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The role of protein kinase C and MARCKS in v-Src- and v-Fps-induced intracellular signaling

Joseph, Cecil Keith, Ph.D.

City University of New York, 1992

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THE ROLE OF PROTEIN KINASE C AND MARCKS IN v-Src- AND v-Fps-INDUCED INTRACELLULAR SIGNALING.

by

CECIL K. JOSEPH

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

1992

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This manuscript has been read and accepted for the Graduate Faculty in Biochemistry in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

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THE CITY UNIVERSITY OF NEW YORK

Abstract

The Role of Protein Kinase C and MARCKS in v-Src- and v-Fps-induced intracellular signaling.

by

Cecil K. Joseph

Adviser: Dr. David A. Foster

Activation of Protein Kinase C (PKC) by tumor promoting phorbol esters leads to the phosphorylation of 67 kilodalton and 80 kilodalton proteins in avian and mammalian cells respectively. We demonstrate that activating the protein tyrosine kinase (PTK) activity of the oncogene products v-Src and v-Fps in cells infected with temperature sensitive (ts) derivatives of v-src and v-fps, results in rapid phosphorylation of a 67 kilodalton protein in chicken embryo fibroblasts (CEF), and an 80 kilodalton protein in murine fibroblasts. This protein known as the myristoylated alanine-rich C kinase substrate (MARCKS), was not phosphorylated when cells expressing v-Src and v-Fps were incubated in the presence of protein kinase inhibitors, or exposed to prolonged treatment with phorbol esters to down-regulate PKC. The kinetics of v-Src-induced increases in the physiological activator of PKC, diacylglycerol, correlated with v-Src-induced phosphorylation of MARCKS. Consistent with v-Src and v-Fps using a PKC-mediated pathway to transduce signals, we find that phosphorylation of MARCKS in response to the PTK activity of v-Src and v-Fps, correlates with expression of the PKC-responsive 9E3 gene in CEF, and the TIS10 gene in murine fibroblasts. We conclude that activation of PKC is an early event in signals which are initiated by v-Src and v-Fps.

In BALB/c-3T3 cells stably transformed by v-Src, phorbol esters were unable to induce phosphorylation of MARCKS. Both PKC protein levels and

kinase activity was unchanged in v-Src-transformed relative to the parental non-transformed BALB/c 3T3 cells. Thus, the inability to induce MARCKS phosphorylation was not due to a lack of PKC. MARCKS protein levels were found to be reduced in v-Src-transformed cells relative to the parental non-transformed cells. MARCKS RNA levels were also correspondingly reduced in v-Src-transformed cells. Nuclear "run-on" assays showed decreased transcription of MARCKS in v-Src-transformed cells. Thus, the absence of MARCKS in v-Src-transformed cells could be explained by a down-regulation of MARCKS transcription. Inhibiting the protein tyrosine kinase activity of v-Src restored MARCKS RNA levels, MARCKS transcription, and MARCKS protein suggesting that down-regulation of MARCKS in v-Src-transformed BALB/c 3T3 cells is a direct effect of the PTK activity of v-Src. These data implicate MARCKS in maintaining cells in the non-transformed state.

PREFACE

This thesis is divided into five sections. Section I is a general introduction. Section II describes the role of PKC in v-Src- and v-Fps-induced signaling. It is a combination of work which has been published in three separate works. Section III focuses on the expression of MARCKS in v-Src-transformed cells. This work has also been published. Section IV is a brief summary. Section V contains materials and methods and the bibliography for the entire document.

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To my wife Brenda. My #1 cheerleader. Without your love and support this would never have been possible.

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LIST OF ABBREVIATIONS

PKC	protein kinase C
PKA	protein kinase A
PMA	phorbol 12-myristate 13-acetate
TPA	12-O-tetradecanoylphorbol 13-acetate
DAG	1,2-Diacylglycerol
PDB(PBt_2)	phorbol 12,13-dibutyrate
OAG	1-oleoyl-2-acetylglycerol
San	Sangivamycin
CEF	chicken embryo fibroblasts
ts	temperature sensitive
EGTA	[ethylenebis(oxyethylenenitrilo)tetraacetic acid
MARCKS	myristylated alanine-rich C kinase substrate
RSV	Rous sarcoma virus
FSV	Fujinami sarcoma virus
PTK	Protein Tyrosine Kinase
NF-kB	Nuclear Factor kappa Binding Protein
Sp1	Stimulator Protein 1

SECTION I

Introduction

Protein-tyrosine kinase (PTK) activity is frequently an early event in the transduction of intracellular signals leading to cell division and transformation (Drucker et al., 1989). The protein tyrosine kinases constitute the largest functional group of oncogenes, with over twenty members divided into two broad families, receptor and non-receptor kinases. Both the receptor and non-receptor protein tyrosine kinases can be viewed as transducers of information within the cell. They respond to activating stimuli by increasing their catalytic activity and transfer information to target molecules by phosphorylation. The stimuli that activate the receptor tyrosine kinases are clearly related to normal cell physiology (Cantley et al., 1991). The catalytic activities of these receptors are increased by the binding of growth factors to their extracellular domains. This results in the phosphorylation of appropriate substrates and the transduction of signals to one or more intracellular targets, eventually responsible for inducing cell proliferation.

v-src and v-fps are oncogenes of the retroviruses Rous Sarcoma Virus (RSV), and Fujinami Sarcoma Virus (FSV) respectively. These retroviral oncogenes are a unique set of genes that function specifically to induce transformation and tumorigenicity without playing a direct role in virus replication. Both oncogenes have been generated by retroviral transduction of host cellular DNA sequences (c-oncs or proto-oncogenes), resulting in modified genes (v-oncs or oncogenes) driven by viral promoters (Stehelin et al., 1976). v-Src and v-Fps are members of the Src family of PTKs and are oncogenic prototypes of the non-receptor class of tyrosine kinases.

The nature of the stimuli that regulate the non-receptor tyrosine kinases is less clear, as these proteins are not exposed to the external environment of the cell. All protein-tyrosine kinases have sequence homology over a stretch of approximately 300

amino acids (sometimes interrupted) that has been defined as the kinase domain (Hanks et al., 1988). The Src subfamily has additional regions of homology not found in the receptor family. These regions include a short amino-terminal sequence required for addition of myristic acid and two additional domains named Src homology 2 and 3 (SH-2 and SH-3). The myristic acid addition is required for membrane localization which is essential for transformation by v-Src (Cross et al., 1985). Evidence is also accumulating that the SH-2 and SH-3 domains are critical for association with phosphotyrosine residues and recruitment of signal-transducing molecules (Cantley et al., 1991).

The signal-transduction pathways and intermediates which are used by oncogene products to transform cells have yet to be established. Although v-src and v-fps encode protein-tyrosine kinases (PTKs) which have been implicated in transmembrane signaling (Hunter and Cooper, 1985), no substrate for v-Src or any other PTK has been shown to be necessary for transformation. Oncogenes act by perturbing the regulatory mechanisms that govern normal cell behavior; deregulation of the mechanisms that control normal cell growth can lead to abnormal cell proliferation and transformation (Cooper, 1990). It is well documented that deregulation of the PTK activity of v-Src and v-Fps leads to cellular transformation (Jove & Hanafusa, 1987). Although these proteins have no extracellular domain for direct binding to growth factors, there is evidence that these kinases are activated by growth factors and other cellular activators. In platelets, thrombin activates protein-tyrosine kinases of the Src family (Ferrell and Martin, 1988). Platelet-derived growth factor (PDGF) activates the PTK activity of $pp60^{c\text{-}src}$ in fibroblasts (Krypta et al., 1990). Protein-tyrosine phosphorylation can be increased in some cells by GTPγS (Nasmith et al., 1989) and by activators of the protein-serine/threonine kinase, protein kinase C (PKC) (Kazlauskas and Cooper, 1988). Thus it is possible that G proteins and/or PKC is an intermediate in the signal cascade from receptor to PTK activation.

Activating the PTK activity of v-Src and v-Fps also results in induction of gene expression in both avian and mammalian cells. One of these genes, 9E3/CEF4 (Sugano et al., 1987; Bedard et al., 1987) is a member of the platelet factor 4 family of inflammatory proteins, and may be the avian homolog of interleukin 8 (Barker and Hanafusa, 1990). Purified 9E3 protein has been found to be chemotactic for both untransformed CEF and CEF transformed with Rous sarcoma virus. However, 9E3 is only slightly mitogenic for chicken embryo fibroblasts, and expression of this gene in CEF in a retroviral vector did not cause transformation (Barker and Hanafusa, 1990). 9E3 has been found associated not only with the cell and in the culture medium of RSV-transformed CEF, but also with the extracellular matrix, suggesting that the role of 9E3 in transformation may be more involved with chemotaxis and morphological alterations than with direct stimulation of mitogenicity. In murine fibroblasts, v-Src and v-Fps induce expression of the "primary response" genes originally designated TIS10 and TIS8, (TPA induced sequences 10 and 8 respectively), (Lim et al., 1987). Both TIS10 and TIS8 cDNAs were cloned as "primary response" genes whose mRNAs rapidly accumulate in 3T3 cells treated with serum, polypeptide growth factors, or phorbol esters. The TIS10 gene encodes a protein with strong similarities to prostaglandin G/H synthase/cyclooxygenase (Fletcher et al., 1992), the rate-limiting enzyme in the biosynthesis of prostaglandins. TIS8 (Egr-1) is a transcription factor with a "zinc finger" motif and sequence-specific DNA binding activity. The secondary effects of v-Src-induced expression of TIS10 and Egr-1 have not been defined. However, v-Src-induced expression of both these genes is sensitive to inhibition of the c-raf-1 proto-oncogene (Qureshi et al., submitted for publication), suggesting that c-raf-1 is an intermediate in signals leading to the induction of TIS10 and Egr-1 by v-Src.

The activation of protein kinase C (PKC) is also an early event in many signaling pathways which lead to cell division (Nishizuka, 1986). PKC comprises a family of closely related serine/threonine protein kinases that are intermediates in signal

transduction pathways (Nishizuka, 1986; Nishizuka, 1988; Ushio et al., 1989). The activation of PKC appears to be an important step in the control of many cellular processes including mitogenesis, exocytosis, differentiation, and neurotransmission (Nishizuka, 1988; Niedel and Blackshear, 1986). Molecular cloning analysis has revealed that, in mammalian tissues, PKC exists as a large family consisting of at least eight subspecies [α, ßI, ßII, gamma, δ, Σ, zeta and ^{n}/(L)] with closely related structures. PKC isozymes exhibit differences in substrate specifities and cofactor requirements, which may reflect different biological functions (Nishizuka, 1988). PKC isozymes α, ß and gamma are markedly activated by phospholipids, diacylglycerol, or phorbol esters and by Ca^{2+}. The PKC isozymes δ, Σ and ^{n}/L in general are activated to a lesser extent by phospholipids and diacylglycerol and are relatively Ca^{2+} independent (Nishizuka, 1988).

Studies on the cellular localization of PKC suggest that in resting cells the enzyme is mainly cytosolic. Activation by PKC agonists results in rapid translocation to the plasma membrane (Nishizuka, 1988). Translocation, however, is not a good indicator of PKC activation. Firstly, PKC, so far as is known, is activated allosterically rather than by autophosphorylation, covalent modification or subunit dissociation, all modes by which other protein kinases are rendered stably active. This means that once cells are broken open for assay of PKC activity, the allosteric activators would be expected to dissociate from the kinase, and thus the kinase activity in a given cellular fraction would represent the amount of enzyme protein present rather than the activity state of the protein before cell disruption. Thus studies of PKC translocation have been difficult to perform and reproduce (Woodgett et al., 1987). Secondly, several studies have found changes in PKC subcellular localization in response to stimuli that do not seem to activate PKC (Cambier et al., 1987). Thirdly, certain isoenzymes of PKC apparently can be translocated to sites other than the plasma membrane upon activation. For example, Mochly-Rosen et al., (1990) found that

activation of PKC in cardiac myocytes and fibroblasts led to translocation of at least one PKC isoenzyme to myofibrils and microfilaments, respectively. Finally, redistribution of PKC within the cell is sometimes impossible to demonstrate in response to stimuli known to activate the kinase (Halsey et al., 1987). Because of these problems with studies of PKC translocation, the best and most reliable means of demonstrating whether a given agonist activates PKC is to evaluate phosphorylation of its specific substrates in intact cells. This is supported by the observation that PKC can phosphorylate substrates in both cytosolic and membrane compartments (Graff et al., 1989b), suggesting that translocation may not be necessary for activation of PKC.

One major cellular substrate for PKC, MARCKS, (myristylated alanine rich C kinase substrate) has been extensively studied. The level of phosphorylation of this protein is rapidly increased upon addition of PKC agonists, or mitogens including platelet-derived growth factor and vasopressin (Rozengurt et al., 1983; Blackshear et al., 1985). The precise function of MARCKS remains to be elucidated. Genomic analysis indicates that MARCKS is transcribed from a single gene, to produce processed and unprocessed messages ranging in sizes from 2.1 to 4.4 kB. (Seykora et al., 1991; Brooks et al., 1991; Stumpo et al., 1989). The 3'-untranslated region of the MARCKS cDNA clone from rat fibroblasts has a relatively high AT content and contains sequence features which are reminiscent of the 3'-untranslated regions of various transiently expressed mRNAs such as those coding for interferons, interleukins, and growth factor-inducible nuclear oncogenes (Erasulimsky et al., 1991), suggesting that MARCKS undergoes rapid turnover. However, it has also been shown that MARCKS mRNA is quite stable in several cell types, with a half-life of disappearance after actinomycin D of 4-6 h. (Harlan et al., 1991). Therefore, it seems possible that most regulation of MARCKS mRNA and protein concentrations occurs at the level of gene transcription. Promoter analysis of human MARCKS shows that it lacks a TATA box, typical of many housekeeping gene promoters, and yet apparently directs tissue-

specific expression of the gene (Stumpo et al., 1989; Blackshear et al., 1986; Albert et al., 1986). This tissue-specific expression is not a typical characteristic of housekeeping genes. The human MARCKS promoter also contains potential binding sites for the transcription factors Spl (Mitchell and Tijan, 1989) and NF-kB (Pessara and Kock, 1990). The latter may be responsible for the TNF-α stimulated MARCKS transcription observed in HL60 and U937 macrophage cells (Harlan et al., 1991).

cDNAs encoding the MARCKS gene from bovine, chicken, murine and human cells predict a protein of molecular mass 30 kDa and a theoretical pI of 4.1. This calculated molecular mass is at variance with the apparent molecular mass of 65-87 kDa obtained from SDS/PAGE analysis. The amino acid composition and the hydropathicity plot suggests that MARCKS is an extended hydrophilic protein with little interior volume. This extended shape together with the unusually high content of negatively charged residues which diminish the binding of SDS may explain, to some extent, the overestimation of the size (65-87 kDa) on SDS-polyacrylamide gels of this family of PKC substrates. In addition, the finding that MARCKS is glycosylated in rat fibroblasts is an additional factor that may contribute to its anomalous migration on gels (Erusalimsky et al., 1991).

To define signaling pathways which may be utilized by the oncogene products v-Src and v-Fps we asked the following: 1) Is activation of PKC an early early event in v-Src- and v-Fps-induced signaling 2) Does activation of PKC correlate with v-Src- and v-Fps-induced gene expression 3) Does the PTK activity of v-Src have any effect on the levels of expression on the major substrate of PKC (MARCKS). We show that a 67-kDa PKC substrate is rapidly phosphorylated in response to the kinase activity of v-Src and v-Fps in CEF. A similar PKC substrate of 80-kDa is rapidly phosphorylated in response to the PTK activity of v-Src in murine fibroblasts. We also present evidence that a PKC-mediated signal-transduction pathway is used by the protein-tyrosine kinase oncogene products, v-Src and v-Fps, to induce expression of the 9E3 gene in CEF, and

the TIS10 gene in murine fibroblasts. Thus, PKC is not only activated in response to the PTK activity of v-Src and v-Fps, but it is required for v-Src- and v-Fps-induced gene expression. Finally, we present data which indicates that the major substrate of PKC, MARCKS, is transcriptionally down-regulated in v-Src-transformed cells.

Section II

v-Src and v-Fps activates PKC in chicken and murine fibroblasts.

Introduction

Oncogenes act by perturbing the regulatory mechanisms that govern normal cell behavior (Cooper, 1990). Several of the proteins reside on the inner surface of the plasma membrane, well situated to transduce signals from outside the cell (Bishop, 1987). We wanted to determine whether v-Src and v-Fps utilize normal cellular regualtory mechanisms to transduce signals. We have shown that forskolin activates adenylate cyclase resulting in increased cAMP levels in mouse fibroblasts stably transfected with ts derivative of v-src (LA90 cells).(Qureshi et al., 1991). Therefore, we explored the posssibility that activation of v-Src and v-Fps might trigger increases in cAMP formation leading to the activation of a cAMP-dependent Protein Kinase A (PKA). However, increased cAMP levels were not detected in response to the PTK activity of v-Src and v-Fps, suggesting that these oncogene products did not activate the cAMP-dependent pathway. Increased levels of DAG (an activator of PKC) have been noted in cells transformed by v-Src (Wolfman and Macara, 1987). Therefore, we tested the hypothesis that activation of PKC is an early event in the signals which are initiated by v-Src and v-Fps, by monitoring increased phosphorylation of a major substrate of PKC (MARCKS) in response to the PTK activity of v-Src and v-Fps. MARCKS protein serves as a convenient marker of PKC activation in many cell types for a number of reasons: 1) it is an excellent substrate for PKC in both its membrane-associated and non-myristoylated, cytosolic forms (Graff et al., 1991); 2) it is phosphorylated within seconds of PKC activation (Rozengurt et al., 1983); 3) the magnitude of the increase in phosphorylation is large, making it a sensitive index of PKC activation; 4) so far as is known, it is a specific substrate for PKC in intact cells; 5) the primary structures of the protein and its phosphorylation sites are known; (Graff

et al.,1990) 6) it can be separated readily from other cellular phosphoproteins by two dimensional electrophoresis and/or immunoprecipitation.

RESULTS

v-Src and v-Fps induce phosphorylation of a Major PKC Substrate. Treatment of fibroblasts with the phorbol ester PMA results in increased phosphorylation of many proteins (Blackshear et al., 1991). Fig. 1. shows that agonists of PKC, but not PKA, lead to the phosphorylation of a 67-kDa protein in CEF. Sagara et al., (1986) have shown that a 67-kDa protein in avian cells is related to or the same as the well-characterized 80-kDa PKC substrate seen in mammalian cells. Thus, activation of PKC results in phosphorylation of a 67-kDa protein.

If v-Src and v-Fps activate PKC then we should see increased phosphorylation of the 67-kDa protein in response to the kinase activity of these oncogene products. Our strategy was to use CEF infected with viruses expressing derivatives of v-Src (Mayer et al., 1986) and v-Fps (Hanafusa et al., 1981) that were temperture sensitive (ts) for protein-tyrosine kinase activity and transformation. We monitored the response to increased protein-tyrosine kinase activity after a shift from the non-permissive to the permissive temperature. To examine the hypothesis that v-Src and v-Fps activate PKC, we tested whether v-Src and v-Fps could induce phosphorylation of the 67-kDa protein which is induced by agonists of PKC in normal CEF. Upon shift from non-permissive to permissive temperatures for both v-Src and v-Fps, phosphorylation of a 67-kDa protein can be detected within 8 min (Fig. 2). Increased phosphorylation of the 67-kDa PKC substrate on serine was previously reported in CEF transformed by v-Src (Sagara et al., 1986). This suggests that v-Src and v-Fps activate PKC as measured by phosphorylation of a major PKC substrate.

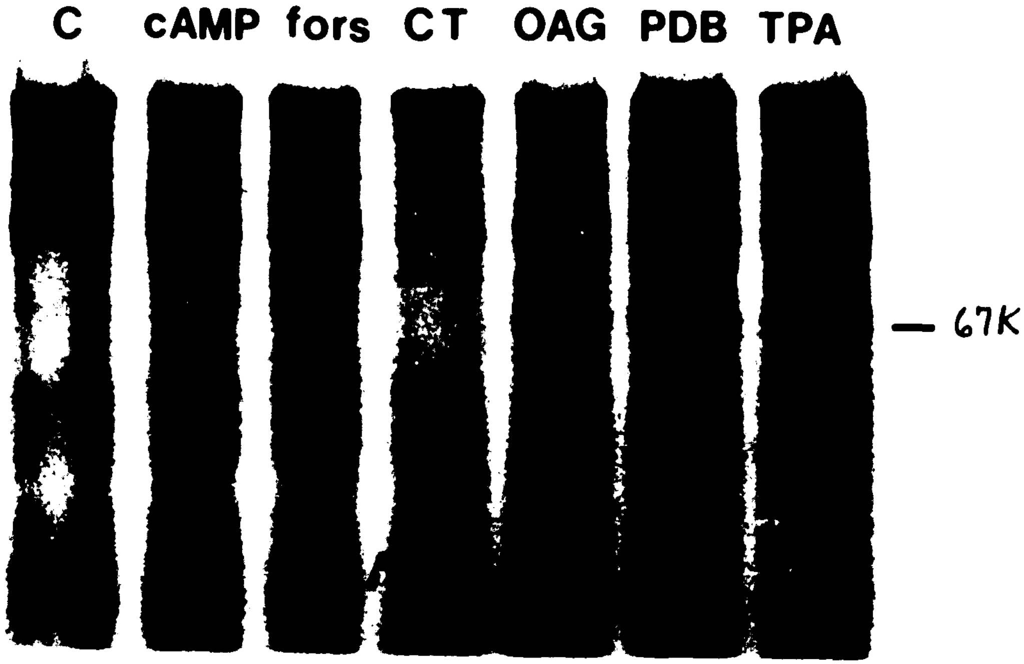
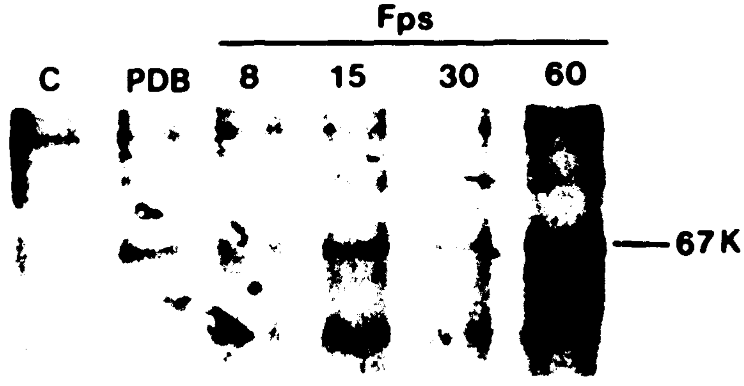


Fig. 1. Phosphorylation of major PKC substrate.
Agonists of both PKA and PKC were added to CEF that were prelabeled with [^{32}P]orthophosphate, and cells were harvested 10 min later as described in "Materials and Methods". Total cell extracts were loaded onto 7.5% SDS gels as described. C, control; cAMP, 8-Br-cAMP (1mM); fors, forskolin (25 μM); CT, cholera toxin (1μg/ml); OAG and PDB (PBt_2) (100 nM); TPA (PMA) (100 nM).



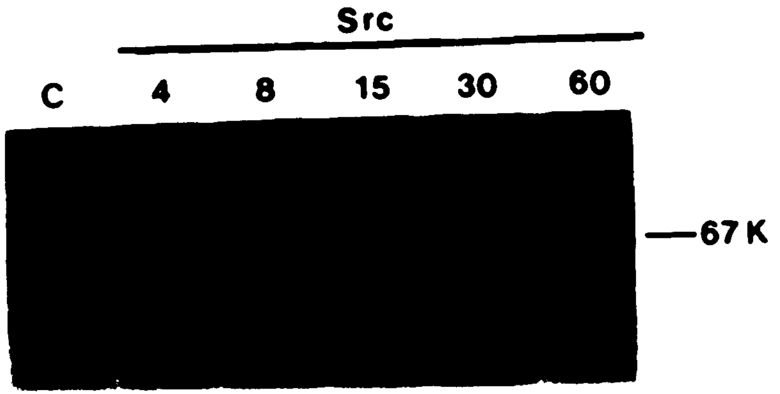


Fig. 2. Induction of phosphorylation of a 67-kDa protein by v-Fps and v-Src. CEF infected with tsNY225 and ts-NY72 were shifted from the nonpermissive to the permissive temperature, and total cell extracts were prepared and processed as in Fig. 6 at the indicated times in min. PBt_2 was added 10 min before processing.

Effect of PKC depletion on v-Src-induced phosphorylation of the 67-kDa protein. Prolonged exposure of cells to the PKC agonist PMA causes loss of PKC activity (Nishizuka, 1986; Rodriguez-Pena and Rozengurt, 1984; McCaffrey et al., 1987; Pike and Eakes, 1987; Davis and Czech, 1987; Ashendel, 1985). In Fig. 3 it can be seen that in cells depleted of PKC by prolonged exposure to PMA, neither PBt_2 nor v-Src induces phosphorylation of a 67-kDa protein. Similar data were obtained for v-Fps (not shown), providing further evidence that the 67-kDa is indeed a major PKC substrate.

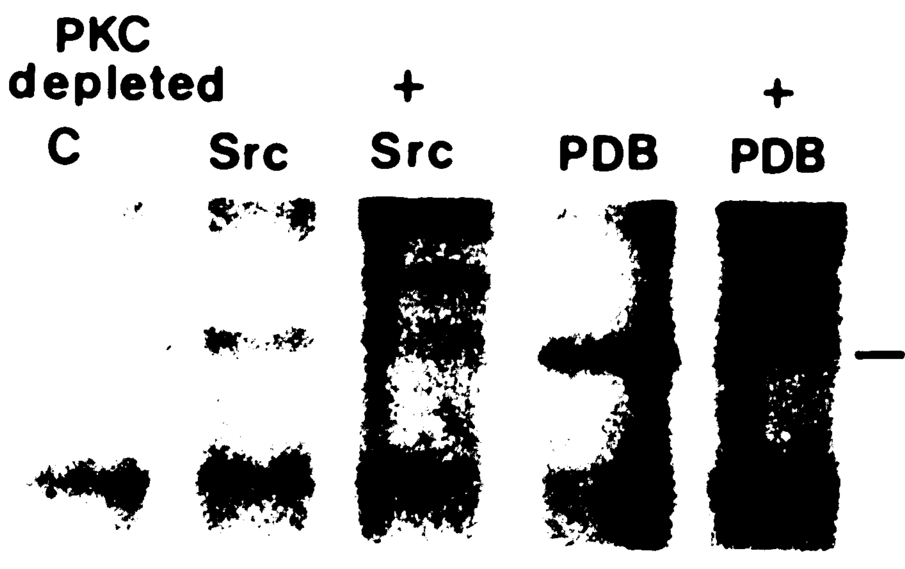


Fig 3. Effect of PKC depletion on v-Src-induced phosphorylation of a 67 kDa protein. CEF or tsNY72-infected CEF were depleted of PKC activity (+) as described, and then induced with PBt_2 or temperature shift as described above and harvested 10 min later.

Immunoprecipitation of the 67-kDa PKC substrate.

To further establish this protein as a PKC substrate, we used an antibody raised against the major 80-kDa PKC substrate from bovine brain (Albert et al., 1987; Aderem et al., 1988). Fig. 4 shows that this antibody immunoprecipitates a 67-kDa protein that is phosphorylated in response to both PBt_2 and v-Fps-further demonstrating that the 67-kDa protein is a PKC substrate.

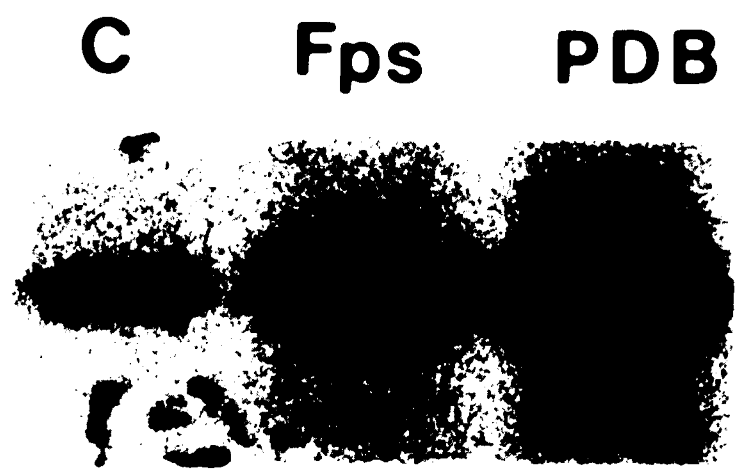


Fig 4. Immunoprecipitation of pp67 by an antibody raised against a major PKC substrate. Total cell extracts from CEF, CEF treated with PBt_2 (10 min), and tsNY225-infected CEF after temperature shift (10 min) were immunoprecipitated with antibody to the major PKC substrate as described (Foster et al., 1985) and then electrophoresed as above.

v-Src and v-Fps use a PKC-mediated signal transduction pathway to induce expression of the 9E3 Gene. Much has been learned about signal transduction by using compounds that block or deplete cells of intermediates in signal-transduction pathways. When using inhibitors, it is imperative to select an appropriate phenotype that is rapidly detectable so as to minimize any indirect inhibitor effects. Recent characterization of a gene (9E3) that responds quickly to the kinase activity of temperature sensitive derivatives of v-Src (Sugano et al., 1987; Bedard et al., 1987) has provided a useful phenotype for characterizing signal-transduction pathways used by v-Src to induce expression of this gene. Expression of 9E3 correlates well with transformation of fibroblasts in culture by v-Src. Mutants of v-Src that retain high levels of kinase activity, but lack the ability to transform, fail to induce 9E3 gene expression. Over-expression of nontransforming c-src does not induce 9E3 gene expression (Sugano et al., 1987). Thus, expression of the 9E3 gene correlates with transformation by v-Src and not only with the kinase activity of Src proteins. The induction of 9E3 by v-Src offers a rapidly detectable transformation-related phenotype. In collaboration with Dr Rudolph Spangler in the lab, we used the rapid induction of v-Src-induced 9E3 gene expression to explore the signal-transduction pathways by v-Src and v-Fps to induce expression of this transformation-related gene in chicken embryo fibroblasts.

9E3 mRNA has been demonstrated to increase at the transcription level in response to the kinase activity of v-Src expressed by tsNY72 (Sugano et al., 1987), and the increased transcription can be detected within 30 min after activation of the kinase activity of v-Src. We confirmed this result and also determined that v-Fps expressed by tsNY225 induced expression of 9E3 mRNA upon temperature shift with the kinetics similar to those observed for v-Src (Fig 5a).

To test the hypothesis that v-Src- and v-Fps-induced gene expression might involve a PKC-mediated signal-transduction pathway, we tested agonists of PKC for

their ability to induce expression of 9E3. PMA, OAG, PBt_2, phospholipase C, and serum, all reported to activate PKC activity (Nishizuka, 1986), strongly activated 9E3 gene expression, whereas dibutyryl-cAMP, which activated the cAMP-dependent protein kinase, protein kinase A (PKA), activated 9E3 gene expression weakly relative to the PKC agonists (Fig 5b). These data show that 9E3 gene expression can be activated by agonists of PKC. Thus, the v-Src and v-Fps induction of 9E3 gene expression could involve a PKC-mediated signal-transduction pathway.

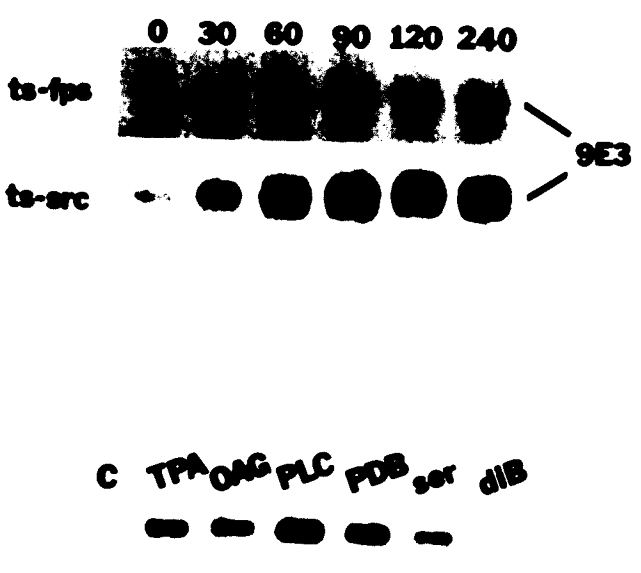


Fig 5. Induction of 9E3 gene expression by v-Src, v-Fps, and protein kinase agonists. (a) Confluent and early-passage CEF infected with tsNY225 (ts-Fps) or tsNY72 (ts-src) were shifted from the nonpermissive temperature (40^0C) to the permissive temperature (34^0C) for the indicated times (min), RNA was extracted and analyzed by Northern blot using radioactively labeled p9E3 DNA as a probe. (b) Protein kinase agonists were added to uninfected CEF for 60 min, and RNA levels were measured as in a. C, control; TPA, PMA (16 nM); OAG (100 μg/ml); PLC, phospholipase C (0.4 unit/ml); PDB, PBt_2 (200 nM); ser, serum, fresh medium added to cultures; diB, dibutyryl-cAMP (3mM; the highest concentration tested).

Depletion of PKC Activity Blocks v-Src- and v-Fps-Induced 9E3 Gene Expression. After treatment of CEF with PMA for 30 hr, the PKC agonist PBt_2 failed to induce 9E3 gene expression (Fig. 6). Similarly, prolonged (30 hr) exposure of virus-infected CEF to PMA resulted in the inability of v-Src and v-Fps to induce 9E3 gene expression (Fig. 6). Removal of PMA by adding fresh medium restored the ability of v-Src and v-Fps to induce expression of 9E3, suggesting that prolonged treatment with PMA did not affect cell viability. These data suggested a PKC requirement for the v-Src and v-Fps induction of 9E3 gene expression.

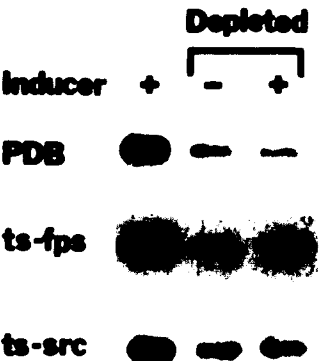


Fig.6. Effect of depleting cells of PKC activity. Confluent CEF were depleted of PKC activity by maintaining them in 800 nM PMA for 30 hr. PBt_2 (200 nM) was added to uninfected CEF (top row). CEF infected with tsNY225 (middle row) or tsNY72 (bottom row) were shifted from 40^0C to 34^0C. RNA was analyzed 60 min after induction.

Protein Kinase Inhibitors Distinguish Signal-Transduction Pathways. Signal-transduction pathways frequently involve protein kinases or protein kinase cascades (Hunter and Cooper, 1985; Rozengurt, 1986a; Rozengurt, 1986b; Ashendel, 1985). Therefore, the effect of protein kinase inhibitors on gene expression was examined to establish patterns of inhibition characteristic of protein kinase-mediated signal transduction. We used four protein kinase inhibitors (H7, H8, HA1004, and sangivamycin), which inhibit a variety of protein kinases with distinguishable K_i values (Hidaka et al., 1984; Kawamoto and Hidaka, 1984; Sasakawa et al., 1986; Ishikawa et al., 1985 and Loomis and Bell, 1988). These inhibitors, which are competitive with ATP, should differentially interfere with signal-transduction pathways by inhibiting different kinases at different concentrations.

Fig 7a shows that at 100 uM, H7 blocks induction of 9E3 by the PKC agonists PMA, PBt_2, OAG and phospholipase C, whereas H8 and HA1004 did so weakly or not at all. Dose response curves for the effects of H7, H8, HA1004, and sangivamycin on PMA-induced 9E3 gene expression are shown in Fig. 7b. H7 blocked PMA-induced 9E3 gene expression with a dose-response curve similar to that of sangivamycin; higher concentrations of H8 were required to block PMA-induced 9E3 gene expression, and HA1004 inhibited only at very high concentrations (Fig 7b).

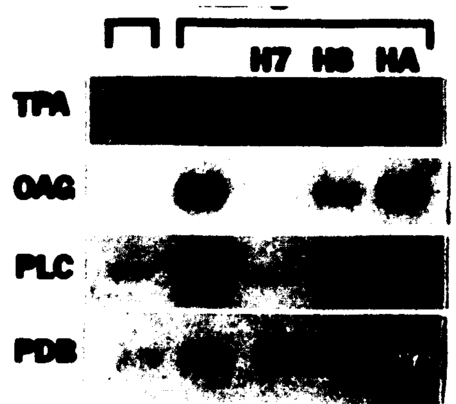
If the inhibition profile seen in Fig. 7 represents a PKC-mediated pathway, then a different profile should be seen when PKC is not involved. We find that PKA agonists-induced expression of the murine TIS10 gene (Lim et al., 1987) is inhibited by protein kinase inhibitors with a profile distinct from that seen for PKC agonist-induced 9E3 gene expression. For 8-Br-cAMP-induction of TIS10, H7 and H8 have similar dose-response curves, whereas sangivamycin inhibits only at very high concentrations (Fig. 8). TIS10, unlike 9E3, is induced strongly by agonists of both PKC and PKA. When TIS10 is induced by the PKC agonist PBt_2, H7 and sangivamycin inhibit at low concentrations with similar dose responses, and H8 is less effective (Fig. 8 open

symbols). Thus, PKC agonist induction of TIS10 gene expression is inhibited by H7, H8, and sangivamycin with the same relative efficiencies as seen for PKC agonist-induced 9E3 gene expression (compare Fig. 7b with Fig. 8 open symbols). The differential inhibition profiles seen for induction of TIS10 gene expression by agonists of PKA and PKC show that protein kinase inhibitors can distinguish different signal transduction pathways. These distinguishable profiles also show that differential effects of these protein kinase inhibitors on gene expression are not due to differences in cell permeability or to non-specific effects of these protein kinase inhibitors on transcription.

Protein Kinase Inhibitors Block v-Src- and v-Fps-Induced 9E3 Gene Expression with the Same Inhibition Profile Seen for PKC Agonist-Induced 9E3 Gene Expression. If the v-Src and v-Fps-induction of 9E3 gene expression is mediated by PKC, then we should observe the same pattern of inhibition of v-Src- and v-Fps-induced 9E3 gene expression by protein kinase inhibitors as that seen for induction of 9E3 by PKC agonists. The effect of H7, H8, and HA1004 (at 100 uM) on v-Src- and v-Fps-induced 9E3 gene expression is shown in Fig. 9a. The pattern of inhibition is very similar to that seen for PKC-agonist-induced 9E3 gene expression (compare Fig. 7a with Fig. 9a). Dose responses to the protein kinase inhibitors H7, H8, HA1004, and sangivamycin for v-Src-induced 9E3 gene expression are shown in Fig. 9b. This pattern of inhibition closely resembles that seen for the PKC agonist PMA (compare Fig. 7b with Fig. 9b). A similar pattern of inhibition was obtained for the v-Fps-induction of 9E3 (data not shown).

The effect of the protein kinase inhibitors is not due to an effect on the v-Src or v-Fps gene products directly, because H7, at 100 uM, had no effect on the autophosphorylation activity of v-Src or v-Fps as measured by *in vitro* kinase assay described in Foster et al., 1985 (Fig. 10). Thus, the profile for v-Src- and v-Fps-

induced 9E3 gene expression with protein kinase inhibitors is consistent with the hypothesis that induction of 9E3 gene expression by these protein-tyrosine kinase oncogenes involves a PKC-mediated pathway.



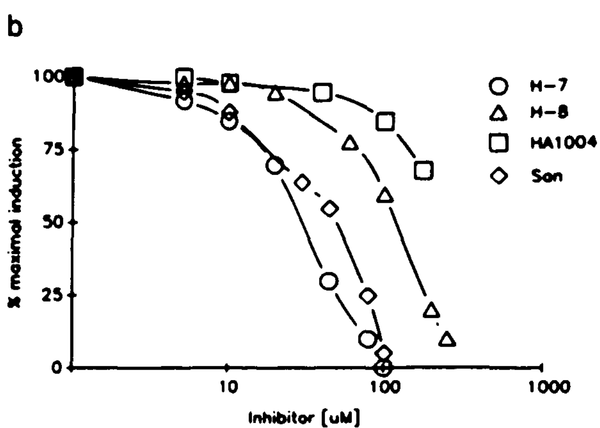


Fig.7. Effect of protein kinase inhibitors on PKC agonist-induced 9E3 gene expression. (a) H7, H8, and HA1004 were added to cell cultures at 100 μM, and PKC agonists were added immediately at the concentrations used in Fig 5b. RNA was extracted 60 min later and subjected to Northern analysis as in Fig. 5. (b) Dose-response curves of the inhibitors H7, H8, HA1004, and sangivamycin (San) for blocking PMA-induced 9E3 gene expression was examined by plotting the level of expression with inhibitor as a percentage of the maximal induced expression seen without inhibitor, as determined by excising gel regions corresponding to 9E3 mRNA and measuring Cerenkov radiation in a scintillation counter.

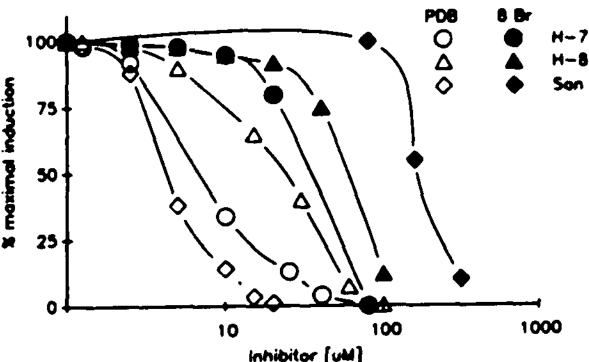
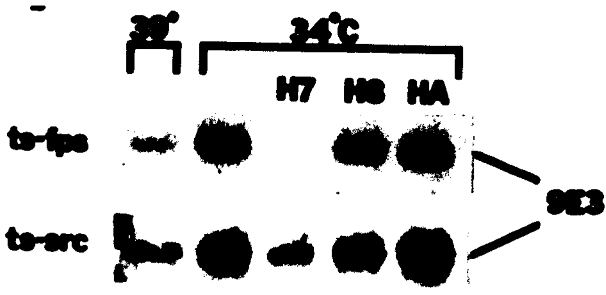


Fig.8. Effect of protein kinase inhibitors on PKC and PKA agonist-induced TIS10 gene expression. TIS10 gene expression was induced in quiescent BALB/c 3T3 cells with 8-Br-cAMP (8 Br, closed symbols) or PBt_2 (PDB, open symbols), and the dose-response curves for the protein kinase inhibitors H7, H8, and sangivamycin (San) were plotted. Analysis was as described in Fig. 7b.



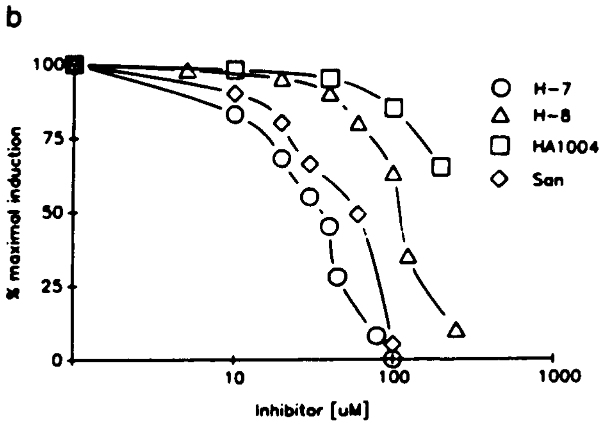


Fig. 9. Sensitivity of v-Src- and v-Fps-induced 9E3 gene expression to protein kinase inhibitors. (a) H7, H8, and HA1004 were added at 100 μM, and cells were shifted immediately from 40^0C to 34^0C. RNA was extracted 60 min later and analyzed as in Fig. 7a. (b) Dose-response curves to protein kinase inhibitors, including sangivamycin (san), for v-Src-induced 9E3 gene expression were generated as in Fig. 7b.

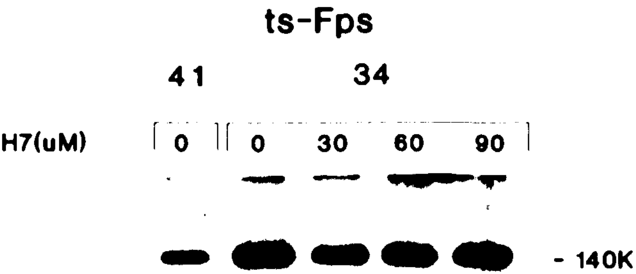


Fig. 10. Effect of Kinase inhibitors on the autophosphorylation activity of Fps. H7 was added at 100 uM to v-Fps-transformed rat fibroblasts. Total protein lysate was extracted and immunoprecipitated with an antibody to v-Fps. *In vitro* kinase activit'' was determined as described by Foster et al., (1985)

Effect of kinase inhibitors on phosphorylation of the 67-kDa PKC substrate. PBt_2-induced 67-kDa phosphorylation was sensitive to sangivamycin and insensitive to H7 at concentrations of these compounds that were effective in blocking PBt_2-induced 9E3 gene expression (Fig. 11b). Similar results were obtained for v-Fps-induced 67-kDa phosphorylation (Fig. 11a). This observation suggests that sangivamycin may act on PKC directly, whereas H7 is probably acting on a kinase functioning downstream from PKC.

Whether phosphorylation of this 67-kDa PKC substrate is required for induction of 9E3 is unclear; however, these data directly implicate PKC in v-Src- and v-Fps-mediated events.

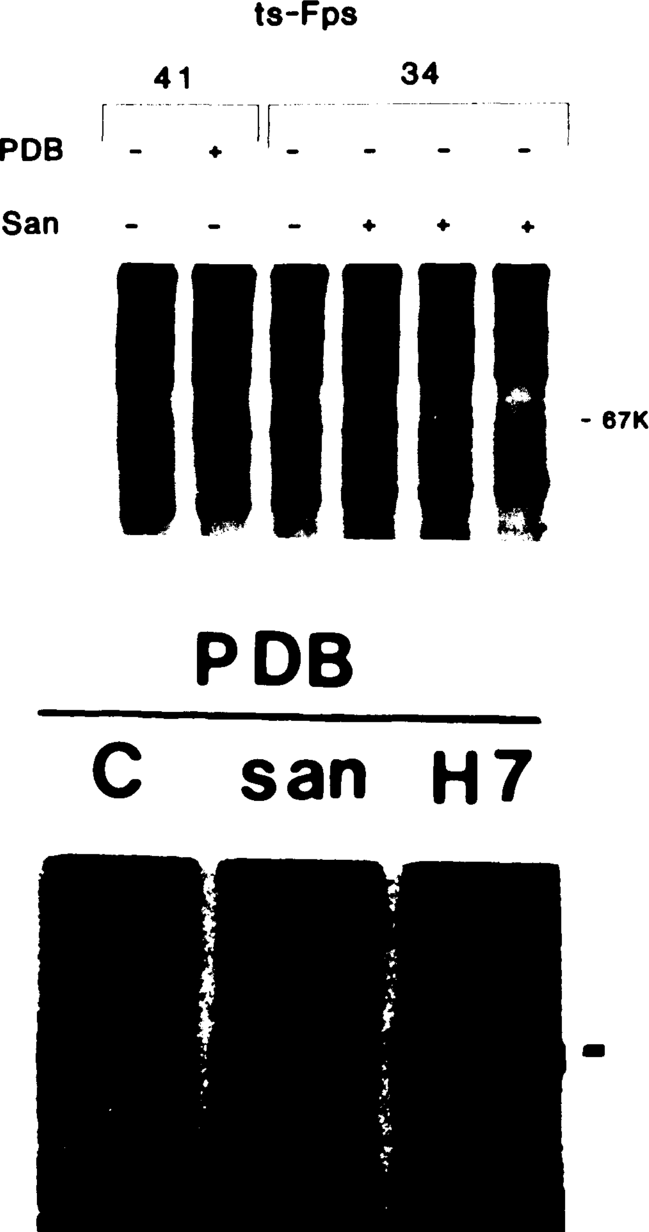


Fig. 11. Effect of kinase inhibitors on phosphorylation of 67-kDa protein. (a) Sangivamycin was added to infected CEF at 40^{0}C. Cells were shifted to 34^{0}C and the effect of the inhibitor on 67-kDa phosphorylation was determined. (b) CEF were treated with PBt_2, and the sensitivity of 67-kDa protein phosphorylation to sangivamycin (san) and H7 was tested at 100 μM each.

v-Src activation leads to the phosphorylation of a major PKC substrate in mouse fibroblasts.

Diacylglycerol, which activates PKC, has been shown to be elevated in murine cells stably transformed by v-Src; however, increased phosphorylation of the 80-kDa PKC substrate was not detected (Wolfman & Macara, 1987). Thus, in murine fibroblasts, the involvement of PKC in v-Src-induced signals, has been unclear. The murine cell line TNR9 (Butler-Gralla et al., 1981) which is resistant to the mitogenic effects of phorbol esters that activate PKC, is also resistant to transformation by v-Src suggesting a requirement for PKC for v-Src-induced transformation (Nori et al., 1990). However, v-Src-induced DNA synthesis and v-Src-induced desensitization of the epidermal growth factor receptor have been reported to be independent of PKC (Han et al., 1990; Gray & Macara, 1988). Because of data both implicating PKC and showing independence of PKC from different laboratories, the involvement of PKC in v-Src-induced signaling in murine fibroblasts has remained in question. It is not clear whether the different conclusions made regarding PKC involvement in v-Src-induced signals are due to different intracellular contexts of different cell types or due to v-Src activating both PKC-dependent and independent signaling pathways in the same cell. Activation of PKC occurs in many tissues and cell types (Nishizuka, 1988). Activation of PKC in mouse fibroblasts leads to phosphorylation of a protein with molecular weight of 80 kilodalton (Rodriguez-Pena & Rozengurt, 1984). Fig. 12. shows that agonists of PKC but not agonists of PKA induce phosporylation of an 80-kDa protein in mouse fibroblasts.

If v-Src activates PKC, then we should see increased phosphorylation of the 80-kDa protein in response to the PTK activity of v-Src. Our strategy was to use BALB c/ 3T3 mouse fibroblasts stably transfected with derivative of v-src (LA90), which is temperature sensitive (ts) for PTK activity. We investigated whether v-Src could induce phosphorylation of the 80 kilodalton MARCKS protein in LA90 cells.

Consistent with the work by Macara and coworkers (Han et al., 1990; Wolfman et al., 1987), we could not detect phosphorylation of MARCKS protein in LA90 cells. We were unable to induce phosphorylation of MARCKS protein in LA90 cells with TPA at either the permissive or non-permissive temperature (Fig. 13); whereas, TPA-induced phosphorylation of MARCKS protein was readily detectable in the parental Balb/c 3T3 cell line (Fig. 12 and Qureshi et al., 1991a). We have found that lack of inducibility of MARCKS phosphorylation in v-Src-transformed cells is due to transcriptional down-regulation of this protein in LA90 cells rather than a lack of v-Src-induced PKC activity or high baseline levels of MARCKS phosphorylation (see section III). Han et al. (1990) have suggested that LA90 cells are somewhat leaky for v-Src at the non-permissive temperature. This could be responsible for the inability to detect phosphorylation of MARCKS in LA90 cells. To circumvent this problem, we employed a transient transfection assay using a plasmid carrying cloned ts LA90 DNA driven by a cytomegalovirus promoter (A. Maroney and J. Brugge, In press). Cloned LA90 DNA was transfected into NIH3T3 murine fibroblasts, which take up and express exogenous DNA very efficiently. We then examined MARCKS protein phosphorylation in these cells after activating v-Src by temperature shift. As shown in Figure 14, increased phosphorylation of an 80-kDa protein is detected in response to treatment with PDB, and within 15 minutes after temperature shift. Phosphorylation of this protein was not detected in cells which had been depleted of PKC by long term treatment with TPA (Fig. 14b). Immunoprecipitation using an antisera raised against MARCKS protein (Rosen et al., 1989), confirmed that the same protein was phosphorylated in response to PDB and v-Src (Fig. 14c). NIH3T3 cells transfected with the vector plasmid without v-Src did not show any change in the phosphorylation state of this protein upon temperature shift (not shown). Thus, activating the PTK activity of v-Src leads to the phosphorylation of a major PKC substrate in NIH3T3 cells strongly suggesting that v-Src activates PKC in murine cells as well as avian cells.

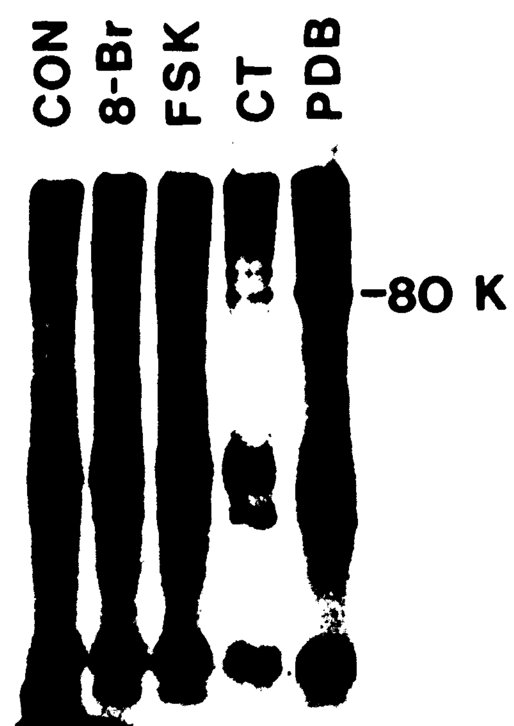


Fig. 12. Effect of PKC and PKA agonists on phosphorylation of an 80 kDA protein. Cholera Toxin, CT (2 ug/ml), PDB (50 ng/ml), 8-Br-cAMP (1 mM), and forskolin, FSK (25 uM) were added to quiescent BALB/c 3T3 cultures, and phosphorylation of the 80-kDa PKC substrate was examined 15 min later as described by Spangler et al., 1989.

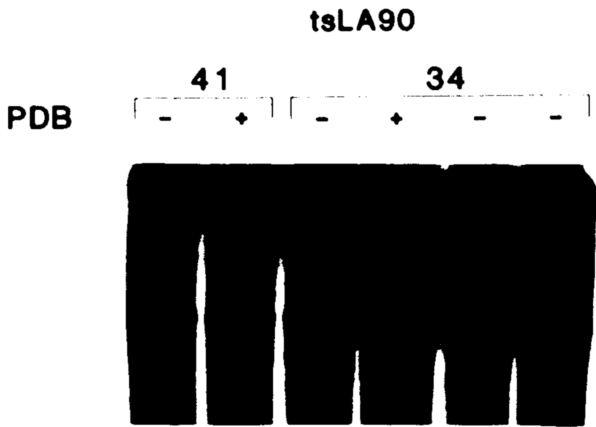


Figure. 13. Lack of Phosphorylation of MARCKS in LA90 Cells. LA90 cells at 40^{0}C were pre-labeled with [^{32}P]Orthophosphate as described in "materials and methods". Cells were treated with PDB 100 ng/ml or shifted to 34^{0}C as indicated.

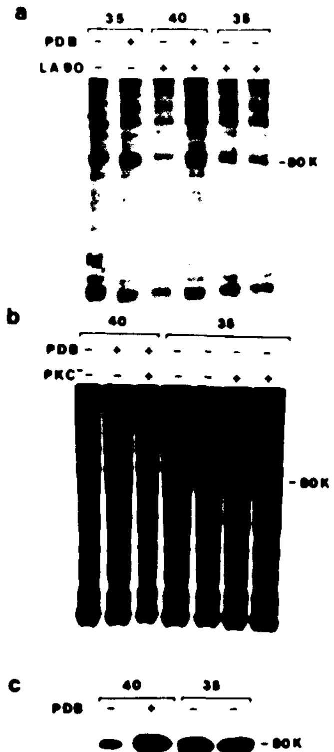


Fig. 14. Phosphorylation of the PKC substrate MARCKS. (a) Molecularly cloned LA90 DNA, expressed under the control of cytomegalovirus promoter was transfected into NIH3T3 cells as described in "Materials and Methods". 16 h after transfection, the cells were washed and fed with medium containing 1% serum. 30 h later, cells were induced either with PDB (100 ng ml^{-1}) or temperature shift. Phosphorylation of the 80 kilodalton MARCKS protein was examined 15 min after the addition of PDB and at 15 and 30 min after shifting to the permissive temperature for v-Src.
(b) Effect of PKC depletion on phosphorylation of the 80 kDa substrate. NIH3T3 cells were transfected with cloned LA90 DNA as described in (a). For PKC depletion (PKC^-) cells were treated with 100 ng ml^{-1} TPA for 26 h prior to PDB stimulation and temperature shift.
(c) Immunoprecipitation of MARCKS. PDB-induced (lane 2), and v-Src-induced (lanes 3 and 4; 15 and 30 min time points, respectively) as described in (a), were immunoprecipitated with antisera raised against MARCKS protein (Rosen et al., 1989) and electrophoresed as described previously (Spangler et al., 1989)

Activation of the kinase activity of v-Src leads to increased levels of DAG.

Since we demonstrated that v-Src activates PKC in both avian and murine fibroblasts (Qureshi et al., 1991 & Spangler et al., 1989). We wished to examine whether DAG, the physiological activator of PKC (Nishizuka, 1986), was produced in response to v-Src. Song et al., (1991) looked for increases in DAG levels in response to the PTK activity of v-Src. The effect of increased v-Src kinase activity was examined in LA90 cells (Gray & Macara, 1988 and Qureshi et al., 1991). Phospholipids of LA90 cells and the parental BALB/c 3T3 cells were metabolically prelabeled with [^{3}H]glycerol for 24h. Quiescent LA90 cells maintained at the non-permissive temperature for v-Src (40^{0}C) were shifted to the permissive temperature (35^0) to activate the PTK activity of v-Src, and the levels of ^{3}H-labeled DAG were examined by TLC. As shown in Fig. 18, ^{3}H-labeled DAG levels rapidly increased in LA90 cells after the temperature shift. The treatment had no effect on ^{3}H-labeled DAG levels in the parental BALB/c 3T3 cell line (Fig. 18). The time course for the generation of DAG was consistent with the time course for v-Src-induced phosphorylation of the PKC substrate MARCKS, strongly suggesting that the DAG produced likely contributes to the activation of PKC by v-Src. Interestingly, the source of DAG was phosphatidylcholine via a type D phospholipase/phosphatidic acid phosphatase mechanism rather than the better known type C phospholipase mediated hydrolysis of PIP_2 (Song et al., 1991).

Fig. 15. v-Src-induced DAG. LA90 and BALB/c 3T3 cells were prelabeled for 24 h with [^{3}H]glycerol. Cells were shifted from the nonpermissive (40^{0}C) to the permissive (34^{0}C) temperature for v-Src, and levels of radioactively labeled DAG were determined at the times shown. Data are averages of three separate experiments.

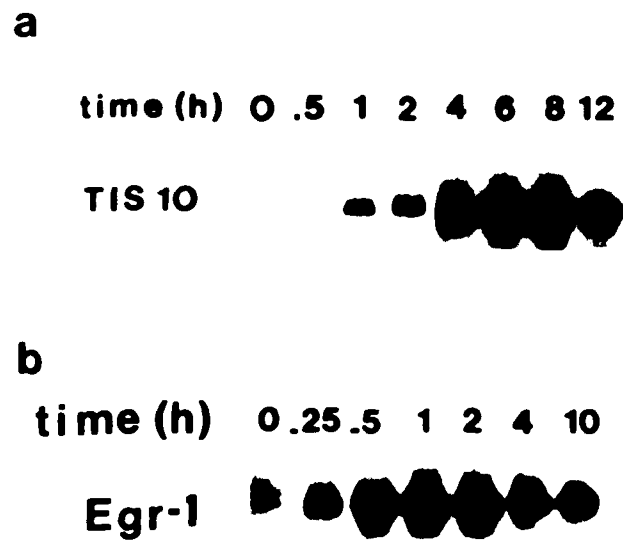


Fig. 16. Induction of TIS10 and Egr-1 expression by v-Src. Quiescent LA90 cells were shifted from the non-permissive (40^{0}C) to the permissive (35^{0}C) temperature for v-Src. RNA was harvested at the times indicated, electrophoresed, and hybridized to radiolabeled DNA as described in Material and methods. **(a)** TIS10. **(b)** Egr-1

v-Src induced expression of TIS10 and Egr-1 are differentially sensitive to PKC depletion.

Cell division signals induce many changes in cellular metabolism including altered transcription patterns. Cellular responses leading to altered transcription patterns are initiated by induction of "primary response" genes (Herschmann, 1989), which are activated in the absence of new protein synthesis. Recently, several primary response genes have been identified that are induced by a variety of stimuli (Almendral et al., 1988; Lau & Nathans, 1987; Lim et al., 1987). TIS10 (Lim et al., 1987) and Egr-1 (Sukhatme et al., 1987) are primary response genes first isolated as phorbol ester- or mitogen-inducible gene sequences.

We wanted to determine if PKC was required for v-Src-induced gene expression. Therefore, in collaboration with Sajjad Qureshi in the lab, we examined the ability of v-Src to induce expression of TIS10 and Egr-1 in murine fibroblasts. We used Balb/c 3T3 cells infected with the LA90 temperature sensitive strain of Rous sarcoma virus (LA90 cells). Shifting from the nonpermissive (40^0C) to the permissive (35^0C) temperature rapidly activates the PTK activity of the v-Src gene product (Gray & Macara, 1988). Shifting LA90 cells from the non-permissive to the permissive temperature led to the induction of both TIS10 and Egr-1 as detected by northern gel analysis (Figure 16). Egr-1 expression peaked at about 1 h and TIS10 peaked at about 4 h. Parental Balb/c 3T3 cells subjected to the same temperature shift did not show increased levels of TIS10 or Egr-1 mRNA (not shown). Pretreatment of cells with cycloheximide (20 μg/ml^{-1}) for 30 min prior to temperature shift did not block v-Src-induced TIS10 or Egr-1 expression (not shown); thus v-Src-induced expression of TIS10 and Egr-1 is independent of new protein synthesis.

Both TIS10 and Egr-1 are induced by phorbol esters which activate PKC (Lim et al., 1987; Sukhatme et al., 1987). Therefore, we looked at the ability of v-Src to induce TIS10 and Egr-1 in cells depleted of PKC activity by prolonged exposure to

phorbol ester (Rodriguez-Pena & Rozengurt, 1984). LA90 cells were treated with increasing concentrations of 12-O-tetradecanoylphorbol 13-acetate (TPA) for 26 h. This treatment reduced the level of PKC protein and PKC activity in LA90 cells, as measured by Ca^{2+}-, phospholipid-, and diacylglycerol-dependent histone phosphorylation, to background levels at 100 $ngml^{-1}$ (Table I). Depleting LA90 cells of PKC blocked more than 70% of v-Src-induced TIS10 gene expression; however, depleting cells of PKC activity had no effect upon v-Src-induced Egr-1 expression (Figure 17). Thus v-Src-induced TIS10 expression is sensitive to PKC-depletion, suggesting that v-Src is activating a PKC-mediated signaling pathway for induction of TIS10. The data also demonstrate that v-Src is activating other signaling pathways since the induction of Egr-1 was insensitive to PKC depletion.

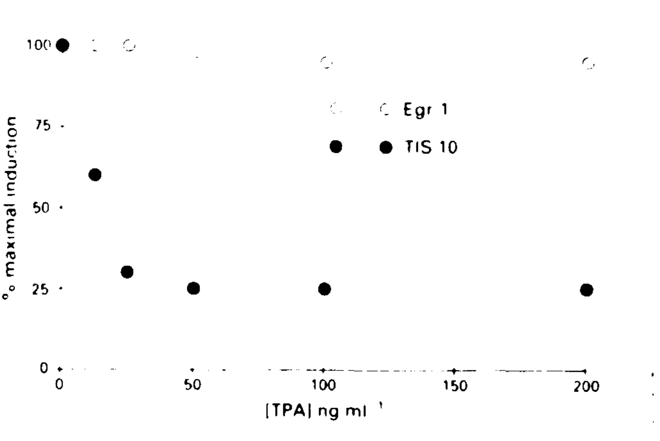


Fig. 17. The effect of depleting cells of PKC on v-Src-induced TIS10 and Egr-1 expression. Increasing concentrations of TPA (ng ml^{-1}) were added to LA90 cell cultures for 26 h prior to either temperature shift or addition of PDB (100 ng ml^{-1}). TIS10 and Egr-1 RNA levels were measured as in Figure 16 after 2 h induction by either temperature shift or PDB.

The induction of TIS10 expression by v-Src and phorbol esters have the same dose response to the protein kinase inhibitors H7 and sangivamycin.

As illustrated in Section II and described elsewhere (Spangler et al., 1989; Qureshi et al., 1991), protein kinase inhibitors can be used to distinguish intracellular signaling pathways. Induction of TIS10 by agonists of PKC was highly sensitive to H7 (Hidaka et al., 1984) and sangivamycin (Loomis et al., 1988), whereas induction of TIS10 by agonists of protein kinase A was relatively insensitive to these compounds (Spangler et al., 1989). The dose responses of v-Src-induced TIS10 and Egr-1 expression to H7 and sangivamycin were compared with those for phorbol ester-induced TIS10 and Egr-1 (Figure 21). Whereas the sensitivity of phorbol ester- and v-Src-induced TIS10 to both H7 and sangivamycin were indistinguishable (Figure 18a and 18b), the sensitivities of phorbol ester and v-Src-induced Egr-1 to these compounds were different (Figure 18c and 18d). The *in vivo* specificities of both H7 and sangivamycin are unknown. Both H7 and sangivamycin have been reported to be effective in blocking PKC *in vitro*, however, this has not held true **in vivo** (Spangler et al., 1989). Thus, it is not clear which protein kinase(s) is required for v-Src-induced Egr-1 that is not required for phorbol ester-induced Egr-1 or v-Src-induced TIS10. However, these data demonstrate that the intracellular signaling pathways activated by phorbol ester and v-Src leading to the induction of Egr-1 are different and that the induction of TIS10 by phorbol ester and v-Src are possibly the same.

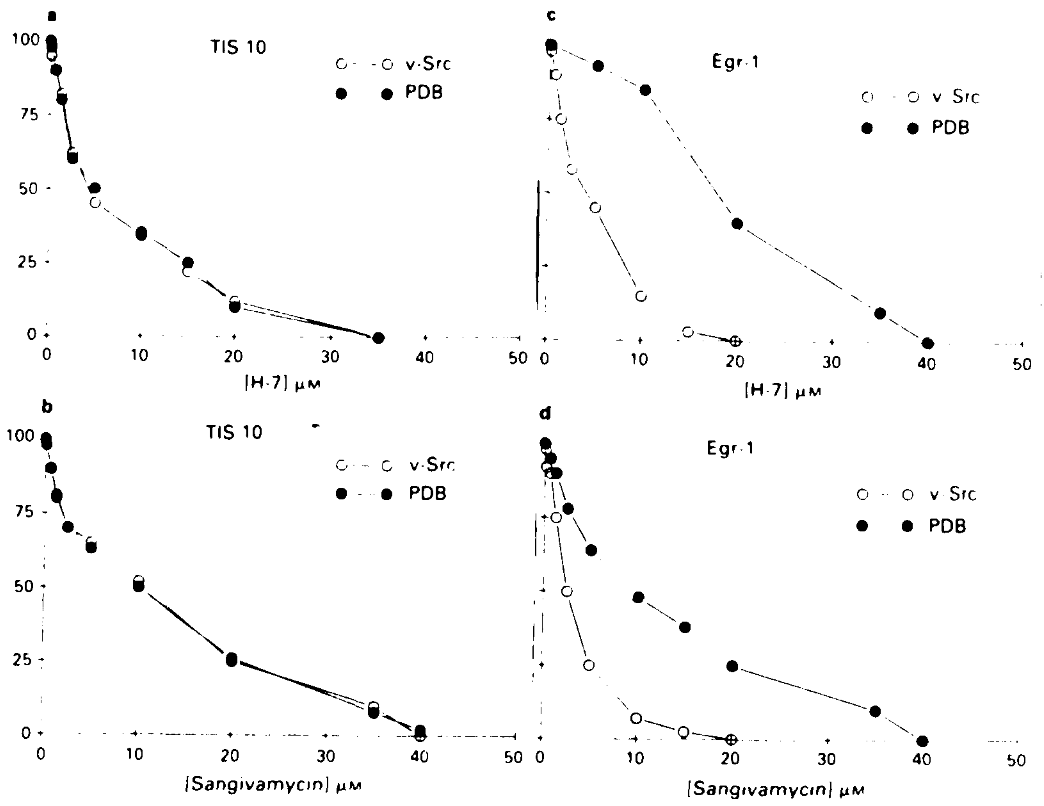


Fig. 18. The sensitivity of v-Src- and phorbol ester-induced TIS10 expression to the protein kinase inhibitors H7 and sangivamycin. LA90 cell cultures were either subjected to temperature shift to activate v-Src or were treated with PDB (100 ng ml^{-1}), H7 and Sangivamycin at the concentrations given (um) were added 10 min prior to stimulus. Northern analysis was performed and densitometric analysis of autoradiographs is presented. TIS10; **(a)** H7, **(b)** sangivamycin. Egr-1; **(c)** H7, **(d)** sangivamycin

DISCUSSION

We have presented evidence that both v-Src and v-Fps rapidly induce phosphorylaton of a major PKC substrate in CEF. In addition, we find that both v-Src and v-Fps use a PKC-mediated signal-transduction pathway to induce expression of a transformation-related gene. We have shown that depletion of PKC activity by prolonged treatment with PMA blocks the ability of v-Src and v-Fps to induce 9E3 gene expression. We also show that agonists of PKC activate 9E3 gene expression and that this activation is sensitive to protein kinase inhibitors with the same inhibition profiles seen for v-Src- and v-Fps-induced 9E3 gene expression. Taken together, these data support a role for PKC in v-Src- and v-Fps-induced 9E3 gene expression.

We have also demonstrated that v-Src induces phosphorylation of MARCKS and expression of the TIS10 gene (Lim et al., 1987) using a PKC-dependent pathway. This finding further establishes a role for PKC in v-Src induced signaling and demonstrates that v-Src is also activating a PKC signaling pathway in murine fibroblasts.

One role for PKC that could be inferred from our data would be a positive effect of PKC on v-Src or v-Fps that is blocked by PKC depletion or inhibition of a PKC-mediated pathway. v-Src has been reported to be phosphorylated on Ser-12 by PKC both *in vivo* and *in vitro* (Gould et al., 1985). However, phosphorylation of v-Src by PKC at this site appears to have no functional significance (Gould et al., 1985). In addition, mutants of v-Src lacking Ser-12 can still transform cells (Pellman et al., 1985). These data suggest that increased protein-tyrosine kinase activity of v-Src and v-Fps gene products induces elevation of PKC activity, which subsequently leads to the induction of 9E3 gene expression. This conclusion is supported by our observation that activating the kinase activity of v-Src and v-Fps rapidly induces phosphorylation of 67-kDa PKC substrate.

A model involving a PKC-mediated pathway predicts that transformation by v-Src and v-Fps might involve phospholipid metabolism to generate the co-factors necessary for activating PKC (Ashendel, 1985). In agreement with this model, v-Src has been reported to increase phospholipase D activity (Song et al., 1991) which generates the second messenger diacylglycerol, which activates PKC activity. Cells transformed by v-Src have been shown to have increased diacylglycerol (Wolfman et al., 1987). This is consistent with our data which shows that increased levels of diacylglycerol can be detected within 5 min of elevating the kinase activity of v-Src (Song et. al., 1991; Martins et al., 1989). A scheme in which v-Src activates PKC predicts that prolonged activation of PKC would down-regulate PKC activity by a mechanism similar to the down-regulation seen when cells are exposed to PMA. Macara and coworkers (1987) have demonstrated a reduced ability to induce phosphorylation of the 80-kDa major PKC substrate in v-Src-transformed murine cells. These data are consistent with our data (see section III) and with a role for PKC in v-Src- and v-Fps-induced expression of the 9E3 gene, and also v-Src-induced expression of the TIS10 gene. Whether the PKC-mediated signaling pathway leading to the induction of TIS10 is required for transformation remains to be determined. However, the TPA resistant cell line, TNR9 (Butler-Gralla & Herschman, 1981), is resistant to transformation by v-Src, suggesting that a PKC-mediated pathway is required for v-Src to transform (Nori et al., 1990). In addition, v-Src-induced expression of Egr-1 is not sensitive to PKC depletion. Thus, v-Src-induced transformation may require activation of PKC-dependent and PKC-independent signaling pathways (Qureshi et al. 1991).

Primary targets for activating the PKC-dependent and independent pathways are not known. However, there are several candidate signaling intermediates that have been implicated as potential substrates or targets of v-Src. These include Ras (Smith et al., 1986), Raf(Morrison et al., 1988), phosphatidylinositol kinase (Kaplan et al., 1987; Courtneidge & Heber, 1987) and GAP (Ellis et al., 1990). In addition to these,

we have demonstrated the involvement of a G-protein of the heterotrimeric class (Alexandropoulos et al., 1991) and a type D phospholipase specific for phosphatidylcholine (Song et al., 1991). Thus it is not inconceivable that v-Src could activate six or more intracellular signaling pathways. The controlling promoter sequences of growth signal responsive genes contain multiple response elements that allow for subtle and complex control of gene expression (Mitchell & Tijan, 1989). It is therefore likely that incoming signals that activate gene expression are also subtle and complex. One way to generate complexity of intracellular signals originating from a single source like v-Src is to act on several signal transducing substrates. Thus, v-Src may induce the transformed phenotype by activating a complex network of several intracellular signaling pathways.

SECTION III

MARCKS Protein is Transcriptionally Down-Regulated in v-Src-Transformed BALB/c 3T3 Cells.*

INTRODUCTION

As described in section II, PKC has been found to play an important role in a wide array of cellular processes. Much less is known, however, about the substrates directly phosphorylated by the activated kinase and their roles in mediating PKC's cellular effects. The MARCKS protein is a prominent cellular substrate for PKC (Rozengurt et al., 1983; Stumpo et al., 1989), and as such may play a role in some of the numerous physiological responses of cells to agents that activate PKC. A role for MARCKS protein, however, has not been firmly established. It has been demonstrated that MARCKS binds calmodulin in a phosphorylation dependent manner (Graff et al., 1989; Hatrwig et al., 1992), and that its regulation of expression may be governed by the levels of PKC (Lindner et al., 1992; Brooks et al., 1991). Thelen et al. (1991) recently proposed that phosphorylation of MARCKS by PKC may lead to the dissociation of actin filaments from the plasma membrane. Thus, MARCKS phosphorylation may contribute to the morphological changes that occur upon transformation. Reduced levels of MARCKS protein might produce the same morphological phenotype.

Although increasing the protein tyrosine kinase activity of v-Src has been shown to cause increased phosphorylation of MARCKS protein in both avian and

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* Joseph, C. K., Qureshi, S. A., Wallace, D. J. and Foster, D. A. (1992). *J. Biol. Chem.* **267**, 1327-1330. Reproduced with permission of the publisher.

murine cells (Spangler et al., 1989; Qureshi et al., 1991c), phosphorylation of MARCKS in fibroblasts stably-transformed by v-Src has been difficult to demonstrate (Wolfman et al., 1987). Reduced phosphorylation of MARCKS has been reported in the phorbol ester resistant 3T3-TNR9 mouse fibroblast cell line and in fibroblasts transformed by benzo(a)pyrene (Biemann and Erikson, 1990; Yang and Pardee, 1986). Recently, Simek et al. (1989) demonstrated that MARCKS RNA levels were reduced in a mouse tumor cell line. Thus, MARCKS might be important for maintaining a nontransformed phenotype; possibly by maintaining appropriate actin-plasma membrane interactions. Consistent with this hypothesis, we report here that MARCKS is transcriptionally down-regulated in murine fibroblasts transformed by v-Src.

RESULTS

Phosphorylation of MARCKS protein in normal and v-Src transformed cells. Treatment of fibroblast cells with the phorbol ester, phorbol 12,13-dibutyrate (PDB), results in the rapid phosphorylation of an 80 kilodalton protein (Fig. 13). However, the same level of phorbol ester that induced phosphorylation of this 80 kilodalton protein in BALB/c 3T3 cells did not significantly increase levels of phosphorylation of an 80 kilodalton protein in v-Src-transformed BALB/c 3T3 cells (Fig. 19a). Also shown in Fig. 19a is that phosphorylation of this protein was not observed in BALB/c 3T3 cells depleted of PKC by long term exposure to 12-O-tetradecanoylphorbol-13-acetate (TPA) (Rodriguez-Pena and Rozengurt, 1984). This suggested that the 80 kilodalton protein being phosphorylated in response to PDB was the well-characterized 80 kilodalton PKC substrate known as MARCKS (Stumpo et al., 1989). To further establish this, we used an antibody raised against murine MARCKS (Rosen et al., 1989). The increased phosphorylation observed on the 80 kilodalton protein recognized by this antibody was similar to the increased phosphorylation of the 80 kilodalton

80 kilodalton protein observed in whole cell lysates (Fig. 19b). This further established that the 80 kilodalton protein being phosphorylated was MARCKS. The level of phosphorylated MARCKS recognized by the antibody in untreated v-Src-transformed cells was reduced to 27% that observed in the parental BALB/c 3T3 cells as determined by densitometer tracings. The data in Fig. 19 suggests that either PKC or its substrate MARCKS is down-regulated in v-Src transformed cells.

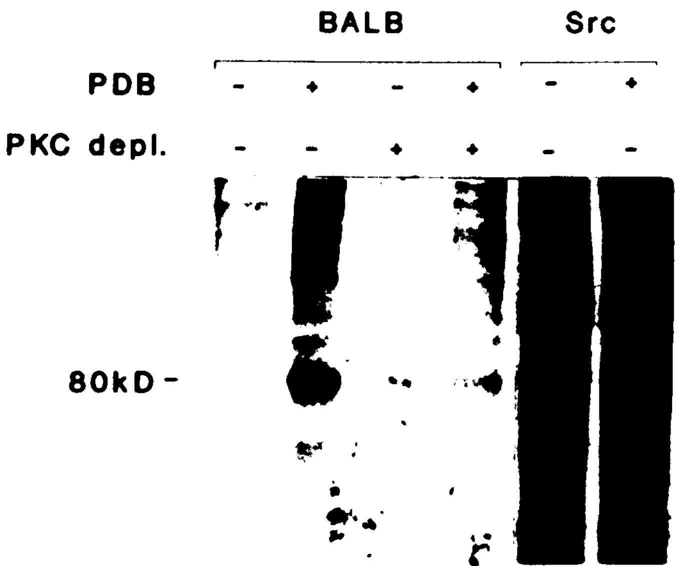


Fig. 19. Phosphorylation of MARCKS protein in BALB/c 3T3 cells and v-Src-transformed BALB/c 3T3 cells. (a) BALB/c 3T3 cells (BALB) and v-Src-transformed BALB/c 3T3 cells (Src) were pre-labeled with [^{32}P]-orthophosphate as described in "Experimental Procedures". Where indicated, cells were induced with PDB (100 ng/ml) and phosphorylated proteins were examined 10 minutes later. For PKC depletion, cells were treated with 100 ng\ml of TPA for 26 hours.

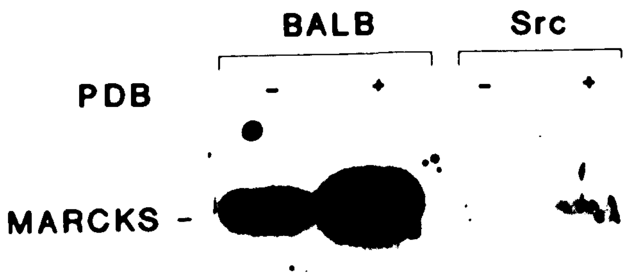


Fig. 19.(b) Lysates from orthophosphate labelled cells were immunoprecipitated with anti-MARCKS antibody (Rosen et al., 1989) and analyzed for MARCKS protein as previously described (Spangler et al. 1989).

PKC levels in normal and v-Src-transformed cells. As shown in Fig 19a, phosphorylation of MARCKS was not observed when cells were depleted of PKC by long term exposure to TPA. Thus, the presence of PKC is essential for phosphorylation of MARCKS. The decreased phosphorylation of MARCKS in v-Src transformed cells could be the result of PKC down-regulation. Therefore, the level of PKC and PKC activity in normal and v-Src-transformed cells was analyzed. Total cell lysates from both normal and transformed cells were subjected to electrophoresis on SDS gels and proteins were subjected to "Western blot" analysis using an antibody raised against a peptide from a highly conserved region of the kinase domain of PKC

(Ballester and Rosen, 1985). The level of PKC in both normal and v-Src-transformed cells were equivalent (Fig. 20). Also shown in Fig. 20 is that the most prominent band recognized by this antibody in both v-Src-transformed and nontransformed BALB/c 3T3 cells disappeared upon prolonged TPA treatment which depletes cells of PKC (Rodriguez-Pena and Rozengurt, 1984). Partial purification of PKC from total cell lysates and analysis of *in vitro* PKC activity using histone type III-S as substrate showed that PKC activity in both v-Src-transformed and nontransformed BALB/c 3T3 cells was also equivalent (Table I). This activity was decreased in both v-Src-transformed and non-transformed cells upon prolonged exposure to TPA. These data demonstrate that the reduced ability to phosphorylate MARCKS protein in v-Src-transformed cells is not due to a lack of PKC.

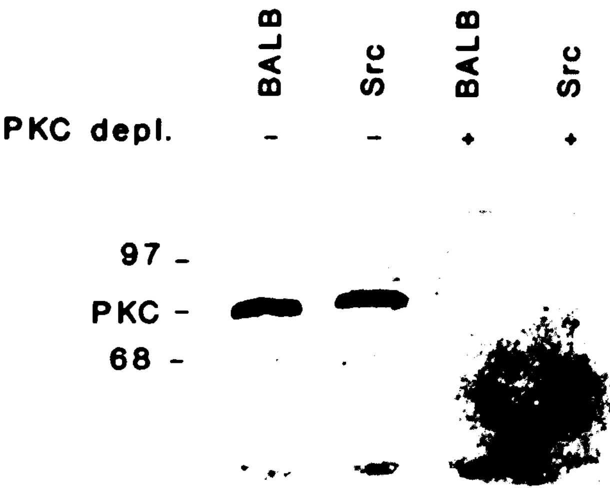


Fig. 20. PKC levels in normal and v-Src transformed cells. Total cell lysates were made from v-Src-transformed (Src) and nontransformed BALB/c 3T3 (BALB) cells that were either untreated (-) or treated (+) with TPA to deplete cells of PKC as in Fig. 19. The lysates were analyzed for PKC protein levels by "western gel" analysis as described in "Materials and Methods".

Table I

PKC activity in normal and v-Src-transformed BALB/c 3T3 cells

Total PKC (membrane plus cytosolic) was isolated from v-Src-transformed (Src) and normal BALB/c 3T3 (BALB) cells as described in "Experimental procedures" and assayed for kinase activity using histone type III-S as substrate. Cells were depleted of PKC by treating with TPA (100 ng/ml) for 26 hours as described previously (Qureshi et al., 1991b). Kinase activity is expressed as the difference between activities in the presence or absence of calcium, phosphatidylserine and diacylglycerol. Values are the mean of three determinations.

	pmoles ^{32}P incorporated into Histone III-S/ g protein/min	
	Untreated	PKC depleted
BALB	14.2 (+/- 0.8)	0.14 (+/- 0.02)
Src	14.0 (+/- 0.9)	0.13 (+/- 0.02)

Reduced levels of MARCKS protein in transformed cells. The lack of phosphorylation of MARCKS protein in v-Src-transformed cells could be explained by reduced levels of expression of MARCKS. Therefore, total cell lysates from nontransformed and v-Src-transformed cells were subjected to Western blot analysis using an antibody raised against murine MARCKS protein (Rosen et al., 1989). In Fig. 21, it can be seen that the level of MARCKS protein in v-Src-transformed cells was reduced relative to the parental BALB/c 3T3 cells. These data suggest that the decreased ability to induce MARCKS phosphorylation in v-Src-transformed cells is due to reduced levels of this protein.

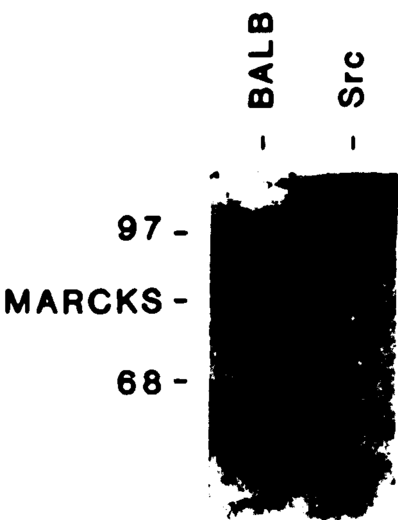


Fig. 21. MARCKS protein levels in normal and v-Src transformed cells. Total cell lysates from v-Src-transformed (Src) and nontransformed BALB/c 3T3 (BALB) cells were analyzed for MARCKS protein levels by "western gel" analysis as described in "Materials and Methods".

MARCKS RNA levels are reduced in v-Src-transformed cells. Decreased protein levels in the transformed cells could be the result of reduced MARCKS RNA levels. Therefore, we performed Northern analysis of total RNA from both normal and transformed cells. As shown in Fig. 22, v-Src-transformed cells contained lower levels of MARCKS RNA when compared with normal cells. The level of ß-actin RNA was unchanged in both cell types. The degree to which the MARCKS RNA was reduced in v-Src-transformed relative to the nontransformed BALB/c 3T3 cells was determined by densitometer tracing of the data in Fig. 22 and the value correlated well with the degree to which MARCKS phosphorylation was reduced in Fig. 19; 27% for phosphorylation and 23% for RNA. These data suggest that the reduced levels of MARCKS phosphorylation seen in Fig. 19 and the reduced levels of MARCKS protein seen in Fig. 21 are due to reduced levels of MARCKS transcripts.

MARCKS transcription is reduced in v-Src-transformed cells. To investigate whether the decrease in the level of MARCKS RNA in v-Src transformed cells was due to decreased RNA stability or decreased transcription, we performed nuclear "run-on" assays (Greenberg and Ziff, 1984). Transcription of the MARCKS gene was decreased in nuclei isolated from v-Src-transformed cells relative to that observed in nuclei isolated from the nontransformed parental BALB/c 3T3 cells (Fig. 23). The observed reduction in MARCKS transcription in v-Src-transformed cells (32% that observed in BALB/c 3T3 cells) correlated with the decrease in MARCKS RNA. Thus, reduced levels of MARCKS RNA are apparently the result of reduced MARCKS transcription in v-Src-transformed cells.

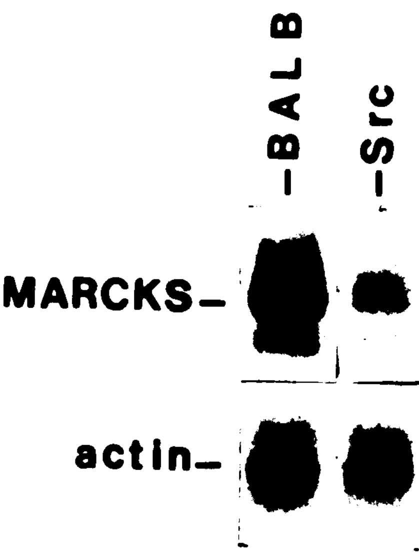


Fig. 22. **Analysis of MARCKS RNA levels in normal and v-Src-transformed BALB/c 3T3 cells.** Total RNA was harvested from BALB/c 3T3 cells (BALB) and v-Src-transformed BALB/c 3T3 cells (Src), electrophoresed, and subjected to northern gel analysis using radiolabeled MARCKS DNA as a probe as described in "Materials and Methods".

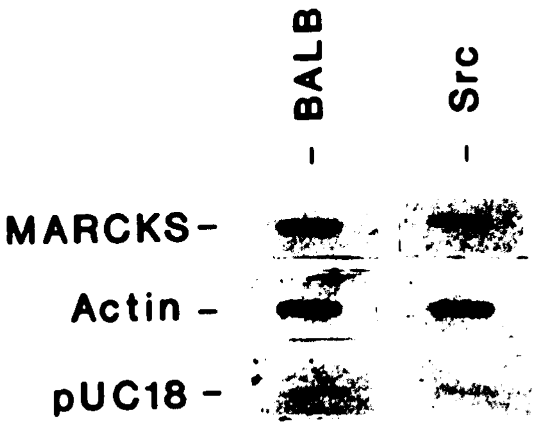


Fig. 23. Analysis of MARCKS RNA levels in normal and v-Src-transformed BALB/c 3T3 cells. Nuclear "run-on" assays with nuclei isolated from v-Src-transformed (Src) and non-transformed BALB/c 3T3 (BALB) cells used in Fig. 4 were performed as described in "Materials and Methods". Transcription of MARCKS is compared with transcription of ß-actin (Actin). pUC-18 vector DNA was used as a negative control.

Herbimycin A restores MARCKS RNA levels, MARCKS transcription, MARCKS protein levels, and phorbol ester-induced MARCKS phosphorylation. Herbimycin A is benzoquinonoid ansamycin antibiotic that was found to reverse oncogenic transformation induced by v-Src (Uehara et al., 1989a; Uehara et al., 1989b). Herbimycin A both inhibits tyrosine phosphorylation mediated by v-Src and increases v-Src turnover (Uehara et al., 1989c). In v-Src-transformed BALB/c 3T3 cells, treatment with 0.5 ug/ml herbimycin A led to a reduction in v-Src protein and *in vitro* v-Src kinase activity that could be detected between 4 and 6 hours (Fig. 24). Therefore, v-Src-transformed BALB/c 3T3 cells were treated with increasing concentrations of herbimycin A for six hours. In Fig. 25a, it is shown that with increased herbimycin A, MARCKS RNA levels, as measured by northern blot analysis, were dramatically increased in v-Src-transformed BALB/c 3T3 cells. This treatment had no effect on MARCKS RNA levels in the parental BALB/c 3T3 cells (Fig. 25a). Similarly, MARCKS transcription was also restored by herbimycin A treatment (Fig. 25b). As shown in Fig. 25c, herbimycin A restored MARCKS protein levels as determined by Western blot analysis with an antibody raised against MARCKS. Herbimycin A also restored the ability phorbol esters to induce phosphorylation of MARCKS in v-Src-transformed cells (Fig. 25d). These data suggest that the protein-tyrosine kinase activity of v-Src has a direct effect on down-regulation of MARCKS.

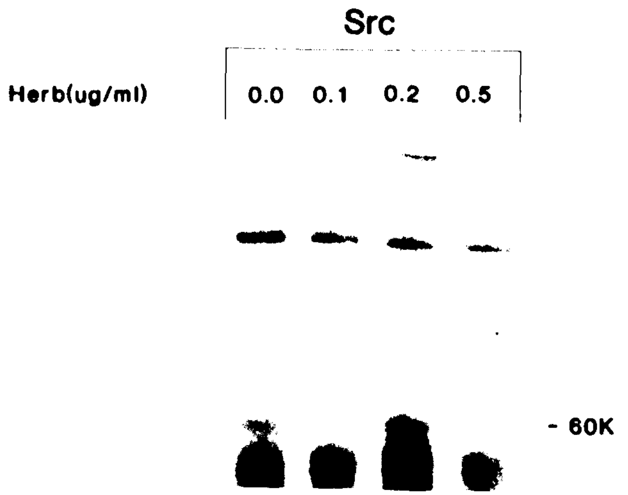
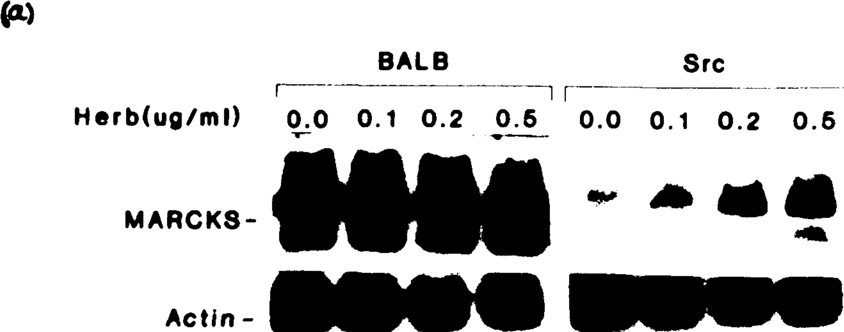


Fig. 24. Effect of herbimycin A on v-Src autophosphoryation activity. v-Src-transformed cells were treated with herbimycin as indicated, prelabeled with [^{32}P]-orthophosphate and total protein lysate was immunoprecipitated with Mab 327.

(a)



(b)

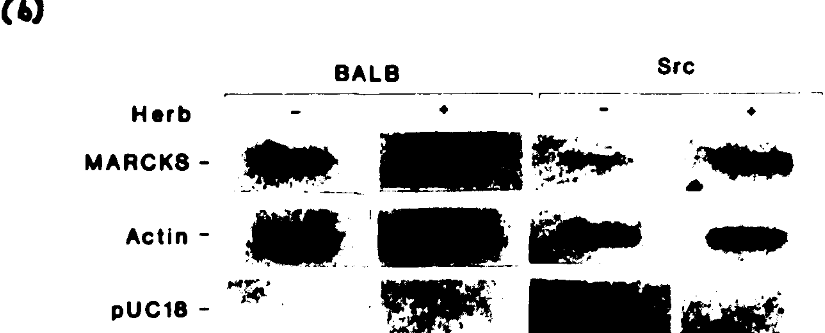
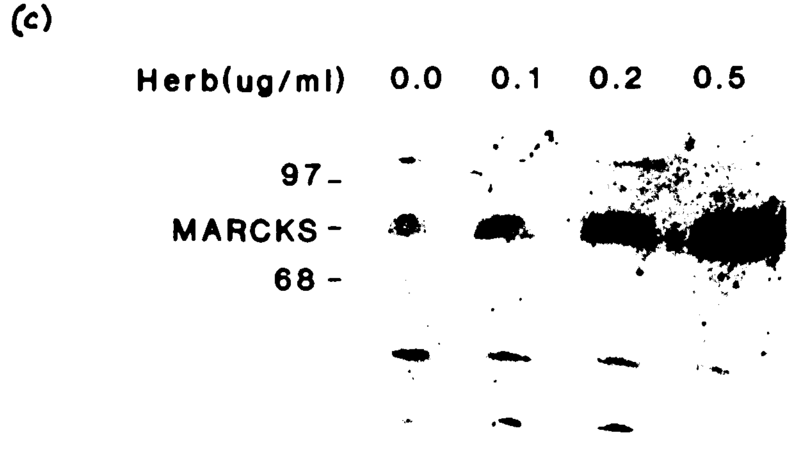


Fig. 25. Effect of herbimycin A on MARCKS expression.
(a) v-Src-transformed (Src) and non-transformed BALB/c 3T3 (BALB) cells were treated with increasing concentrations (ug/ml) of herbimycin A (Herb) for 6 hours and then analyzed for MARCKS RNA levels using northern blot analysis as in Fig. 12. After probing for MARCKS, filters were washed and reprobed with radiolabeled ß-actin DNA. **(b)** MARCKS transcription was examined as in both v-Src-transformed (Src) and non-transformed BALB/c 3T3 (BALB) cells as in Fig. 16. in the presence (+) and absence of herbimycin A (0.5 ug/ml for 6 h).



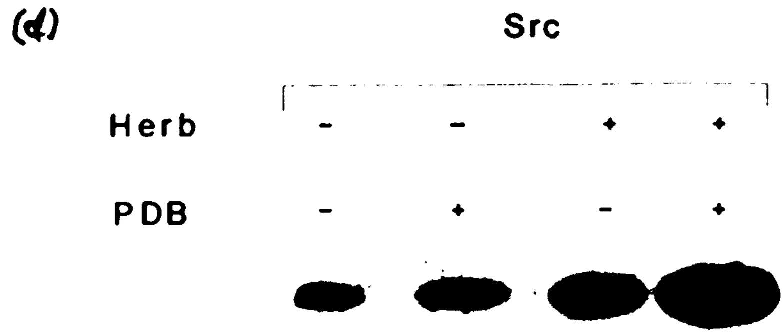


Fig. 25. (**c**) MARCKS protein levels were examined by Western blot analysis in v-Src-transformed BALB/c 3T3 cells treated with increasing concentrations (ug/ml) of herbimycin A (Herb) for 6 hours. (**d**) The ability of PDB (100 ng/ml) to induce phosphorylation of MARCKS was examined by immunoprecipitation with anti-MARCKS antibody of extracts from [^{32}P]-PO_4-labeled v-Src-transformed BALB/c 3T3 cells in the presence and absence of herbimycin A (herb) [6 hour treatment as in (c)].

DISCUSSION

We have demonstrated that phorbol esters do not induce phosphorylation of the PKC substrate MARCKS in v-Src-transformed cells. The inability to induce MARCKS phosphorylation was not due to a lack of PKC. The levels of MARCKS protein, MARCKS RNA, and MARCKS transcription were all reduced in v-Src-transformed cells relative to the nontransformed parental BALB/c 3T3 cells, suggesting that MARCKS is transcriptionally down regulated in v-Src-transformed cells. Inhibiting the protein-tyrosine kinase activity of v-Src restored the levels of MARCKS protein, MARCKS RNA, MARCKS transcription, and phorbol ester-induced MARCKS phosphorylation in the transformed cells, suggesting that the down-regulation of MARCKS in v-Src-transformed cells is a direct effect of v-Src protein-tyrosine kinase activity.

Since transformed cells proliferate in reduced levels of MARCKS, it seems that high levels of expression of MARCKS may not be essential for cell growth. The uncontrolled growth of transformed cells suggests the possibility that MARCKS may be important for control of normal cellular proliferation. Transcriptional down-regulation of MARCKS may be the result of persistent v-Src kinase activity.

This is consistent with our observation that an early effect of the tyrosine kinase activity of v-Src is the activation of PKC and phosphorylation of MARCKS (Spangler et al., 1989; Qureshi et al., 1991b). It was recently proposed that phopsphorylation of MARCKS by PKC leads to a dissociation of actin from the plasma membrane (Thelen et al., 1991; Hartwig et al., 1992). In this context, the lack of MARCKS in v-Src-transformed cells might generate the same phenotype as phosphorylation of MARCKS and lead to the dissociation of actin filaments from the plasma membrane. This could be important for the altered morphology seen in transformed cells and suggests that the presence of an unphosphorylated MARCKS is important for maintaining the typical flat non-transformed morphology. Whether down-regulation of MARCKS is required for

progression to the transformed state remains to be determined. However, correlations between transformation and reduced MARCKS protein have been reported in other cell types (Simek et al., 1989). Thus, expression of MARCKS might inhibit transformation by v-Src or other transforming stimuli.

SECTION IV

SUMMARY

We have shown that activation of PKC is an early event in the transduction of intracellular signals by the oncogene products v-Src and v-Fps. Activation of PKC correlated with expression of the 9E3 gene in CEF and the TIS10 gene in murine fibroblasts. We have also shown that a major substrate of PKC (MARCKS) is transcriptionally down-regulated in v-Src-transformed cells.

A role for PKC in v-Src- and v-Fps-induced transformation remains unclear. This may be complicated by the observation that although PKCα is the predominant isozyme in fibroblasts, expression of PKCδ and PKCΣ has been detected in these cells (Nishizuka, 1988). The murine cell line TNR9 (Butler-Gralla et al., 1981) which is resistant to the mitogenic effects of phorbol esters that activate PKC, is also resistant to transformation by v-Src suggesting a requirement for PKC for v-Src-induced transformation (Nori et al., 1990).

MARCKS protein is a substrate for all PKC isozymes tested to date (Blackshear et al., 1991), and may mediate many of the effector functions of PKC. As noted above MARCKS is associated with plasma membranes (Graff et al., 1989a) and binds both calmodulin (Graff et al., 1989b) and actin (Thelen et al., 1991;). PKC-dependent phosphorylation removes MARCKS from the plasma membrane (Aderem et al., 1988; Thelen et al., 1991). The process is reversible. The cycles of membrane attachment and detachment of MARCKS are thought to provide a PKC-sensitive, reversible link between the actin cytoskeleton and the plasma membrane (Hartwig et al., 1992). Thus down-regulation of MARCKS might disrupt this link and have profound effects on cell morphology. PKC-dependent phosphorylation of MARCKS also promotes its dissociation from calmodulin (Graff et al., 1989b). Thus down-regulation of MARCKS would increase and prolong the effect of Ca^{2+}/calmodulin-dependent processes by raising the concentration of free calmodulin.

We have not established the signals which might mediate the down-regulation of MARCKS in v-Src-transformed cells. Increased expression of MARCKS has been observed in tsLA90 cells stably transfected with a dominant-negative inhibitory mutant of c-raf-1 (Qureshi et al., manuscript in preparation), suggesting that c-raf may be involved in regulation of expression of MARCKS. We have also observed increased expresssion of MARCKS in v-Src-transformed cells which have been depleted of PKC by prolonged treatment with phorbol esters (Joseph et al., unpublished). This observation is consistent with the results of others (Lindner et al., 1992; Erusalimsky et al., 1991), who proposed that the level of MARCKS may be governed by the state of activation of PKC in normal mouse fibroblasts. Thus, a complex interaction of many signals may be involved in the regulation of expression of MARCKS.

SECTION V

Materials and Methods

Cells and Viruses. Generating and maintaining chicken embryo fibroblasts (CEF) and the ts derivatives of Rous sarcoma virus (tsNY72) and Fujinami sarcoma virus (tsNY225) have been described (Foster et al., 1985; Mayer et al., 1986; Hanafusa et al., 1981). NIH3T3 (obtained from the American Type Culture Collection), BALB/c 3T3 and BALB/c 3T3 cells infected with Rous Sarcoma virus (Schmidt Ruppin D strain) were maintained in Dulbecco's modified Eagle medium supplemented with 10% newborn calf serum (GIBCO). Confluent cell cultures were made quiescent by maintaining cells for three days in 1% newborn calf serum.

Chemicals. H7, H8, and HA1004 were obtained from Seikegaku of America, St Petersburg, FL. Sangivamycin was obtained from the National Cancer Institute (NSC-65346). Herbimycin A was obtained from Oncogene Science, New York. Phorbol 12-myristate 13-acetate (PMA), phorbol dibutyrate (PBt_2), phospholipase C (Sigma P7633), dibutyryl-cAMP, 8-Br-cAMP, 1-oleoyl-2-acetylglycerol (OAG), cholera toxin, and forskolin were obtained from Sigma. [^{3}H]glycerol,[^{32}P]orthophosphate and [^{32}P]dCTP were obtained from New England Nuclear.

RNA Analysis. RNA was extracted as described by Hatch and Bonner (1987), with modifications described by Qureshi et al. (1991a). Briefly, an equal quantity of cells was washed and resuspended in isotonic buffer with 2% (wt/vol) bentonite, dissolved with hot formaldehyde/formamide/SDS and microcentrifuged. Supernatants were loaded directly onto 1% agarose gels. RNA recovery was monitored by ethidium bromide-stained ribosomal RNA on the transfer membrane and by hybridization to the GADPH or ß-actin gene (Bedard et al., 1987), which is expressed at a constant level in the cell. Northern (RNA) blot transfer to nylon membrane, nick translation (for

preparation of 9E3 probes), and hybridization were done as described (Davis et al., 1986).

Nuclear "run-on" analysis was performed as described by Lineal et al., (1985), with modifications as described previously (Qureshi et al., 1991c).
Briefly, cells were washed three times in with ice-cold PBS, collected in PBS, pelleted at 500g for 5 minutes, and lysed in 1 ml of NP40 lysis buffer (10 mM Tris-HCl pH 7.4/10 mM NaCl/3 mM $MgCl_2$/0.5% (v/v) NP40). The nuclear pellet was collected by pelleting at 500g for 5 minutes, resuspended in 200 μl storage buffer (50 mM Tris-HCl pH 8.3/40% (v/v) glycerol/5 mM $MgCl_2$/0.1 mM EDTA and frozen in liquid N_2. Nuclei were thawed for run-off transcription assay and mixed with 100 μl of reaction buffer (10 mM Tris-HCl pH 8.0/5 mM $MgCl_2$/300 mM KCl/0.5 mM each of ATP, CTP, and GTP and 100 μCi of [α-^{32}P]UTP (3000 Ci $mmol^{-1}$, NEN) and reacted at 30^0C for 30 min. The ^{32}P-labelled RNA was isolated and purified as described previously (Qureshi et al., 1991a) and hybridized to DNA immobilized on nitrocellulose filters. Post-hybridization washes and exposure to Kodak X-OMAT film was performed as described in Qureshi et al., (1991a).

Plasmids and Probes. ts-LA90 DNA was cloned by polymerase chain reaction amplification of genomic DNA from RSV ts-LA90 virus transformed BALB/c 3T3 fibroblasts (Maroney et al., In Press). The PCR-amplified clone of the tsLA90 gene was inserted into the Cla1 cloning site of the mammalian expression vector, pLNCX (Miller and Rosman, 1989) which contains the G418 resistance gene. Molecularly cloned tsLA90 was provided by Dr Joan Brugge, University of Pennsylvania.

The following probes were used for RNA analysis. A 1.7 kb Pst I fragment of bovine MARCKS cDNA (nucleotides 531-2229 of a 5 kbp cDNA clone) (Stumpo et al., 1989) subcloned into bluescript$^+$ (pBS80K-4), a kind gift from Dr. Perry Blackshear of Duke University Medical Center, North Carolina. 1.6 and 1.4 kbp

EcoRI fragments of TIS10 and TIS8 (Egr-1) cDNAs respectively, cloned into pGEM-4, kindly provided by Dr Harvey Herschmann of UCLA School of Medicine. 1.2 kbp EcoRI fragment of 9E3 cDNA subcloned into pUC18, a gift from Dr Hidesaburo Hanafusa of The Rockefeller University, New York. Cloned cDNAs were cut with restriction endonucleases and fragments were isolated from 1.0% agarose gels using a Geneclean Kit (New England Biolabs) according to the vendor's protocol. Isolated inserts were labeled with [α-^{32}P]dCTP (DuPont/NEN) using the random primers DNA labeling kit from US Biochemical Laboratories to a specific activity of $> 10^9$ cpm/ug of DNA.

Protein Analysis. For analysis of phosphorylated proteins, cells were lysed in a Nonidet P-40 lysis buffer (0.2 % Nonidet P-40/50 mM NaCl/10 mM Tris.HCl, pH 8.0/50 mM NaH_2P0_4/10 mM sodium pyrophosphate/50 mM NaF/1 mM Na_3V0_4/1 mM EDTA/1 mM phenylmethylsulfonyl fluoride/1% aprotinin) and clarified by centrifugation in a microcentrifuge for 15 min. Supernatants were normalized for total protein by Bio-Rad assay and then precipitated with acetone. Samples were then prepared for polyacrylamide gel electrophoresis by resuspending precipitates in sample buffer, and assayed by electrophoresis on 7% polyacrylamide gels by the buffer system described by Laemmli (1970). Phosphate labelling was for 3 hr with 250 μCi/ml [^{32}P]orthophosphate (9000 Ci/mmol; 1 Ci = 37 GBq) in phosphate-free Dulbecco's modified Eagle's medium in cells pretreated with phosphate-free Dulbecco's modified Eagle's medium (0.1% dialyzed calf serum) for 16 hr.

For analysis of v-Src levels, cells were lysed in RIPA buffer (1% Triton X-100 /150 mM NaCl/1% Sodium Doxycholate/0.1% Sodium dodecyl sulphate/10 mM Tris hydrochloride [pH 7.2]/1mM phenylmethylsulfonyl fluoride/1% aprotinin) and clarified as above.

Immunoprecipates were performed on clarified total cell lysates by adding 2 μl of anti-pp60 monoclonal antibody MAb327 (generously donated by Joan Brugge, University of Pennsylvania), or 10 μl anti-MARCKS antibody (generously donated by Alan Aderem, The Rockefeller University, New York). Titration curves had revealed that these conditions provide antibody excess. Immune complexes were allowed to form for 1 hr at 4^0C. Complexes were collected on 50 μl of protein A-containing S. *aureus* bacteria for 1 hr at 4^0C. S. aureus bacteris used as immunoabsorbent for the $pp60^{src}$ immunoprecipitations with monoclonal antibody were precoated with 1 μg of goat anti-mouse IgG (Sigma) per reaction. Bacterial pellets containing the immune complexes were washed four times with RIPA or NP-40 lysis buffer, and three times with 20 mM HEPES/10% glycerol. The final pellets were resuspended in 50 μl electrophoresis sample buffer and assayed by electrophoresis on polacrylamide gels (8.5% for v-Src; 7% for MARCKS) by the buffer system described by Laemmli (Laemmli, 1970). v-Src kinase activity was measured in immune complexes as described previously (Foster et al., 1985).

Cell Transfection and Protein Analysis. NIH3T3 cells were plated at $5x10^5$ cells per 100 mm dish 16 h prior to transfection. Transfections were performed using $CaPO_4$ as a coprecipitate as described by Chen and Okayama (1987). Each transfection mixture contained 20 ug of DNA. 20 ug of ts-LA90 was transfected into NIH3T3 cells. Phosphorylation of the 80 kilodalton PKC substrate was characterized as described previously (Spangler et. al., 1989) using antibody to MARCKS protein (Rosen et al., 1989).

Purification and Assay of PKC Activity. Total PKC activity was determined after partial purification of cellular extracts as described by Ballester and Rosen (1985). Briefly, 150 mm culture dishes were washed twice with ice-cold isotonic buffer, and

then 5 ml of homogenization buffer (20 mM Tris [pH 7.5], 5mM NaCl, 1 mM EDTA, 5mM $MgCl_2$, 2mM DTT, 200uM PMSF, 20 ug/ml aprotinin, 500 ng/ml leupeptin and 1% Triton X-100) was added. The cells were disrupted with 30 strokes in a Dounce homogenizer (type B pestle) and centrifuged at 100,000 g for 1 h. The supernatant was loaded on a DEAE column (Sigma) pre-equilibrated with TEE buffer (20mM Tris [pH 7.5], 2mM EDTA and 0.5 mM EGTA), and eluted with TEE containing 100 mM NaCl. Under these conditions, PKC activity eluted in the first 1 ml of eluent. Fractions containing equal amounts of protein were assayed for PKC activity using Histone type III-S as a substrate, with or without calcium, phosphatidylserine and diacylglycerol.

Western Analysis. Extraction of proteins from cultured cells was performed as previously described (Spangler et al. 1989). Equal amounts of protein were subjected to SDS-PAGE using a 7% acrylamide separating gel. Transfer to nitrocellulose was performed as described by Towbin et al. (1979). Blocking was at 4^oC overnight with 5% bovine serum albumin in isotonic phosphate buffered saline (PBS: 136 mM NaCl, 2.6 mM KCl, 1.4 mM KH_2PO_4, 4.2 mM Na_2HPO_4). Nitrocellulose filters were washed three times for five minutes in 0.2% bovine serum albumin in PBS and then incubated with a polyclonal antibody raised against MARCKS (Rosen et al., 1989) or a polyclonal antibody raised against a peptide from a conserved region of the kinase domain of PKC (Ballester and Rosen, 1985). Blots were washed as described above and incubated with alkaline phosphatase conjugated goat anti-rabbit antiserum (Sigma). Visualization of MARCKS and PKC was performed as described by Unger et al., (1986).

Lipid Analysis. Analysis of lipids was performed as described by Song et al., (1991).

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