenic v-Ras and v-Src (Diaz-Laviada et al., 1990; Song et al., 1991). The later phase increased DG maintains the sustained activation of PKC that is essential for long term cellular responses such as differentiation and proliferation (Aihara et al., 1991; Asaoka et al., 1991). Several mechanisms have been proposed for the formation of DG from PC (Nishizuka, 1992a; 1992b). A PLC which utilizes PC as a substrate has been suggested. However, PLC reactive with PC has not been purified. Several lines of evidences indicates that PC is hydrolyzed by PLD in an agonist dependent manner, resulting in formation of

phosphotidic acid (PA) that is converted to DG by PA phosphotase (PAP). Furthermore, mammalian PLD was detected in various tissue and cell types (Chalifa et al., 1990; Olson et al., 1991). Human PLD (Hammond et al., 1995) and plant PLD (Ueki et al, 1995)have been cloned recently.

The potential for specific PKC isoform activation by unique DG species is now being considered. It has been demonstrated that DG production does not always lead to PKC activation (Kujubu et al., 1991; Moscat et al., 1989) and that different stimuli generate different DG species (Cazaubon and Parker, 1993). DG species generated from PI and PC are different in their fatty acid composition (Foster, 1993). DGs with different fatty acid composition can activate PKC to different degrees (Ford et al., 1989; Leach et al., 1991). In addition, other lipids, such as arachidonic acids (McPhail et al., 1984), cis-unsaturated fatty acids (el Touny et al., 1990; Murakami et al., 1986; Sekiguchi et al., 1987), phosphatidylinositol(3,4)biphosphate (PI(3,4)P2) (Lee and Bell, 1991) and phosphatidylinositol(3,4,5)triphosphate (PIP3) (Nakanishi et al., 1993) are potentially selective activators of PKC isoforms *in vitro*. Therefore, it is plausible that PKC isoforms are differentially regulated by various combinations of DG, Ca^{2+}, PS and other lipid metabolic products.

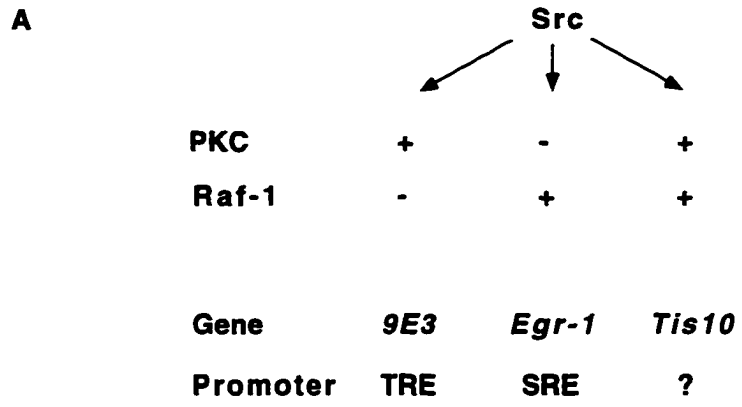
Protein modification has been suggested to be involved in regulation of PKC isoforms. It has been shown that PKC δ tyrosine phosphorylated is induced by various stimuli (Denning et al., 1993; Denning et al., 1996; Gschwendt et al., 1994; Li et al., 1994a; 1994b). However, how this phosphorylation affects kinase activity of PKC δ and what is the role of tyrosine phosphorylated PKC δ *in vivo* are unclear (see Chapters IV and V). No tyrosine phosphorylation has been found in other PKC isoforms. Thus, this

protein modification may be specific for PKC δ. In addition, phosphorylation of the α isoform on threonine is essential for the catalytic activity of PKC α (Cazaubon and Parker, 1993). Therefore, protein modifications could define the functional or regulatory specificities for different PKC isoforms.

4. PKC in v-Src induced signaling

In our lab, we are studying the intracellular signals induced by oncogenic tyrosine kinase v-Src. We and others have implicated PKC in the transduction pathway initiated by v-Src (Halsey et al., 1987; Nori et al., 1990; Qureshi et al., 1991; 1992; Sagara et al., 1986; Spangler et al., 1989; Wolfman et al., 1987). As summarized in figure 3A, PKC is required for the gene expression controlled by promoter TPA response element (TRE) (Qureshi et al., 1992) and the expression of *TIS10* gene (Qureshi et al., 1991) in v-Src-transformed cells. *TIS10* gene expression is not mediated by TRE (Kujubu et al., 1991). Thus, PKC is involved in the regulation of at least two transcriptional control elements - TRE and the transcriptional control element(s) that regulate(s) *TIS10* gene expression. The existence of multiple isoforms of PKC in the same cell could contribute to the activation of a subset of transcriptional control elements.

Consistent with PKC involvement in v-Src-induced intracellular signals, we found that v-Src induces an increase in DG, the physiological activator of PKC (Song et al., 1991). We further demonstrated that v-Src generates DG via a PLD that is specific for a subpopulation of PCs lacking arachidonic acid (Song and Foster, 1993). Further more, our recent data have shown that the induction of PLD activity by v-Src is dependent on



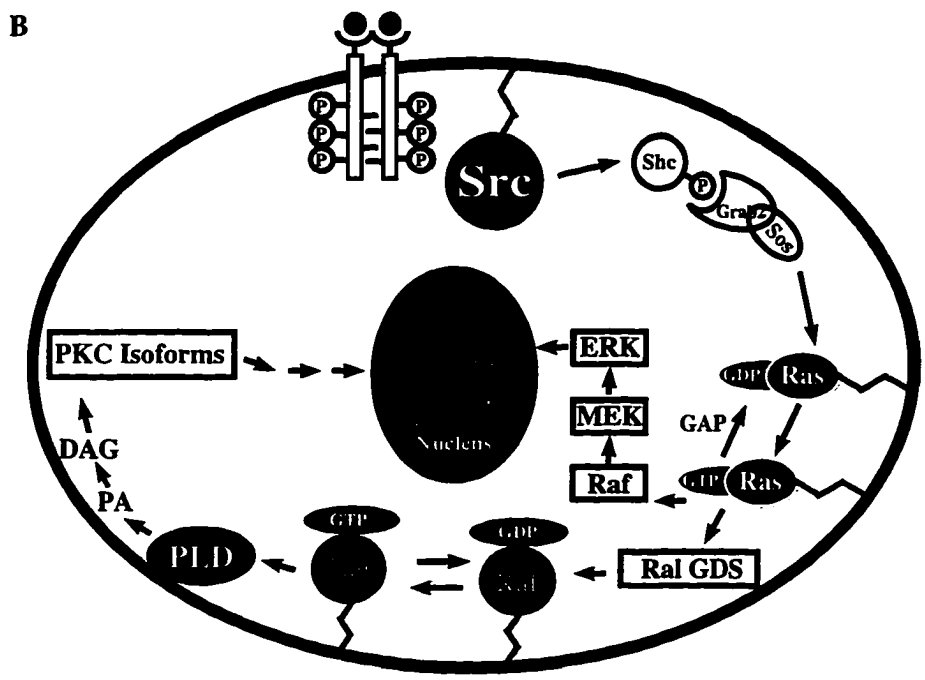


Figure 3. **A. v-Src induced gene expression.** v-Src induces the expression of genes *9E3*, *Egr1* and *Tis10*, and the genes controlled by promoters TRE and serum response element (SRE). We previously reported that those gene expressions are PKC (or Raf-1) dependent (+) or independent (-). **B. Model of v-Src induced signaling.** v-Src induces PLD activity via Ras and Ral. Activation of PLD results in increase of DG which activates PKC isoforms.

the function of small G proteins Ras (Jiang et al., 1995a) and Ral (Jiang et al., 1995b) (Figure 3B).

Thus, to better understand the role of PKC isoforms in the signaling pathways initiated by v-Src, we have examined the differential activation and regulation of PKC isoforms in v-Src-transformed rat fibroblasts.

Chapter II. Materials and Methods

1. Cells and cell culture conditions

Rat fibroblasts 3Y1, v-Src transformed 3Y1 cells (3Y1-NY72^{ts}) which express the NY72 temperature-sensitive mutant of v-Src in a Moloney murine leukemia virus vector (Mayer et al., 1986), mouse fibroblasts Balb/c 3T3, Balb/c 3T3 cells transformed with the Schmidt Rupin D (SRD) strain of Rous sarcoma virus were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% newborn calf serum (HyClone). The 3Y1-NY72^{ts} cells were maintained at the permissive temperature for transformation (35°C).

In some cases, Ca^{2+} was depleted by incubation with 25 μM BAPTA/AM (CalBiochem) or 10 mM EGTA (Sigma) for 30 minutes. The cells were treated with 100 μg/ml 1,2-dioctanoyl-*sn*-glycerol (DiC_8) (Sigma) and/or 5 μM ionomycin (Sigma) for 5 minutes. Genestein (Sigma) treatment, when used, was at 300 μM for 4 hours. TPA was added in the culture at 200 nM for 30 min to activate PKC or 800 nM for 24 hours to down-regulate PKC.

2. Antibodies.

For westernblot, monoclonal antibodies to PKC α and phosphotyrosine (4G10) were obtained from Upstate Biotechnology Inc. Monoclonal anti-PKC β was obtained from Seikagaku. Polyclonal antibodies to PKC δ, ε and ζ were obtained from Gibco BRL. Polyclonal antibodies to PKC γ, θ, and η were obtained from Santa Cruz Biotechnology.

The anti-vSrc monoclonal antibody was from Oncogene Sciences. For immunoprecipitation, polyclonal antibody to PKC δ was purchased from Calbiochem. Monoclonal anti-phosphotyrosine (PY20) was from Transduction Laboratories and the monoclonal antibody against v-Src was from UBI.

For the secondary antibodies, rabbit anti-mouse IgG was purchased from Zymed. Peroxidase conjugated anti-mouse IgG and anti-rabbit IgG were from Sigma.

3. Determination of DG levels

Confluent 60 mm culture dishes were prelabeled with isotopes for overnight in 2 ml of media containing 0.5% newborn calf serum. Isotopes were added to the culture media as follows: [^{3}H]-myristate, 2 μCi (40 Ci/mmol); [^{3}H]-arachidonate, 2 μCi (240 Ci/mmol). [^{3}H]-myristate (NET-830), and [^{3}H]-arachidonate (NET-2982) were obtained from New England Nuclear. Extraction of lipids was performed as described previously (Song and Foster, 1993). DG levels were characterized by TLC (silica gel 60A plates) using the following solvent system: hexane:diethylether:methanol: glacial acetic acid (90:20:3:2;v/v). Lipid standards were visualized by treating TLC plates with iodine vapor. To quantitate metabolically labeled DG, the TLC plate was subjected to autoradiography and the intensity of DG bands was determined using a Molecular Dynamics densitometer.

4. Subcellular fractionation

To examine the subcellular localization of PKC isoforms, cultures were allowed to get to approximately 85% confluence and then shifted to 0.5% DMEM for 24 hours. For

subcellular fractionation, cells from 150 mm culture dishes were washed three times with ice-cold isotonic buffer [phosphate-buffered saline; 136 mM NaCl, 2.6 mM KCl, 1.4 mM KH_2PO_4, 4.2 mM Na_2HPO_4, pH 7.2 (PBS)] and then scraped in 1 ml homogenization buffer (20 mM Tris-HCl [pH 7.5], 5 mM NaCl, 1 mM EDTA, 5 mM $MgCl_2$, 2 mM dithiothreitol). The cells were disrupted with 20 strokes in a Dounce homogenizer (type B pestle) and the lysate was centrifuged at 100,000 X g for 1 hour. The supernatant was collected and considered to be the cytosolic fraction. The membrane pellet was suspended in the same volume of homogenization buffer with 1% Triton-100. After incubation for 30 minutes at 4°C, the suspension was centrifuged at 100,000 g for 1 hour. The supernatant was collected as the membrane fraction. To prepare the total cell lysates, cells were lysed in homogenization buffer with 1% Triton-100. Lysates were clarified for 1 hour centrifugation at 100,000 g. Protein concentration was measured by Bio-Rad assay.

5. Western Analysis

Equal amounts of protein from both cytosolic and membrane fractions or total cell lysate were subjected to SDS-polyacrylamide gel electrophoresis using an 8% acrylamide separating gel and transferred to nitrocellulose. After blocking at 4°C overnight with 5% non-fat dry milk in PBS, the nitrocellulose filters were incubated with appropriate antibodies. The results were detected using the ECL system (Amersham) and SuperSignal™ western blotting reagent (Pierce).

6. Immunoprecipitation

Equal amount of cell lysates or subcellular fractions were incubated with proper antibodies at 4^0C for overnight. 40 µl protein A-agarose beads (Santa Cruz Biotechnology) was added to the lysates mixture for 3 hours to precipitate immunocomplex. Immunoprecipitates were washed three times with the immunoprecipitation buffer (10 mM Tris pH 7.5, 1 mM EDTA, 1 mM EGTA, 150 mM NaCl, 0.5% NP-40, 1% Triton-100, 0.1 mM PMSF, 10 µg/ml leupeptin and 10 µg/ml aprotinin) and were denatured with SDS sample buffer.

7. *in vitro* PKC assay

PKC activity of tyrosine-phosphorylated and non-tyrosine-phosphorylated PKC δ was determined according to protocols described before (Denning et al., 1993). Cell lysates from v-Src-transformed cells were immunoprecipitated with anti-phosphotyrosine antibody and phosphotyrosine-containing proteins were recovered with protein A-agarose beads. The supernatant was used as the source of non-tyrosine-phosphorylated PKC δ. The anti-phosphotyrosine immunoprecipitate pellet was resuspended in homogenization buffer containing 30 mM phenylphosphate to release the tyrosine-phosphorylated proteins. The antibodies were recovered by centrifugation and the supernatant was used as the source of tyrosine-phosphorylated PKC δ. Both the tyrosine-phosphorylated and non-tyrosine-phosphorylated preparations were then immunoprecipitated with anti-PKC δ antibody. The immunprecipitates were washed three times with immunprecipitation buffer and twice with (20 mM HEPES pH 7.5, 10 mM $MgCl_2$) followed by resuspension in 100 µl kinase buffer (20 mM HEPES pH 7.5, 10 mM $MgCl_2$, 1mM DTT, 100 µM ATP, 1 mg/ml histone type IIIS, 60 µg/ml phosphatidylserine, and TPA at 1 µM if included. PKC

activity was then determined as described previously (Joseph et al., 1992). The PKC δ levels in the assays was determined by western blot analysis and activity was normalized to these levels.

8. Transfections and plasmid vectors

3Y1 cells were plated at a density of 10^5 cells/100 mm dish 18 h prior to transfection. Transfections were performed using lipofectamine reagent (GIBCO) according to the vendors instructions. The plasmid expression vectors contained the G418-resistance marker and transfected cultures were selected in 400 ng/ml G418 for 8-10 days at 37°C. At that time colonies were examined for morphology, picked and expanded for further analysis. The c-Src mutants transfected into 3Y1 cells are as follows: c-Src 527F has a mutation of Tyr to Phe at position 527 (Kmiecik and Shalloway, 1987); c-Src 527F-A12 has an additional change at Ser 12 to Ala (Yaciuk et al., 1989); the LN mutation has 4 additional amino acids at the amino terminus (MAAA) (Bagrodia et al., 1993) and was placed in the c-Src 527F context as described for the A12 mutation (Yaciuk et al., 1989); the SH2 deletion of c-Src 527F-ΔSH2 has a disruption of the SH2 domain in which amino acids 148-187 have been deleted (Seidel-Dugan et al., 1992) and this mutation was placed in the c-Src 527 context as with the LN and A12 mutations (Yaciuk et al., 1989). All Src constructs were in the pEVX expression vector (Johnson et al., 1985; Kriegler et al., 1984).

Chapter III. PKC isoforms are differentially activated in v-Src-transformed fibroblasts

Introduction

As described in Chapter I, PKC is a serine/threonine kinase family with multiple distinct members and has been implicated in a wide variety of signaling mechanisms (Nishizuka, 1992a). We and others have implicated PKC in the transduction of intracellular signals initiated by tyrosine kinase v-Src (Halsey et al., 1987; Nori et al., 1990; Sagara et al., 1986; Spangler et al., 1989; Wolfman et al., 1987). We have further demonstrated that v-Src induced gene expression in murine fibroblasts via two distinguishable signaling pathways: one dependent upon PKC and another that is independent of PKC (Qureshi et al., 1991). The existence of multiple isoforms of PKC in the same cell could contribute to the PKC-dependent signals.

Consistent with the PKC involvement in v-Src signaling, we also found that v-Src stimulates a phospholipase D (PLD) activity, which subsequently leads to an increase in DG, the physiological activator of most PKC isoforms (Song et al., 1991). In order to understand the role of PKC isoforms in v-Src signaling, we started to examine the expression of PKC isoforms and their activation in v-Src-transformed rat and murine fibroblasts.

Results

1. PKC isoforms expression in 3Y1 and v-Src-transformed 3Y1 cells.

In order to determine which PKC isoforms are present in rat fibrobasts 3Y1 cells and 3Y1 cells expressing v-Src. Whole cell lysates from 3Y1 and 3Y1-v-Src cells were examined by western blot analysis. Using antibodies specific for the α, β, γ, δ, ε, η, θ, and ζisoforms, we determined that the α, δ, ε, and ζ were present in both the 3Y1 and 3Y1-v-Src cells (Figure 4); we were unable to detect the β, γ, η or θ isoforms (data not shown). By proceeding the same experiment in mouse fibroblasts BALB/c 3T3 cells and v-Src-transformed BALB/c 3T3 cells, we obtained the similar result as that in 3Y1 cells (data not shown).

These data are consistent with previous reports where the α, δ, ε, and ζ isoforms were shown to be expressed in rat (Borner et al., 1992a) and murine fibroblasts (Olivier and Parker, 1994). As shown in figure 4, the α, δ, ε, and ζ isoforms were present in approximately equal amounts in both parental cells and in v-Src-transformed cells. Thus, v-Src does not increase gene expression of these PKC isoforms in 3Y1 cells.

2. DG levels are elevated in v-Src-transformed cells

The cPKCs and nPKCs are activated by the lipid second messenger DG, which is generated by the hydrolysis of membrane phospholipids (Foster, 1993).

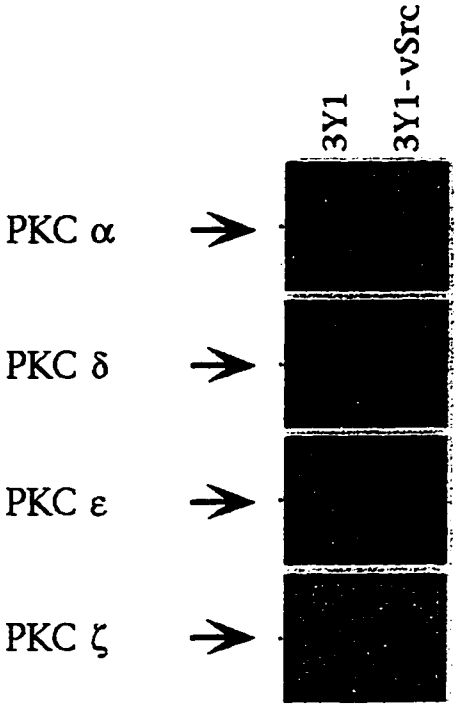


Figure 4. **Expression of the α, δ, ε and ζ PKC isoforms in 3Y1 and v-Src-transformed 3Y1 (3Y1-vSrc) cells.**

Whole cell extracts were normalized for protein amount and subjected to western blotting analysis using antibodies to specific PKC isoforms.

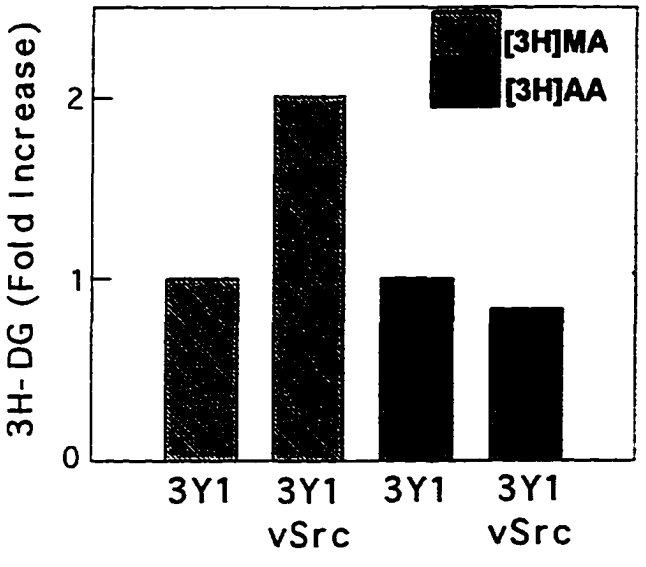


Figure 5. **DG levels in 3Y1 and v-Src-transformed 3Y1 cells.**

3Y1 and 3Y1-vSrc cells were prelabeled with either [^{3}H]-myristate (MA) or [^{3}H]-arachidonate (AA) as shown and DG levels were determined. Data are presented as the percent DG of the total cpm incorporated into the 3Y1 and 3Y1-vSrc cells using the [^{3}H]-myristate or [^{3}H]-arachidonate labels as shown. Data are the average of duplicates from a representative experiment that was repeated at least twice.

We previously demonstrated that v-Src induces increase of DG level by the phospholipase D/phosphatidic acid phosphohydrolase pathway (Song and Foster, 1993; Song et al., 1991). The phospholipase D activated by v-Src is specific for PC species lacking arachidonic acid but rich in myristic acid (Song and Foster, 1993). We therefore compared DG levels in 3Y1 cells and v-Src-transformed 3Y1 cells. Cells were prelabeled with either [^{3}H]-myristate or [^{3}H]-arachidonate; [^{3}H]-myristate is incorporated almost exclusively into PC, whereas [^{3}H]-arachidonate is incorporated into all of the major membrane phospholipids including PC (Song and Foster, 1993). As shown in figure 5, there were significant elevated levels of DG in v-Src-transformed 3Y1 cells relative to that in parental 3Y1 cells. If the cells were labeled with [^{3}H]-arachidonate, no such difference between the two cell types was detected. Thus, there is an elevation of DG in v-Src-transformed cells that is derived from PC, the source of v-Src-induced increases in DG (Song et al., 1991).

3. PKC isoform activation in v-Src-transformed 3Y1 cells.

Since the PKC translocation from cytosolic to membrane fractions has been used as a measurement of PKC activation in various systems (Borner et al., 1992b; Ha and Exton, 1993; Olivier and Parker, 1994). We therefore compared the subcellular distribution of the PKC isoforms in the 3Y1-v-Src and parental 3Y1 cells. PKC α is dependent upon both Ca^{2+} and DG for activation (Nishizuka, 1992). As a positive control for PKC α activation, we examined the effect of short term TPA treatment on the subcellular distribution of PKC α. As shown in figure 6, short term treatment with 200 nM TPA for 30 minutes caused the complete association of PKC α with the membrane

fraction in 3Y1 cells. Prolonged treatment with 800 nM TPA for 24 hours, which down-regulates most PKC isoforms, eliminated PKC α in these cells. The distribution of PKC α between the membrane and the cytosol in 3Y1 and 3Y1-v-Src cells is shown in figure 6. The α isoform is almost completely associated with the cytosolic fraction in the 3Y1 cells, suggesting that PKC α is not activated in these cells. However, in the v-Src transformed 3Y1 cells, a substantial portion of PKC α is associated with the membrane fraction. These data suggest that the α isoform of PKC is activated in response to v-Src.

We next examined the subcellular distribution of the Ca^{2+}-independent PKC isoforms δ and ε in the 3Y1 and 3Y1-v-Src cells. As demonstrated for the α isoform, short term TPA treatment lead to a complete association of both the δ and ε isoforms with the membrane fractions and long term TPA treatment lead to down-regulation of both of these isoforms (Figure 6). Analysis of the subcellular distribution of the δ isoform in 3Y1 and 3Y1-v-Src cells revealed a substantially enhanced membrane association of PKC δ in the v-Src transformed 3Y1 cells relative to the parental 3Y1 cells (Figure 6). In contrast, the ε isoform had the same subcellular distribution in both the 3Y1-v-Src and 3Y1 cells, in which this isoform fractionated almost exclusively with the cytosol (Figure 6). Thus, the Ca^{2+}-independent nPKCs δ and ε appear to be differentially activated in response to the kinase activity of v-Src.

The ζ isoform of PKC is both Ca^{2+}- and phorbol ester-independent (Nakanishi and Exton, 1992; Ways et al., 1992). Unlike the α, δ, and ε isoforms, a change in the subcellular distribution for the ζ isoform has not been demonstrated to correlate with or represent activation. The subcellular distribution of PKC ζ is not dramatically affected by either short term or long term TPA treatment (Figure 6). There were no significant

differences in the subcellular distribution of the ζ isoform in the 3Y1 and 3Y1-v-Src cells (Figure 6).

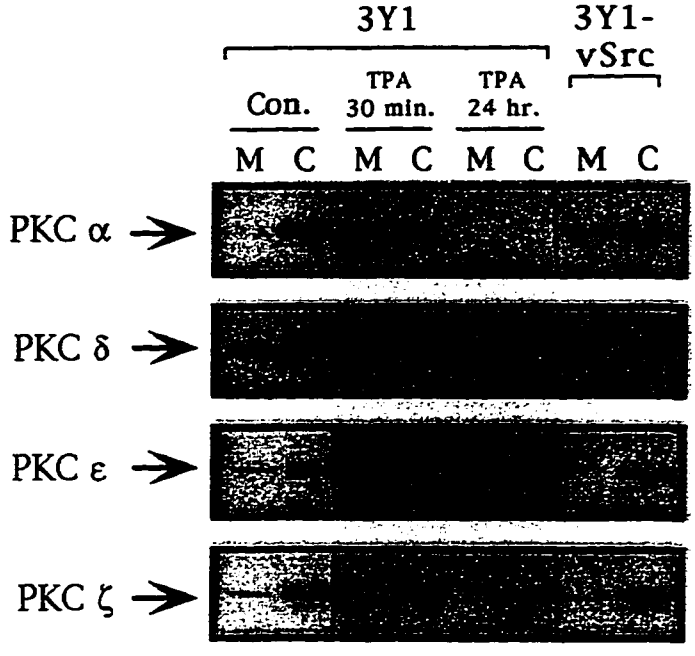


Figure 6. **Subcellular distribution of PKC isoforms in 3Y1 and v-Src-transformed 3Y1 cells**

Cytosolic (C) and membrane (M) fractions of the cells were prepared and subjected to western blot analysis using antibodies raised against the α, δ, ε, and ζ PKC isoforms as shown. As a positive control for activation, the parental cells were treated with TPA (200 nM, 30 min). The effect of prolonged exposure to TPA (800 nM, 24 hr) on the PKC isoforms in parental cells is also shown.

In order to illustrate if the activation of PKC α and δ is due to the kinase activity of v-Src, the effect of tyrosine kinase inhibitor, genistein, on the translocation of α and δ in 3Y1-v-Src cells was examined. As shown in figure 7, genistein treatment blocked the increased membrane association of the δ and α isoforms in the v-Src transformed 3Y1cells.

4. Ca^{2+}-dependence of PKC α membrane association in 3Y1-v-Src cells

The activation of the α isoform of PKC is dependent upon both DG and Ca^{2+}, whereas the nPKC isoforms δ and ε are Ca^{2+} independent (Nishizuka, 1992a). A role for Ca^{2+} in the signals activated by Src is not well characterized. We therefore examined whether the activation of the α isoform by v-Src required Ca^{2+}. To establish a role for Ca^{2+} in the activation of PKC α by v-Src, we tested the sensitivity of increased membrane association of the α PKC isoform in 3Y1-v-Src cells to the membrane-permeant Ca^{2+} chelator BAPTA/AM and the extracellular Ca^{2+} chelator EGTA. As shown in figure 7, the membrane association of the α isoform in 3Y1-v-Src cells was sensitive to both BAPTA/AM and EGTA. As expected, neither BAPTA/AM nor EGTA had any effect on the subcellular distribution of the δ isoform in either 3Y1 or 3Y1-v-Src cells. The same result was also obtained in BALB/c 3T3 and v-Src-transformed BALB/c 3T3 cells (data not shown). These data suggest that the increased membrane association of the α isoform of PKC in response to v-Src requires Ca^{2+}. Although the mechanism for generation of the Ca^{2+} needed for the v-Src-induced activation of PKC α requires further investigation, it is

likely to involve an influx of extra-cellular Ca^{2+} since EGTA, which chelates only extra-cellular Ca^{2+}, inhibited the membrane association of PKC α in the 3Y1-v-Src cells.

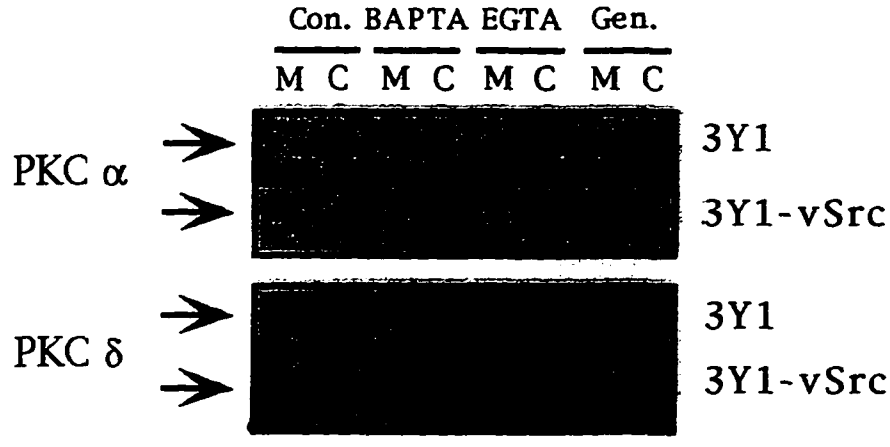


Figure 7. **Effect of Ca^{2+}-chelators and genistein on PKC α and δ activation in 3Y1 and v-Src-transformed 3Y1 cells.**

Ca^{2+} was depleted by incubation of the cells with intracellular Ca^{2+} chelator BAPTA/AM (25 μM) or the extracellular Ca^{2+}-chelator EGTA (10 mM). Protein-tyrosine kinase activity was inhibited by genistein treatment (300 μM). The control cells (Con.) were untreated with any of the compounds. BAPTA/AM or EGTA was added 30 minutes prior to harvesting of cells; genistein, 4 hr prior to harvest . The subcellular distribution of the α and δ isoforms was determined as in Fig. 6.

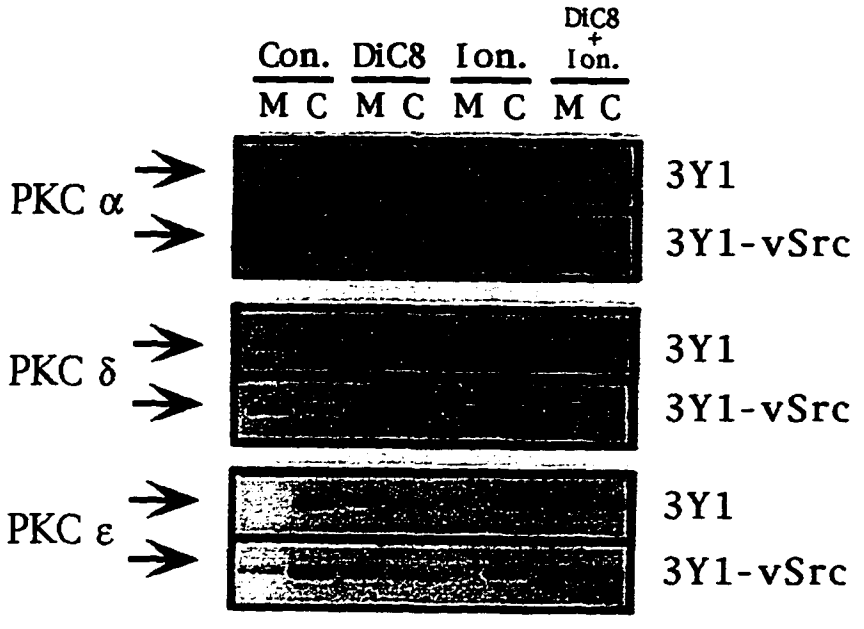


Figure 8. **Activation of PKC isoforms in 3Y1 and 3Y1-v-Src cells by DiC_8 and ionomycin.**

3Y1 and 3Y1-v-Src cells were either untreated (Con.) or treated with the soluble DG DiC8 (100 μg), ionomycin (5 μM) (I), and both DiC8 and ionomycin (D + I) as shown. DG and/or ionomycin were added 5 min prior to harvesting of cells. The subcellular distribution of the α, δ and ε isoforms was then determined as in Fig. 6.

To confirm the requirement of DG and/or Ca^{2+} for the activation of the PKC isoforms in 3Y1 and 3Y1-v-Src cells, we examined the effects of 1,2-dioctanoyl-*sn*-glycerol (DiC_8), a soluble DG, and the Ca^{2+} ionophore, ionomycin, on the subcellular distribution of the α, δ and ε isoforms. As shown in figure 8, incubation with DiC_8 alone induced the translocation of both PKC δ and ε but not α in 3Y1 and 3Y1-v-Src cells. Ionomycin had no effect on the translocation of any of the PKC isoform. However, DiC_8 and ionomycin induced complete translocation of PKC α in 3Y1 and 3Y1-v-Src cells. Since both DiC_8 and TPA were able to induce the membrane association of PKC ε, the lack of PKC ε activation in response to v-Src was not due to lack of responsiveness to diacylglycerol. The differential responsiveness of PKC δ and ε in v-Src transformed cells suggests more complex mechanism involved in regulation of novel PKCs.

5. PKC isoform activation in v-Src-transformed Balb/c 3T3 cells

In order to determine whether the phenomena of differential activation of PKC isoforms in response to v-Src observed in the 3Y1 rat fibroblasts could also be observed in another cell line transformed by v-Src. We therefore compared the subcellular distribution of the PKC isoforms in v-Src-transformed and parental murine BALB/c 3T3 cells. As shown in figure 9, there were substantial increases in the membrane association of the α and δ isoforms in the v-Src-transformed cells relative to the Balb/c 3T3 cells; whereas, there was no difference in the membrane association of PKC ε in the two cell lines. Thus, qualitatively, v-Src induces the same pattern of PKC isoform activation in the murine Balb/c 3T3 cells as that observed in the rat 3Y1 cells. A small but reproducible increase in the membrane association of the ζ isoform was observed in the v-Src-transformed

BALB/c 3T3 cells. However, as discussed above, it is not clear that such changes in membrane distribution correlate with activation of this PKC isoform.

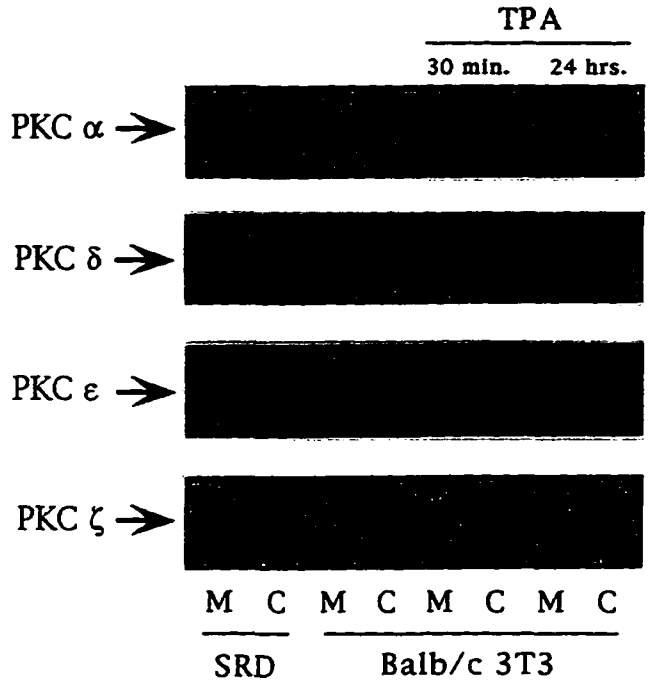


Figure 9. **Subcellular distribution of PKC isoforms in BALB/c 3T3 and v-Src-transformed BALB/c 3T3 (SRD) cells.**

Cytosolic (C) and membrane (M) fractions from v-Src-transformed (SRD) and parental Balb/c 3T3 (BALB) cells were prepared and subjected to Western analysis using antibodies raised against the α, δ, ε, and ζ PKC isoforms as shown. As a positive control for activation, the BALB/c 3T3 cells were treated with TPA (200 nM, 30 min). The effect of prolonged exposure to TPA (800nM, 24 hr) on the PKC isoforms in BALB/c 3T3 cells is also shown. Because of the weak expression of PKC δ in these cells, we enriched this isoform by partial purification using chromatography as described previously (Joseph, 1992). In the PKC ζ detection, the high molecular weight band present in the membrane fraction of Balb/c 3T3 cells after short term TPA treatment is likely PKC α which is weakly cross-reactive with ζ antibody [Ha, 1993 #927].

Discussion

The role of PKC in the transduction of many diverse intracellular signals is well established (Nishizuka, 1992a); however, the molecular mechanisms for the activation of the different PKC isoforms are more complex than originally proposed. In this part of our work, we have shown that in cells expressing the oncogenic protein-tyrosine kinase v-Src, there is a selective activation of the α and δ isoforms of PKC. The ε isoform is not activated by v-Src despite the production of DG. We were not able to detect substantial differences in the cellular distribution of the ζ isoform in v-Src-transformed 3Y1 cells, but there was a small increase in membrane association of the ζ isoform in v-Src-transformed Balb/c 3T3 cells. It is not known whether membrane association represents activation of the ζ isoform; however, since PKC ζ has been implicated in mitogenic signaling (Berra et al., 1993), a role for PKC ζ in v-Src-induced signaling can not be ruled out.

It was previously shown that the nPKC isoforms δ and ε are co-activated in response to bombesin and platelet-derived growth factor (Olivier and Parker, 1994). Additionally, the δ and ε isoforms were shown to be co-activated in response to both serum and EGF in 3Y1 rat fibroblasts (Ohno et al., 1994). However, we found that the δ and ε isoforms are not co-activated in response to v-Src. A possible explanation for the selective activation of the δ isoform in response to v-Src is the generation of unique DG species. It was recently shown that a differential activation of the α and ε PKC isoforms could be explained simply by the presence or absence of Ca^{2+} and not by different DG species that might be generated (Ha and Exton, 1993). However, the preferential activation of the δ over the ε isoform observed in v-Src-transformed cells can not be

explained by a difference in Ca^{2-} production since both of these isoforms are independent of Ca^{2+}. Thus, it is possible that the DG species generated in response to v-Src might preferentially activate the δ isoform. We previously demonstrated that v-Src generates DG via a phospholipase D that is specific for a subpopulation of PC lacking arachidonic acid (Song and Foster, 1993). While the basis for this apparent aliphatic specificity is not understood, it is possible that specific DG species are generated in order to activate specific PKC isoforms. Several reports have demonstrated that different DG species are differentially phosphorylated by DG kinase (Ford and Gross, 1990; Lee et al., 1991; Song et al., 1994) Additionally, it has been demonstrated that DG production does not always lead to PKC activation (Daniel et al., 1993; Moscat et al., 1989) and that different stimuli generate different DG species (Pessin et al., 1990). Thus, there is precedent for biological specificity at the level of the aliphatic composition in the generation, metabolism and effector function of DG which could contribute to the differential activation of the δ and ε PKC isoforms observed here.

Whether activation of either PKC α or δ plays any role in the transformed phenotype induced by v-Src has not been established. PKC α has been implicated in the regulation of cell proliferation since overexpression of the α isoform has been reported to enhance cell growth potential (Liao et al., 1994). However, it was reported that overexpression of the δ isoform in NIH 3T3 cells caused cells to grow more slowly and to lower cell densities; whereas the ε isoform caused opposite effects (Mischak et al., 1993). Thus, how v-Src overcomes the inhibitory effect of PKC δ to maintain cell transformation becomes a big question. It was recently reported that tyrosine phosphorylation of PKC δ can suppress its enzymatic activity in murine keratinocytes overexpressing v-Ras (Denning

et al., 1993) or stimulated with EGF (Denning et al., 1996). However, PKC δ that was phosphorylated on tyrosine by various tyrosine kinases *in vitro* had enhanced its kinase activity (Li et al., 1994a). Therefore, the regulation of PKC δ in v-Src-transformed cells may involve both DG and post-translational modification. Overall, specific roles for PKC α and δ in the signals initiated by v-Src remain to be determined.

Chapter IV. PKC δ is tyrosine phosphorylated and associated with v-Src in v-Src-transformed 3Y1 cells

Introduction

In both murine and rat fibroblasts transformed by the oncogenic tyrosine kinase v-Src, there is an increased membrane association of the α and δ, but not the ε or ζ PKC isoforms (Chapter III). Since the δ and ε PKC isoforms both belong to the Ca^{2+}-independent class of PKC, the preferential increase in membrane association of the δ over the ε isoform suggests that regulation of this class of PKC isoform involves more than simply elevating DG levels.

The selective increase in membrane association of the δ over the ε isoform of PKC in v-Src-transformed cells was also surprising because of previous reports that overexpression of PKC δ inhibits cell proliferation (Borner et al., 1995; Mischak et al., 1993). These observations suggested the possibility that PKC δ might have a different effect in v-Src-transformed cells than in the non-transformed parental cells. Alternatively, membrane association of PKC δ in v-Src-transformed cells may not correlate with an activation of its kinase activity since it has been demonstrated that PKC isoforms α and ε can affect phospholipase D (Conricode et al., 1992; Singer et al., 1996) and phosphatidate phosphohydrolase (Jiang et al., 1996) activity independent of the kinase activity of the α and ε isoforms respectively.

Tyrosine phosphorylation of PKC δ in response to several different stimuli has recently been reported (Denning et al., 1993; 1996; Haleem-Smith et al., 1995; Li et al., 1994a; 1994b). The biological significance of the tyrosine phosphorylation of PKC δ is unclear. It has been reported that tyrosine-phosphorylated PKC δ has a reduced kinase activity in Ras-transformed cells (Denning et al., 1993). Similarly, epidermal growth factor receptor activation also resulted in a decrease in the kinase activity of tyrosine-phosphorylated PKC δ (Denning et al., 1996). In contrast, PKC δ that was phosphorylated on tyrosine by either Fyn or the insulin receptor *in vitro* had elevated kinase activity (Li et al., 1994a). In response to antigen activation of the IgE receptor, PKC δ becomes tyrosine phosphorylated and phosphorylation apparently alters its substrate specificity (Haleem-Smith et al., 1995). Thus, the effect of tyrosine phosphorylation on PKC δ activity is apparently complex and may involve other cellular factors.

The tyrosine kinase(s) responsible for PKC δ phosphorylation is(are) not known. *in vitro* studies have shown that PKC δ can be phosphorylated by Src family and receptor tyrosine kinases (Gschwendt et al., 1994; Li et al., 1994a). In this chapter, we will show tyrosine phosphorylation of PKC δ and describe a functional interaction between Src and PKC δ in v-Src-transformed cells.

RESULTS

1. PKC δ is tyrosine-phosphorylated in v-Src-transformed 3Y1 cells.

In v-Src-transformed 3Y1 cells, the δ isoform of PKC is preferentially associated with the membrane relative to the parental 3Y1 cells (Zang et al., 1995). It was recently reported that PKC δ can be phosphorylated on tyrosine (Denning et al., 1993; 1996; Haleem-Smith et al., 1995; Li et al., 1994a; 1994b) and that Src family kinases can phosphorylate PKC δ on tyrosine *in vitro* (Gschwendt et al., 1994; Li et al., 1994a). We therefore investigated tyrosine phosphorylation of PKC δ in v-Src-transformed 3Y1 rat fibroblasts where the expression of v-Src results in increased membrane association of PKC δ. 3Y1 cells and v-Src-transformed 3Y1 cells were lysed and subjected to immunoprecipitation with antibodies against either phosphotyrosine (P-Tyr) or PKC δ. The immunoprecipitates were then subjected to western blot analysis using either anti-P-Tyr or anti-PKC δ antibody. As shown in figure 10A, anti-P-Tyr antibody precipitated a protein from v-Src-transformed 3Y1 cells that could be recognized by the anti-PKC δ antibody, and reciprocally, the 80 kD protein precipitated by the anti-PKC δ antibody from the v-Src-transformed cells was recognized by the anti-P-Tyr antibody. These results were observed only in the v-Src-transformed cells. As expected, PKC depletion by prolonged treatment with phorbol ester abolished precipitation of PKC δ by the anti-P-Tyr antibody, and treatment with phenyl phosphate, a phosphotyrosine analog, abolished precipitation of PKC δ by anti-P-Tyr antibody. As expected, the peptide used to generate the PKC δ antibody abolished the ability of the anti-PKC δ antibody to precipitate PKC δ.

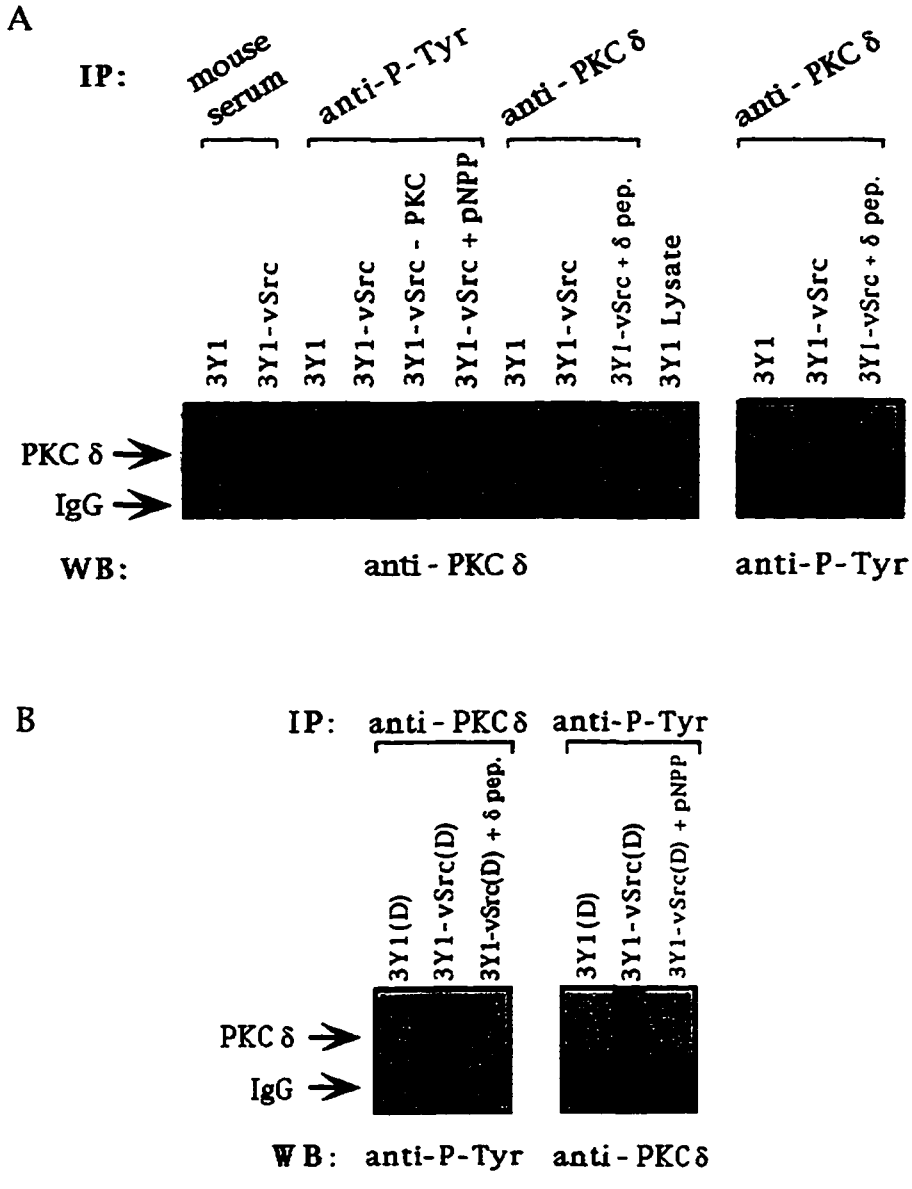


Figure 10. **PKC δ is tyrosine-phosphorylated in v-Src-transformed 3Y1 cells.**

(A) Cell lysates were generated from either 3Y1 cells or 3Y1 cells transformed by v-Src. Cell lysates were immunoprecipitated (IP) with a control mouse serum or antibodies raised against P-Tyr or PKC δ, and immune complexes were recovered with protein A agarose and subjected to western blot (WB) analysis using antibodies against either P-Tyr or PKC δ as shown. 3Y1-v-Src - PKC; cells were depleted of PKC by prolonged treatment with TPA (800 nM, 24h). 3Y1-v-Src + δ pep; the peptide against which the PKC δ antibody had been raised was included in the immunoprecipitation to neutralize the anti-PKC δ antibody. 3Y1-v-Src + pNPP; 30 mM phenylphosphate was included to neutralize the anti-phosphotyrosine antibody. (B) Identical to (A) except that the lysates were denatured (D) by treatment with 1% SDS and boiling for 10 min prior to immunoprecipitation.

To establish that the data shown in figure 10A was not due to contamination with a co-precipitating tyrosine-phosphorylated 80 kD protein, we repeated the experiments using denatured cell lysates in which protein-protein interactions were disrupted. As shown in figure 10B, the same results as observed in figure 10A were obtained using lysates that were treated with 1% SDS and heated at 100^0C for 10 min prior to immunoprecipitation. We conclude that PKC δ is tyrosine-phosphorylated in v-Src transformed 3Y1 cells. Tyrosine phosphorylation of PKC isoforms α and ε was not detected in similar experiments (data not shown), suggesting that the v-Src-induced tyrosine phosphorylation is specific for the δ isoform of PKC.

2. Tyrosine-phosphorylated PKC δ is primarily associated with the membrane fraction.

We demonstrated previously that there are substantial increases in membrane association of PKC δ in v-Src-transformed cells (Zang et al., 1995). We therefore wished to determine whether the tyrosine phosphorylated PKC δ is preferentially membrane-bound. v-Src-transformed cells were fractionated into membrane and cytosolic fractions, and lysates from each fraction were immunoprecipitated with anti-PKC δ antibody and subjected to Western blot analysis using anti-P-Tyr or anti PKC δ antibody.
As shown in figure 11, almost all of the PKC δ that is recognized by the anti-P-Tyr antibody is present in the membrane fraction, whereas the PKC δ antibody recognized substantial amounts of PKC δ in both the membrane and cytosolic fractions. As a control, when the cells were stimulated with TPA for 30 min, all PKC δ was found in the

membrane fraction. These data indicate that most of the tyrosine-phosphorylated PKC δ is membrane-associated.

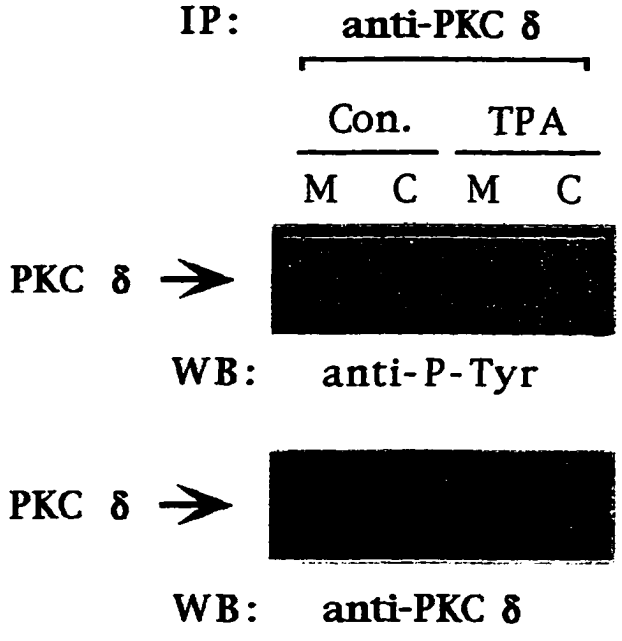


Figure 11. **Tyrosine-phosphorylated PKC δ is primarily associated with the membrane fraction.**

Lysates from membrane (M) and cytosolic (C) cell fractions from v-Src-transformed cells were prepared as described in Materials and Methods and immunoprecipitated with anti-PKC δ antibody. The immunoprecipitates were then subjected to Western blot analysis using anti-PKC δ or anti-P-Tyr antibodies as indicated. Cells were either treated (TPA) or were untreated (Con) with 100 nM TPA for 30 min prior to preparation of subcellular fractions.

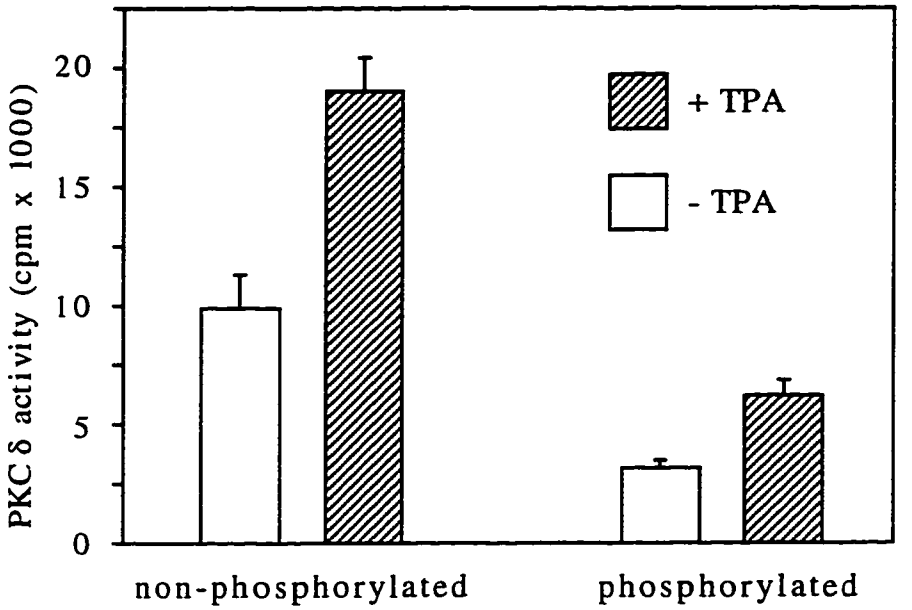


Figure 12. **Tyrosine-phosphorylated PKC δ from v-Src-transformed cells has reduced enzymatic activity.**

Cell lysates were subjected to immunoprecipitation with anti-phosphotyrosine antibody. Tyrosine-phosphorylated proteins were recovered with protein A agarose and then eluted with phenylphosphate as described in Materials and Methods. PKC δ was then precipitated with anti-PKC δ antibody from both the eluted tyrosine-phosphorylated proteins and the supernatant of the anti-phosphotyrosine immunoprecipitation. The amount of PKC δ in the immunoprecipitates was determined by Western blot analysis and the PKC activity in the immunoprecipitations normalized to the relative amount of PKC δ was examined in the presence and absence of TPA (1 μM in kinase assay buffer) as shown.

3. Tyrosine-phosphorylated PKC δ from v-Src-transformed cells has reduced enzymatic activity.

Tyrosine phosphorylation of PKC δ has been reported to both enhance (Li et al., 1994a; 1994b)and reduce (Denning et al., 1993; 1996) the kinase activity of PKC δ. We therefore compared the kinase activity of tyrosine-phosphorylated and non-tyrosine-phosphorylated PKC δ. Sequential immunoprecipitation with anti-P-Tyr and anti-PKC δ antibodies was used to separate tyrosine-phosphorylated and non-tyrosine-phosphorylated PKC δ isolated from v-Src-transformed cells as described in Materials and Methods. We then examined the *in vitro* kinase activity as described previously (Joseph et al., 1992). As shown in figure 12, the tyrosine-phosphorylated PKC δ possessed about 3-fold lower basal and TPA-induced enzymatic activity than non-tyrosine-phosphorylated PKC δ. These data suggest that tyrosine phosphorylation of PKC δ reduces the enzymatic activity of PKC δ in v-Src-transformed cells.

4. PKC δ associates with v-Src.

Since v-Src was shown previously to be able to phosphorylate PKC δ directly *in vitro* (Gschwendt et al., 1994), we further explored the possibility that PKC δ may be a substrate of v-Src *in vivo* by examining for a direct association between PKC δ and v-Src. The results of co-immunoprecipitation experiments are shown in figure 13. When cell lysates were immunoprecipitated with v-Src antibody and then western blotted with anti-PKC δ antibody, PKC δ was detected in v-Src immunoprecipitates from v-Src transformed 3Y1 cells, but not the parental 3Y1 cells. In the reciprocal experiment, where anti-PKC δ immunoprecipitates were western blotted with anti-v-Src antibody, the PKC δ antibody

co-precipitated v-Src protein. (Figure 13). For controls, the signal was competed away by PKC δ peptide and v-Src was undetectable in anti-PKC ε immunoprecipitates. The amount of v-Src in the anti-PKC δ immunoprecipitates is estimated to be about 1 - 2% of the total v-Src and the amount of PKC δ in the anti-v-Src precipitates is also estimated to be about 1-2% of the total PKC δ.

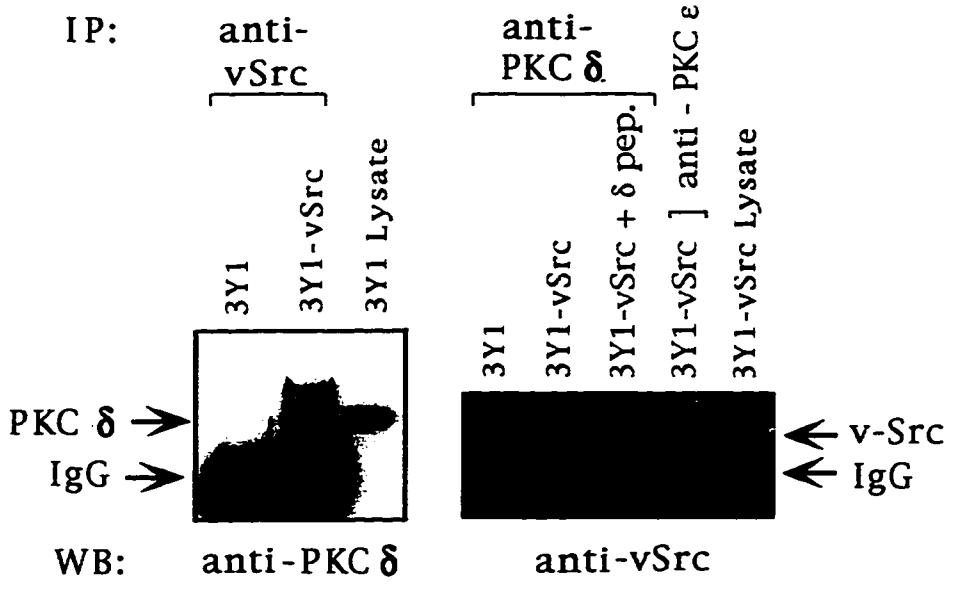


Figure 13. **PKC δ associates with v-Src.**

Lysates from 3Y1 or v-Src-transformed 3Y1 cells were prepared as described in Materials and Methods, immunoprecipitated (IP) with either anti-v-Src, anti-PKC δ, or anti-PKC ε antibody and then subjected to western blot analysis using the anti-PKC δ and anti-v-Src antibodies as shown. The 3Y1 and 3Y1-vSrc lysates were subjected to Western blot analysis without prior immunoprecipitation and represented 2% of the lysate used for the immunoprecipitations.

5. Interaction between PKC δ and Src mutants.

To further investigate the interaction between Src and PKC δ, we characterized the interaction between PKC δ and Src in cells overexpressing c-Src and several c-Src mutants (Figure 14A). Cell lines that overexpress the c-Src genes were established and expression levels of the c-Src proteins were determined by western blot analysis (Figure 14B). We first examined the interaction between PKC δ and c-Src and an activated mutant of c-Src that has the Tyr at 527 converted to Phe (c-Src 527F) (Bagrodia et al., 1993; Kmiecik and Shalloway, 1987). As shown in figure 14C, very little Src protein was present in anti-PKC δ immunoprecipitates from cells overexpressing c-Src. Consistent with this observation, little or no tyrosine phosphorylation of PKC δ was detected in the c-Src-overexpressing cells (Figure 14C). In contrast, activated c-Src 527F was associated with PKC δ, and PKC δ was tyrosine phosphorylated, although not quite to the level observed in cells expressing v-Src. However, c-Src 527F was active as v-Src in inducing tyrosine phosphorylation of PKC δ if TPA was added to stimulate membrane association of PKC δ.

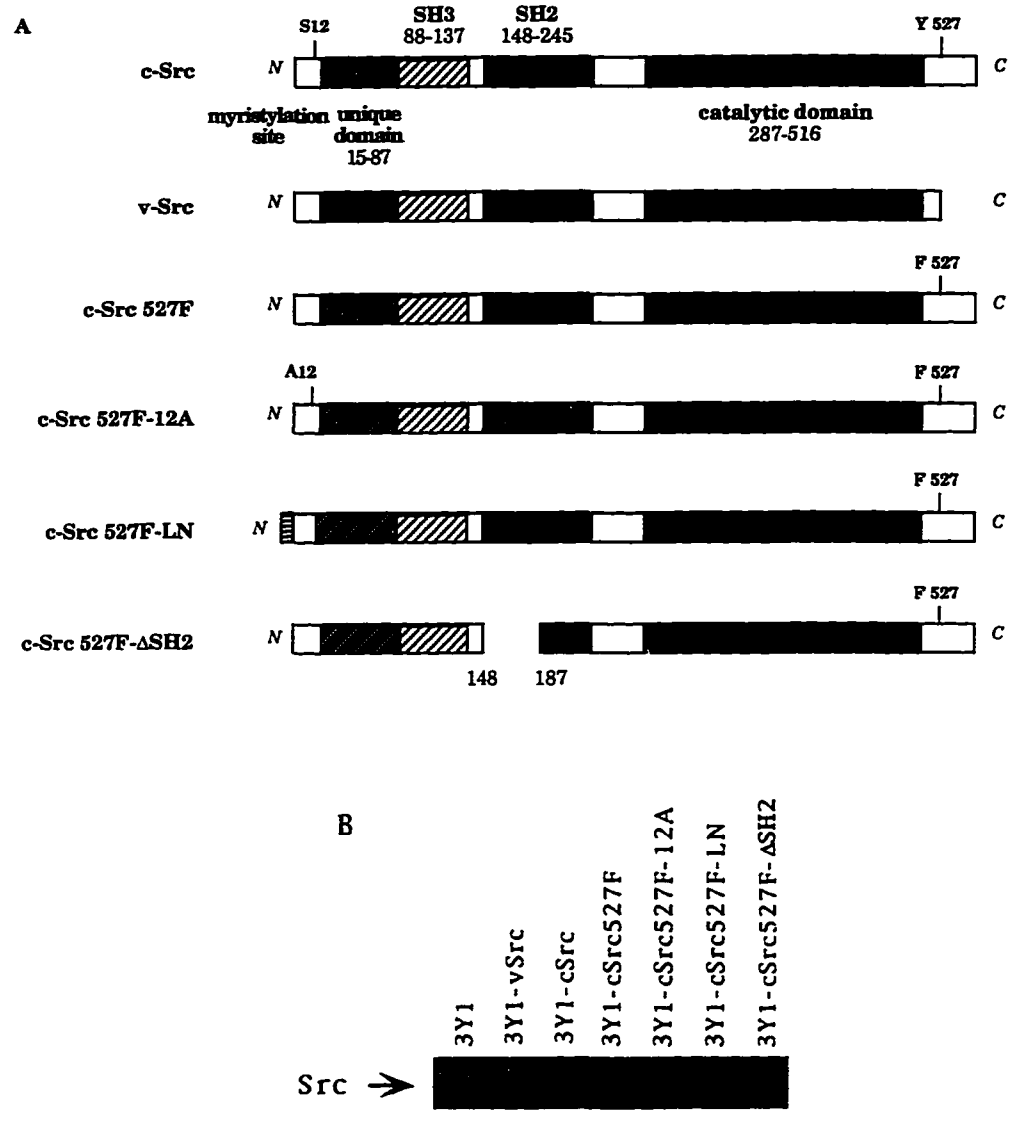


Figure 14 (A and B). **PKC δ tyrosine phosphorylation and PKC δ association with Src in 3Y1 cells expressing c-Src and mutants of Src.**

Cell lines expressing wild type or mutant c-Src were established as described in Materials and Methods. The Src mutants used are shown schematically in (A). c-Src 527F has a mutation of Tyr to Phe at position 527 which activates the tyrosine kinase. C-Src 527F-A12, in addition to the change at Tyr 527, has Ser 12 changed to Ala. c-Src 527F-LN has 4 additional amino acids (MAAA) at the amino terminus that prevents myristoylation and membrane association. C-Src 527F-ΔSH2 has the activating Tyr 527 mutation and a disruption of the SH2 domain in which amino acids 148-187 have been deleted. (B) Expression levels of the Src proteins in the transfected cell lines was analyzed by western blot analysis.

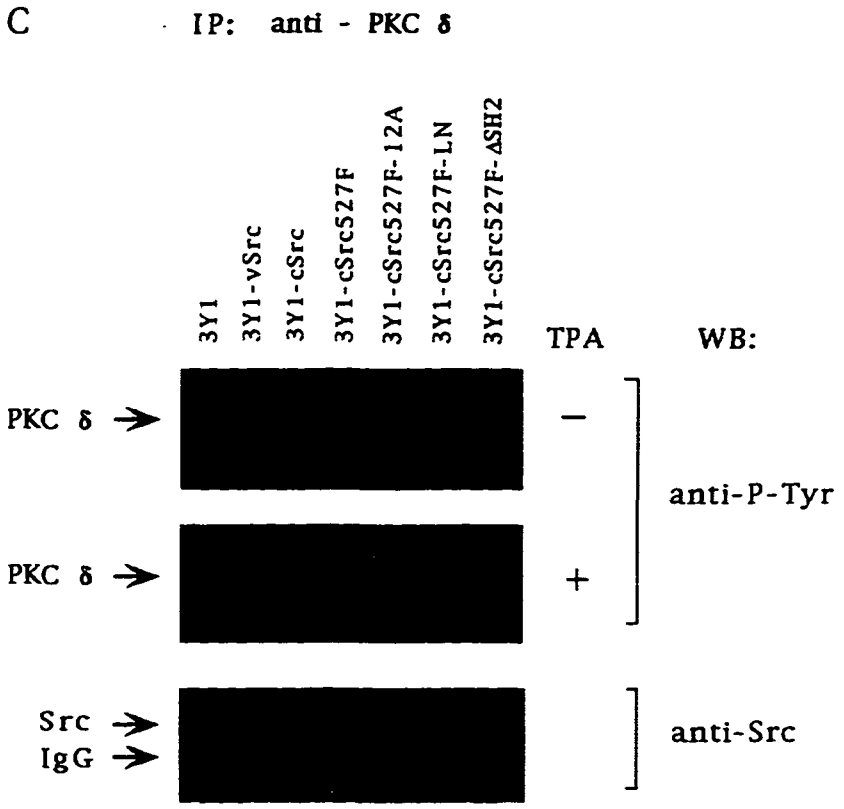


Figure 14C. **PKC δ tyrosine phosphorylation and PKC δ association with Src in 3Y1 cells expressing c-Src and mutants of c-Src.**

(C) Lysates from 3Y1 cells and 3Y1 cells expressing v-Src, c-Src and the c-Src mutants were immunoprecipitated (IP) with anti-PKC δ antibody and then subjected to Western blot (WB) analysis using anti-phosphotyrosine and anti-Src antibodies as shown. The phosphotyrosine analysis was also performed upon cells that had been treated with TPA (100 nM) for 30 min prior to lysis of cells.

We also investigated the effect of a mutation at Ser 12, a phosphorylation site for PKC (Gould et al., 1985). As shown, changing Ser 12 of c-Src 527 to Ala (c-Src 527-A12) substantially enhanced the association between PKC δ and Src and the level of tyrosine phosphorylation of PKC δ. A mutation to the SH2 domain of c-Src-527 had little or no effect upon either tyrosine phosphorylation of PKC δ or the association between Src and PKC δ (Figure 14C). Lastly we examined the effect of an amino terminal modification of c-Src 527 that prevents membrane association but not kinase activity. This mutant (c-Src 527-LN) failed to associate with PKC δ and did not stimulate tyrosine phosphorylation of PKC δ. These data indicate that PKC δ tyrosine phosphorylation and the interaction between Src and PKC δ require both Src tyrosine kinase activity and membrane localization. Phosphorylation of Src at Ser 12 by PKC may lead to the dissociation of a Src/PKC δ complex since a mutation at Ser12 of Src increased the Src/PKC δ interaction.

DISCUSSION

We have demonstrated that in cells transformed by v-Src, PKC δ is phosphorylated on tyrosine and is associated with v-Src. This interaction requires constitutively activated, membrane-localized Src kinase. The association between Src and PKC δ was not significantly affected by SH2 deletion, but was greatly enhanced by a mutation to the PKC

phosphorylation site on Src at Ser 12, suggesting that phosphorylation of Ser 12 may destabilize the interaction. This may explain why relatively low amounts of v- and c-Src and PKCδ are found in the protein complex.

Tyrosine-phosphorylated PKC δ has reduced kinase activity relative to the non-tyrosine-phosphorylated PKC δ. We previously reported that PKC δ becomes preferentially associated with the membrane in response to the kinase activity of v-Src (Zang et al., 1995). The increase in membrane association of PKC isoforms has been widely used to demonstrate PKC isoform activation. The finding here that tyrosine phosphorylation of PKC δ inhibits its kinase activity suggests that regulation of PKC activity involves more than simply generating DG and membrane association.

It was previously reported that PKC δ could be phosphorylated on tyrosine in response to phorbol esters that activate PKC (Li et al., 1994a). However, in 3Y1 cells and in 3Y1 cells overexpressing wild type c-Src or activated c-Src that was not membrane localized, we did not see an increase in PKC δ tyrosine phosphorylation in response to TPA. On the other hand, in cells expressing activated membrane-bound c-Src 527F, we did detect a TPA-induced increase in PKC δ tyrosine phosphorylation. These data suggest that tyrosine phosphorylation of PKC δ in response to TPA is dependent upon an active membrane-bound tyrosine kinase and is consistent with the hypothesis that TPA-induced tyrosine phosphorylation of PKC δ is a secondary effect of TPA-induced membrane localization.

Overexpression of PKC δ has previously been reported to inhibit cell growth (Mischak et al., 1993). Our previous observation that PKC δ became membrane

associated in response to the mitogenic stimuli of v-Src (Zang et al., 1995) was surprising since membrane association of PKC isoforms has widely been used to imply activation. The finding here that PKC δ becomes phosphorylated and has a reduced kinase activity in v-Src-transformed cells is perhaps consistent with the previous reports that PKC δ is an inhibitor of cell growth. The increased DG levels observed in response to v-Src (Song et al., 1991) may reflect a requirement for activation of the α PKC isoform, which also becomes membrane bound in response to v-Src (Zang et al., 1995). PKC α has been reported to phosphorylate Raf, which contributes to the activation of Raf (Kolch et al., 1993). And since Raf is required for transformation by v-Src (Qureshi et al., 1993), it is possible that activation of PKC α and phosphorylation of Raf is required for the mitogenic signals activated by the tyrosine kinase activity of v-Src. The increased DG that results in PKC α activation brings PKC δ to the membrane. However, since PKC δ inhibits mitogenic signals, there may be a mechanism whereby tyrosine phosphorylation, which correlates well with mitogenic signals, results in down-regulation of the enzymatic activity of PKC δ.

Although PKC δ becomes membrane associated in v-Src-transformed cells, there is no change in the subcellular distribution of the ε PKC isoform, which is also a DG-dependent, Ca^{2+}-independent PKC isoform (Zang et al., 1995). The preferential increase in membrane association of PKC δ over PKC ε observed in v-Src-transformed cells suggests that there may be some functional significance for the observed membrane association of PKC δ in response to v-Src. Several recent reports have suggested kinase independent roles for PKC isoforms (Conricode et al., 1992; Jiang et al., 1996; Singer et

al., 1996). It is possible that increased membrane association of PKC δ and down-regulation of its enzymatic activity indicates a kinase-independent function for PKC δ. Alternatively, Src could be a critical substrate for PKC δ and that upon phosphorylation of Ser 12, there is a reciprocal tyrosine phosphorylation that serves as a negative feedback control mechanism for PKC δ. A mutation to c-Src at ser 12 was previously shown to be required for the enhanced responsiveness to β-adrenergic agonists in cells overexpressing c-Src (Moyers et al., 1993). Thus, the interaction between Src and PKC δ may also be important for regulating other indirect effects of Src.

The effect of the Ser 12 mutant on both association and tyrosine phosphorylation further supports the hypothesis that PKC δ is a direct substrate of Src. The dependency of the association on an active kinase suggests that interaction occurs only when Src has been activated. It is still not clear as to what role(s) c-Src plays in cell physiology, and while the data presented here with cells overexpressing activated forms of Src do not prove that PKC δ is a normal cellular target of c-Src, the data do show that PKC δ could be regulated by Src or perhaps a related Src-family kinase. Perhaps more importantly, the data presented here in cells transformed by v-Src, demonstrate that v-Src can associate with and down-regulate a protein kinase that has been strongly implied in inhibiting cell growth. The ability to down-regulate this inhibitory PKC isoform may be important for the transforming ability of v-Src.

Chapter V. Summary

In v-Src-transformed 3Y1 rat fibroblasts, we have demonstrated that PKC α and δ are selectively translocated from cytosol to membrane. On the cell membrane, PKC δ interacts with v-Src and is tyrosine phosphorylated. The tyrosine phosphorylation of PKC δ leads to down-regulation of the kinase activity of PKC δ.

We previously reported that PLD activity is elevated in v-Src-transformed cells (Song et al., 1991). Our recent research showed that this induction of PLD is dependent on a GTPase cascade of Ras (Jiang et al., 1995a) and Ral (Jiang et al., 1995b). This v-Src induced signaling pathway is summarized in figure 15. The PLD activated by v-Src specifically hydrolyses PC and generates DG species that likely brings both PKC α and δ to membrane in v-Src-transformed cells.

The function of PKC α and δ in v-Src-transformed cells is still unclear. Overexpression of PKC α promotes cell growth in soft agar (Liao et al., 1994). It was also reported that PKC α phosphorylates Raf-1 and therefore results in the activation of Raf-1 (Kolch et al., 1993). Thus, PKC α may contribute to the v-Src initiated mitogenic response by activating Raf-1 and subsequently turning on gene expression.

The involvement of PKC δ is rather more complicated. Overexpression of PKC δ in NIH 3T3 cells (Mischak et al., 1993) and CHO cells (Hirai et al., 1994) results in decreased cell growth rate. Although PKC δ associates with membrane in v-Src-transformed cells, we assume that PKC δ may be important for some effect related to v-Src signaling rather than maintain cell transformation. As the model summarized in figure

16, our data showed that PKC δ is tyrosine phosphorylated and associates with v-Src. The result of *in vitro* PKC assay suggests that the tyrosine phosphorylation is a negative regulation for PKC δ kinase activity. The direct interaction between PKC δ and v-Src gives the evidence for the hypothesis that v-Src is the tyrosine kinase for PKC δ. Therefore, the mechanism for v-Src to overcome the inhibitory effect of PKC δ on cell growth is to phosphorylate this PKC isoform and down-regulated its activity on cell membrane. However, the accurate effect of tyrosine phosphorylation on the biological function of PKC δ awaits analysis of phosphopeptide mapping and site-directed mutagenesis. Besides, we can not exclude the possibility that PKC δ is a substrate of multiple tyrosine kinases, or it may be phosphorylated by different kinases at different tyrosine residues.

It has been shown that v-Src is serine phosphorylated by PKC at serine 12 (Gould et al., 1985). It is still unclear for the biological significance of this phosphorylation, and which PKC isoform is responsible for the phosphorylation is unknown. Our data showed that mutation at serine 12 of activated c-Src (cSrc 527F) causes enhanced tyrosine phosphorylation of PKC δ and the association between PKC δ and Src. Therefore, it is possible that PKC δ is at least one of the PKC isoforms which phosphorylate v-Src at serine 12. This serine phosphorylation may cause the conformational change of v-Src caused by this serine phosphorylation blocks the association between PKC δ and Src.

In summary, in v-Src-transformed cells, elevated DG brings PKC α and δ to the cell membrane. On cell membrane, PKC δ associates with v-Src and is tyrosine

phosphorylated by v-Src. The tyrosine phosphorylation down-regulates PKC δ activity, therefore, the cells can maintain the transformation phenotype.

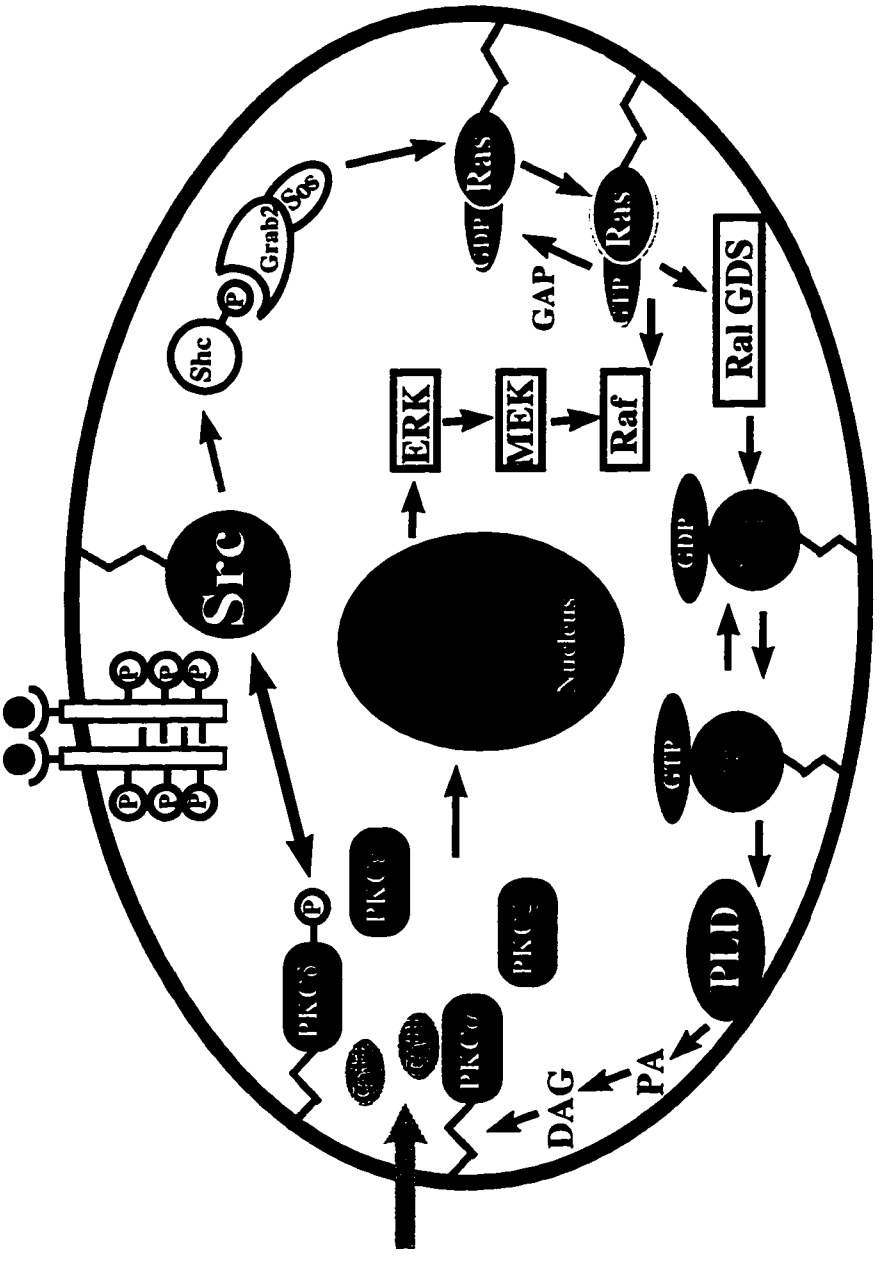


Figure. 15. **Summary of v-Src induces signal transduction pathway.**

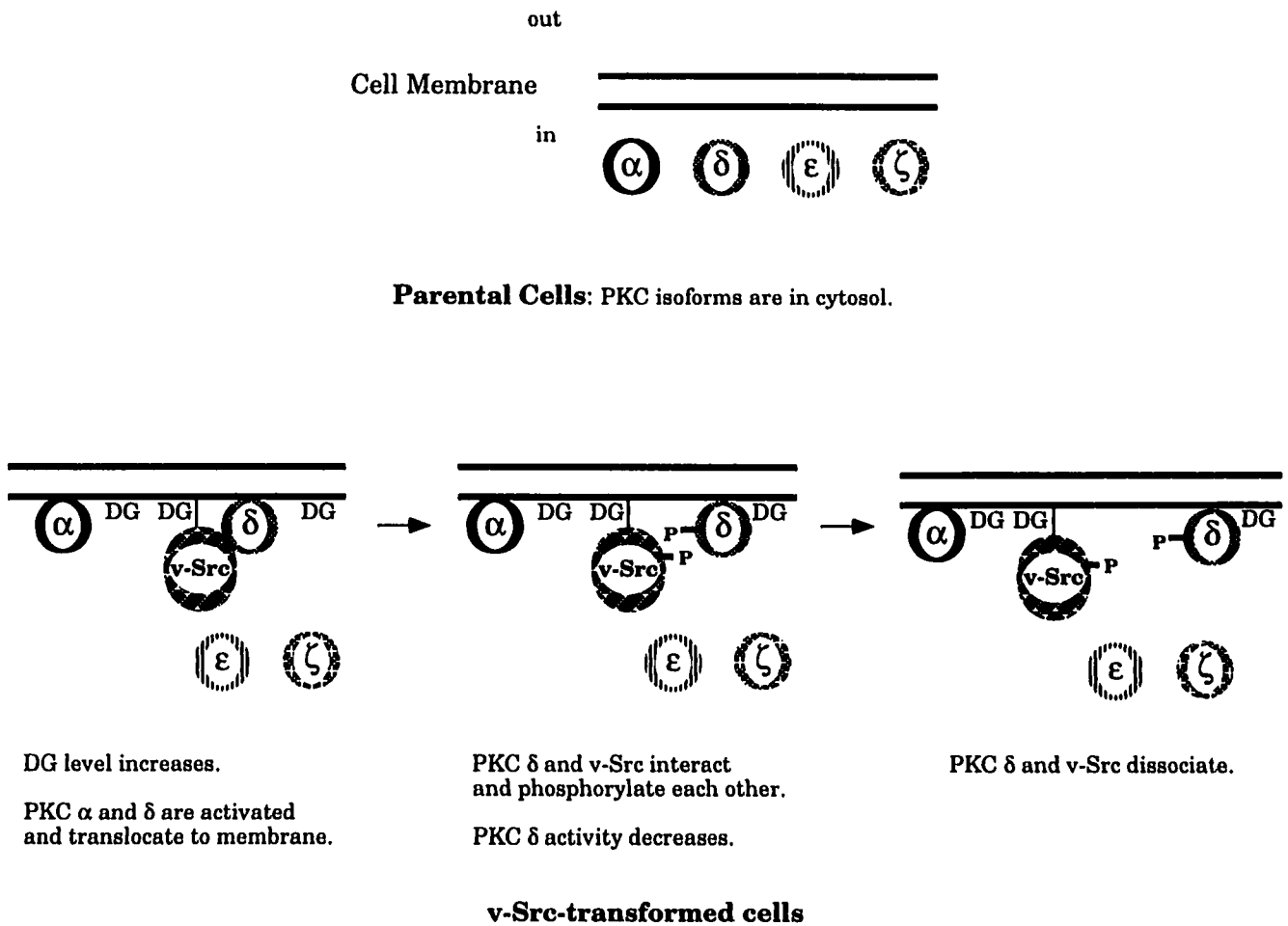


Figure. 16. **Model of PKC isoforms regulation in v-Src-transformed cells.**

Chapter VI. References

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