The Role of Protein Kinase C and Phospholipase D in Tumor promotion and Apoptosis

by

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Abstract

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Advisor: Dr. David A. Foster

Human tumorigenesis involves multiple genetic alterations with successive rounds of mutation (initiation) and selected amplification (promotion) of mutated cells. Our laboratory demonstrated previously that the tumor-promoting phorbol ester TPA (12-O-tetradecanoylphorbol-13-acetate) cooperates with c-Src overexpression to transform 3Y1 rat fibroblasts. The 3Y1 cells overexpressing c-Src are therefore capable of detecting tumor-promoting compounds able to cooperate with a signaling oncogene such as an overexpressed tyrosine kinase - a common genetic defect in human cancers - to transform cells.

Because of the likelihood that tamoxifen has tumor-promoting properties beyond its estrogen-mimetic capability, we examined the effect of tamoxifen on the $3Y1^{c\text{-}Src}$ cells. Our study showed that tamoxifen, like TPA, causes the down regulation of protein kinase C δ (PKCδ) and has tumor promoting effects that cooperate with an overexpressed tyrosine kinase to transform rat fibroblasts.

When $3Y1^{c\text{-}Src}$ cells subjected to serum withdrawal, they undergo apoptosis via a cytochrome c/caspase 9 pathway. If PKC δ was down-regulated, the apoptotic phenotypes induced by serum withdrawal in the $3Y1^{c\text{-}Src}$ cells were suppressed. The apparent survival

signal generated by PKC δ down- regulation was independent of the phosphatidylinositol-3- kinase (PI3K)/Akt survival pathway.

3Y1 cells expressing the activated Src kinase (v-Src) are resistant to the apoptotic stimulus of serum withdrawal. v-Src stimulates, whereas c-Src does not stimulate phospholipase D (PLD) activity in 3Y1 cells. These data suggest the possibility that PLD provides a survival signal that overcomes apoptotic signals induced by serum withdrawal. Consistent with this hypothesis, elevated expression of either PLD1 or PLD2 in the $3Y1^{c\text{-}Src}$ cells prevented apoptosis upon serum withdrawal. Moreover, if PLD activity was inhibited in the v-Src-transformed 3Y1 cells, they underwent apoptosis in response to serum withdrawal. Surprisingly, if PLD activity was elevated in the parental 3Y1 cells, these cells became sensitive to the apoptotic stimulus of serum withdrawal. Thus, in the context of tyrosine kinase overexpression, PLD activity provides a survival signal, whereas in the absence of another cell division signal, elevated PLD activity leads to apoptosis.

The data presented in this thesis suggest that the survival signals provided by downregulation of PKC δ or upregulation of PLD may be a critical aspect of tumor progression. Therefore PKC δ and PLD could be targets for therapeutic intervention in cancers.

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CHAPTER I

INTRODUCTION

Proliferation, tumor promotion and apoptosis in cancer

Cancer is a genetic disease caused by a multistep process involving activation of oncogenes, loss of function of tumor suppressor genes, and alterations of modifier genes, for example, genes involved in DNA repair and genomic stability. Many types of cancers are diagnosed in the human population with an age-dependent incidence implicating four to seven rate-limiting, stochastic events (Renan *et al.*, 1993) Pathological analyses of a number of organ sites reveal lesions that appear to represent the intermediate steps in a process through which cells evolve progressively from normalcy via a series of premalignant states into invasive cancers (Foulds *et al.*, 1954).

Transformation of cultured cells is itself a multistep process: rodent cells require at least two introduced genetic changes before they acquire tumorigenic competence, while their human counterparts are more difficult to transform (Hahn *et al.*, 1999). Transgenic models of tumorigenesis have repeatedly supported the conclusion that tumorigenesis in mice involves multiple rate-limiting steps (Bergers *et al.*,1998). Taken together, observations of human cancers and animal models argue that tumor development proceeds via a process formally analogous to Darwinian evolution, in which a succession of genetic changes, each conferring one or another type of growth advantage, leads to the progressive conversion of normal human cells into cancer cells (Foulds *et al.*,1954; Nowell *et al.*,1976).

In Weinberg's recently review, He suggested that the vast catalog of cancer cell genotypes is a manifestation of six essential alterations in cell physiology that collectively dictate malignant growth (Figure 1.1) self-sufficiency in growth signals, insensitivity to growth-inhibitory (antigrowth) signals, evasion of programmed cell death (apoptosis),

limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis. Each of these physiologic changes—novel capabilities acquired during tumor development—represents the successful breaching of an anticancer defense mechanism hardwired into cells and tissues. They propose that these six capabilities are shared in common by most and perhaps all types of human tumors. This multiplicity of defenses may explain why cancer is relatively rare during an average human lifetime. In this part, I will mainly focus on proliferation and apoptosis which are highly related to my thesis study.

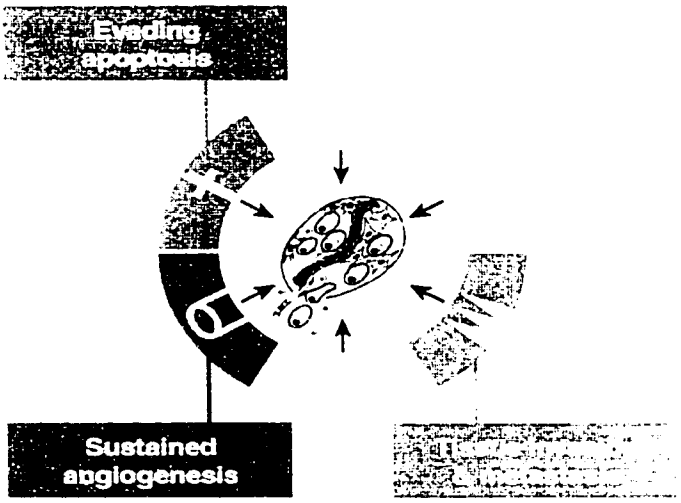


Figure 1.1. Acquired Capabilities of Cancer

They suggest that most if not all cancers have acquired the same set of functional capabilities during their development, albeit through various mechanistic strategies.

Self-sufficiency in growth signals

Normal cells are totally dependent for their proliferation upon receipt of appropriate mitogenic signals. These signals are transmitted into the cell by transmembrane receptors that bind distinctive classes of signaling molecules: diffusible growth factors, extracellular matrix components, and cell-to-cell adhesion/interaction molecules. Many of the oncogenes in the cancer catalog act by mimicking normal growth signaling in one way or another. In these ways, tumor cells generate many of their own growth signals, thereby reducing their dependence on stimulation from their normal tissue microenvironment. This liberation from dependence on exogenously derived signals disrupts a critically important homeostatic mechanism that normally operates to ensure a proper behavior of the various cell types within a tissue. Three common molecular strategies for achieving autonomy are evident, involving alteration of extracellular growth signals, of transcellular transducers of those signals(EGFR), or of intracellular circuits(Ras Src) that translate those signals into action. These oncogenic growth signals are analogous to the accelerators in a car. A mutation in an oncogene is tantamount to having a "stuck accelerator": even when the driver releases his foot from the accelerator pedal, the car continues to move. Likewise, cells with mutant oncogenes continue to grow even when they are receiving no growth signals. Examples are Ras, activated in pancreatic and colon cancers; EGFR, is upregulated in stomach, brain, and breast tumors.

Insensitivity to Antigrowth Signals

When the accelerator is stuck to the floor, the driver can still stop the car by using the brakes. Cells have brakes, too. Within a normal tissue, multiple antiproliferative signals

operate to maintain cellular quiescence and tissue homeostasis; these signals include both soluble growth inhibitors and immobilized inhibitors embedded in the extracellular matrix and on the surfaces of nearby cells. These growth-inhibitory signals, like their positively acting counterparts, are received by transmembrane cell surface receptors coupled to intracellular signaling circuits.

At the molecular level, many and perhaps all antiproliferative signals are funneled through the retinoblastoma protein (pRb) and its two relatives, p107 and p130. When in a hypophosphorylated state, pRb blocks proliferation by sequestering and altering the function of E2F transcription factors that control the expression of banks of genes essential for progression from G1 into S phase.Disruption of the pRb pathway liberates E2Fs and thus allows cell proliferation, rendering cells insensitive to antigrowth factors that normally operate along this pathway to block advance through the G1 phase of the cell cycle(Weinberg *et al.*, 1995). Soluble signaling molecule TGFβ acts in a number of ways, most still elusive, to prevent the phosphorylation that inactivates pRb; in this fashion, TGFβ blocks advance through G1. In some cell types, TGFβ suppresses expression of the *c-myc* gene, which regulates the G1 cell cycle machinery in still unknown ways (Moses *et al.*, 1990). More directly, TGFβ causes synthesis of the $p15^{INK4B}$ and p21 proteins, which block the cyclin:CDK complexes responsible for pRb phosphorylation (Hannon and Beach, 1994; Datto *et al.*, 1997).

Tumor promotion

Antiproliferative signals can be inhibited by tumor promoter. In the highly-defined two-stage model of initiation/promotion of skin carcinogenesis (Armuth & Berenblum,1974) in the mouse, developed in the early 1940's, a low dose of a well-

studied chemical carcinogen (dimethylbenzanthracine) is applied to the skin on the back of a mouse. This exposure alone will not cause any statistically significant increase in the number of skin tumors (papillomas) over the lifetime of the animal. A number of environmental chemicals have been determined to be "promoters" of tumor formation in this and other models. A promoting agent is capable of increasing significantly the incidence of papillomas following a single exposure of initiating agent where application of the promoting agent itself causes no increase in cancer.

The latency between exposure to a carcinogen and the onset of cancer can be explained in part by the results of animal experiments that demonstrate that skin carcinogenesis in the rabbit and mouse can be divided into two stages, tumor initiation and promotion. Following single exposure to a subcarcinogenic dose of a carcinogen ("initiation"), the latent period can be shortened and the tumor number or yield increased with certain "promoting agents" (croton oil, benzene, gasoline, UV treatment, phorbol esters, pesticides, and others). Promoters are not carcinogenic in themselves, or only weakly so, but cause significantly elevated cell proliferation in target tissue. These cells are transformed. Transformed cells display many properties of tumor cells and some actually form tumors when injected back into animals. The properties of transformed cells include immortalization, decreased anchorage dependence, decreased dependence on exogenous growth factors, and loss of contact inhibition of growth.

Initiation and promotion are two stages in the development of tumors. Chemical, physical, or biological agents that irreversibly and heritably alter the cell genome typically cause initiation. However, the mechanism of promotion is not well understood. There are many kinds of promoting agents with diverse molecular structures: phorbol

esters, estrogen, prolactin, other endogenous hormones, and drugs. Some of the promotors exhibit specific interaction with cell receptors. For example, phorbol esters bind with the intracellular receptor protein kinase C, a serine-threonine kinase, and activate it. The structure of a phorbol ester, TPA, which is a potent activator of PKC is provided. Short-term treatment with TPA leads to the activation of PKC while prolonged treatment down regulates this protein. Consequently, this substance has been used extensively throughout many years in an effort to assess the involvement of PKC in signaling pathways.

Deregulation of apoptosis

Evolution of cancer is more complex than the straightforward linear accumulation of oncogenic mutations. Potentially oncogenic proliferative signals are coupled to a variety of growth-inhibitory processes, such as the induction of apoptosis, differentiation or senescence, each of which restricts subsequent clonal expansion and neoplastic evolution. Tumour progression occurs only in the very rare instances where these anti-growth mechanisms are thwarted by compensatory mutations.

Proliferation-deregulating oncoproteins seem to promote apoptosis through the activation of several downstream pro-apoptotic effector pathways. For example, Myc has a profound effect on the mitochondrion, triggering release of cytochrome *c* and activation of caspase 9. This pathway is inhibited by members of the Bcl-2/Bcl-x_L anti-apoptotic family and by survival factors, both of which have been shown to potentiate the oncogenic action of c-Myc(Fanidi, *et al*, 1992;Bissonnette *et al*.,1992; Wanger *et al*.,.1993;Harrington *et al*.,1994 & Strasser *et al*.,1990) E2F1 can directly influence apoptotic signalling from death receptors(Phillips *et al*.,1999), whereas Myc greatly

enhances sensitivity to signalling through the CD95 (Huebber,1997), TNF(Klefstrom *et al.*, 1994) and TRAIL(Lutz *et al.*, 1998) death receptors. Another common pathway through which a wide variety of proliferative signals influence the apoptotic programme is through induction of ARF, an alternate product of the *INK4a* locus, one of whose functions is to trigger upregulation of p53 through its inhibitory action on MDM-2 (Sherr *et al.*, 2000).

In my thesis study, we showed that when $3Y1^{c\text{-}Src}$ cells were subjected to serum withdrawal, they underwent apoptosis via a cytochrome C/caspase 9 pathway. In contrast, neither parental nor v-Src-transformed 3Y1 cells underwent apoptosis when subjected to serum withdrawal. If PKC δ was downregulated or activatate PLD, the apoptotic phenotypes induced by serum withdrawal in the $3Y1^{c\text{-}Src}$ cells were suppressed.

APOPTOSIS

Apoptosis was initially described by its morphological characteristics, including cell shrinkage, membrane blebbing, chromatin condensation and nuclear fragmentation (Kerr *et al.*, 1972; Wyllie *et al.*,1980 & Kerr *et al.*,1994). The realization that apoptosis is a gene-directed program has had profound implications for our understanding of developmental biology and tissue homeostasis, for it implies that cell numbers can be regulated by factors that influence cell survival as well as those that control proliferation and differentiation. Moreover, the genetic basis for apoptosis implies that cell death, like any other metabolic or developmental program, can be disrupted by mutation. In fact,

defects in apoptotic pathways are now thought to contribute to a number of human diseases, ranging from neurodegenerative disorders to malignancy (Thompson *et al.*, 1995). Subsequent studies revealed a high frequency of apoptosis in spontaneously regressing tumors and in tumors treated with cytotoxic anticancer agents (Kerr *et al.*, 1994). These observations suggested that apoptosis contributed to the high rate of cell loss in malignant tumors and, moreover, could promote tumor progression. Nevertheless, the importance of apoptosis in cancer remained under-appreciated for >15 years.

Mechanisms of apoptosis

At the molecular level, the best understood cell death pathways involve those initiated by 'death receptors', including Fas/CD95, TNFR1, DR3, DR4 and DR5 (Ashkenazi *et al.*, 1998). Upon binding, tumor necrosis factor α(TNF-α) trimerizes its ligand TNFR1 and results in the subsequent recruitment of the signal transducing molecules TRADD through conserved protein interaction regions known as 'death domains'. TRADD recruits RIP and TNF receptor-associated factor (TRAF-2), leading to activation of nuclear factor κB (NF-κB), which suppresses TNF-α-induced apoptosis (Takeuchi *et al.*, 1996). While the recruitment of FADD by TRADD results in apoptosis through activation of a cell death protease, caspase-8. Activated caspase-8 initiates a protease cascade that cleaves cellular targets and results in apoptotic cell death (Ashkenazi *et al.*, 1998). Hence, disruption of *FADD* can prevent activation of caspase-8, thereby producing defects in receptor-mediated cell death (Yeh *et al.*, 1998). This pathway is rarely the target of oncogenic mutations, but, if anything, it is enhanced during tumor development.

Growth factors, cytokines and DNA damage appear to signal cell death through the mitochondria, and this pathway is the target of many oncogenic mutations. These diverse signals affect the function of Bcl-2 family members which, in turn, can modulate the mitochondrial function though the permeability transition pore (PTP), a proposed channel evolved in mitochondria following necrotic or apoptotic signals. The PTP is thought to be composed of clustered components of the mitochondrial membranes, including the voltage-dependent anion channel and adenine nucleotide translocator; and the opening of PTP results in the release of cytochrome c from mitochondria (Green,1998). Consistent with this idea, the crystal structure of Bcl-x_L is reminiscent of pore-forming proteins of some bacterial toxins (Muchmore *et al.*, 1996). Enforced expression of the pro-apoptotic molecules Bax or Bak can result in increased mitochondria membrane potential and release of cytochrome c, which can be blocked by overexpression of Bcl-2 (Tsujimoto *et al.*, 1998). Cytosolic cytochrome c can interact with Apaf-1 and pro-caspase-9 to initiate a protease cascade similar to that described above (Zou, *et al.*, 1996;Li *et al.*, 1997& Srinivasula *et al.*, 1998).

As eluded to above, a series of enzymes known as caspases are considered the engine of apoptotic cell death. Caspases are cysteine proteases that are expressed as inactive pro-enzymes, and can be broadly classified into 'signaling' or 'effector' caspases (Thornberry *et al.*, 1998). Signaling pro-caspases associate with specific adapter molecules that facilitate caspase activation by induced proximity (Hu *et al.*, 1998; Muzio *et al.*, 1998& Yang *et al.*, 1998). For example, caspase-9 associates with Apaf-1, and oligomerization of this complex in the presence of cytochrome c can activate the downstream caspase cascade. Other adapter/caspase complexes include FADD/caspase-8 and RAIDD/caspase-

2 (Ashkenazi *et al.*, 1998). In mitochondrial pathways, most caspases act downstream of cytochrome c release, and some evidence suggests that disruption of caspases only delays cell death (Xiang, *et al.*, 1996& McCarthy *et al.*, *1997*). However, in other circumstances, loss of these proteases produces pathological increases in cell numbers (Soengas *et al.*, 1999; Woo *et al.*, 1998; Hakem *et al.*,1998; Kuida *et al.*, 1998; Yoshida *et al.*, 1998 & Cecconi *et al.*, 1998). To date, little is known about the involvement of caspase mutations in cancers. Nevertheless, disruption of Apaf-1 is associated with Noonan's Syndrome, caspase-10 mutations contribute to autoimmune lymphoproliferative syndrome type II (Cecconi *et al.*,1998 ;Wang *et al.*, 1998), and frameshift mutations in caspase-5 can occur in hereditary nonpolyposis colorectal cancers, gastrointestinal and endometrial tumors (Schwartz *et al.*, 1999).

Certain signal transduction pathways alter the probability with which pro-apoptotic signals induce apoptosis. For example, cytokines such as IL-6 can suppress p53-induced apoptosis in certain cell types (Yonish-Rouach *et al.*,1991). Also, TNF-α-induced apoptosis is modulated by TRADD's ability to bind TRAF2, which facilitates NF-κB-mediated cell survival (Takeuchi *et al.*, 1996). Finally, PI-3 kinase pathway mediates cell survival signaling from extracellular cytokines receptors. These receptors activate Ras and a kinase cascade involving PI-3 kinase and Akt leading to the ultimate phosphorylation and inactivation of pro-apoptotic molecules such as BAD and caspase-9 (del Peso *et al.*, 1997). PTEN acts as a lipid phosphatase to inactivate 3-phosphorylated phosphoinositides, thereby downregulating this pathway (Myers *et al.*, 1998). Together, these studies imply that the ultimate decision to initiate apoptosis results from a complex integration of internal and external pro- and anti-apoptotic signals.

Apoptotic programs cannot simply be described as two parallel programs converging on a common caspase machinery. First, genetic studies using caspase-deficient mice demonstrate that the requirement for different death effector molecules during apoptosis is highly variable, being cell-type and stimulus specific (Woo *et al.*, 1998; Hakem *et al.*, 1998; Kuida *et al.*, 1998; Yoshida *et al.*, 1998 & Cecconi *et al.*, 1998). Second, a large degree of 'cross-talk' can exist between pathways. For example, p53 can transactivate genes encoding death receptors (Wu *et al.*,1997). Also, receptor-mediated activation of caspase-8 can cleave and activate BID, a pro-apoptotic Bcl-2 family member that can facilitate cytochrome c release from the mitochondria (Wang *et al.*, 1996). Animal studies indicate that the caspase-8/BID pathway is highly cell-type dependent (Yin *et al.*, 1999). It is likely that more complexities will be identified; although confounding to the experimentalist, cell type and stimuli specificities provide avenues for designing selective therapeutics.

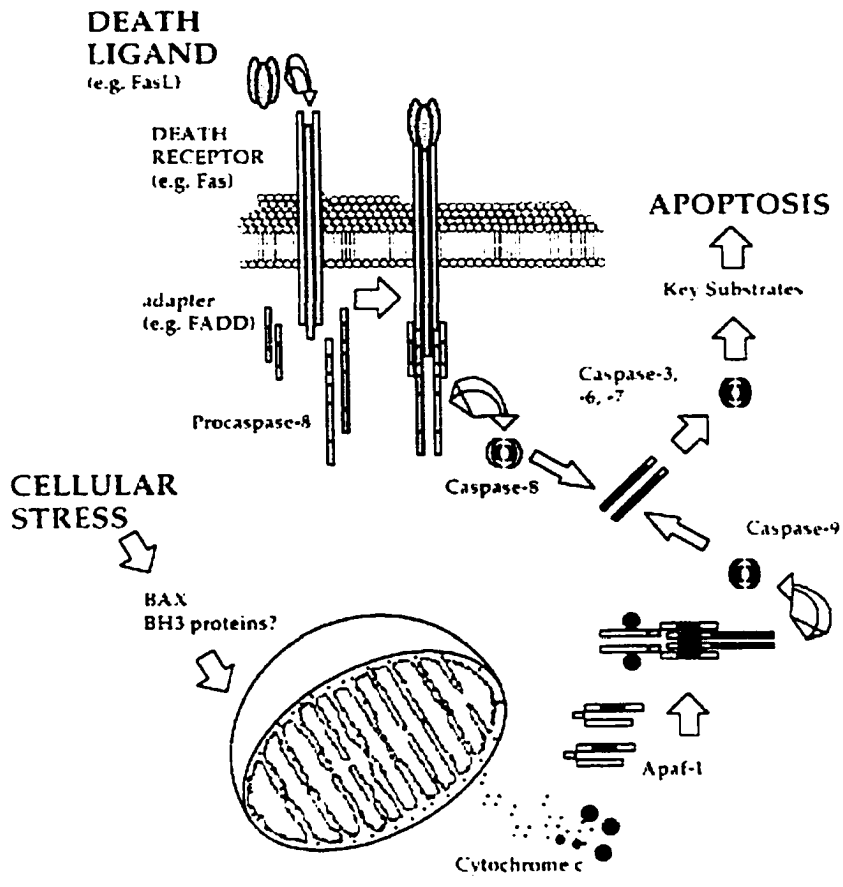


Fig 1.2 Two Pathways of Caspase Activation and Apoptosis

Two independent apoptosis pathways are presented that converge on the activation of "downstream" caspases (-3, -6, -7), key substrate cleavage, and apoptotic death. The first involves ligation of death receptors by their ligands, resulting in the recruitment of adaptor proteins and procaspase molecules. The complex is an "apoptosome" in which the aggregated procaspase transactivates. The active caspase (e.g., caspase-8) then acts to cleave and activate the downstream caspases. In the second pathway, various forms of cellular stress trigger mitochondrial release of cytochrome c, which binds to Apaf1, which in turn self-associates and binds procaspase-9, resulting in an apoptosome. Transactivation of the complexed procaspase-9 to active caspase-9 follows, and the caspase then cleaves and activates downstream caspases. In this model, there is no cross-talk between the pathways, and ligation of death receptors does not affect mitochondrial function in a relevant manner.

Oncogenic mutations can promote apoptosis

Some oncogenic changes promote, rather than suppress apoptosis. Although this finding has ramifications for multistep carcinogenesis and cancer therapy (see below), the initial insights came from studies on adenovirus. Adenovirus encodes several oncoproteins that, when expressed together, transform rodent cells (Branton *et al.*,1985). The E1A oncoprotein induces quiescent cells to enter S phase (presumably to make the host cell permissive for virus replication) and, as a result, acts as a potent oncogene. The E1B 19 and 55K oncoproteins are required for efficient virus replication and cooperate with E1A in transformation. Adenovirus mutants lacking the E1B 19K protein induce an E1A-dependent 'cytocidal' phenotype that is associated with 'degradation' of both viral and host-cell DNA (White *et al.*, 1984). As a result, E1B mutant adenoviruses produce poor virus yield. Subsequent studies explained these puzzling observation: E1A induces apoptosis, and the E1B 19K oncoprotein acts like Bcl-2 to suppresses apoptosis (Rao *et al.*,1996). In virus-infected cells, E1B prevents E1A-induced apoptosis, allowing viral replication to proceed. This implies that apoptosis can act to counter virus replication and provides a biologic basis for cooperation between E1A and E1B in adenovirus transformation.

Studies on the c-*myc* oncogene highlight the importance of oncogene-induced apoptosis in human cancer (Evan *et al.*, 1998). In normal cells, ectopic c-Myc expression drives proliferation and prevents cell-cycle arrest upon serum withdrawal. However, while c-Myc-expressing cells continue to proliferate in low serum, cells do not accumulate because they die by apoptosis (Evan *et al.*,1992). Importantly, survival factors

such as IGF-1 can suppress c-Myc-induced cell death without producing substantial effects on c-Myc-induced proliferation (Harrington *et al.*, 1994). The observation that c-Myc actively promotes apoptosis explains the potent cooperative effects observed between c-*myc* and *bcl-2* in murine lymphomagenesis (Strasser *et al.*, 1990). In fact, much like E1A and E1B, c-Myc cooperates with Bcl-2 to transform rodent fibroblasts (Bissonnette *et al.*, 1992). The ability of c-Myc to cooperate with Bcl-2 in transformation could be viewed very much like E1A and E1B; Bcl-2 allows c-Myc-induced proliferation to proceed without apoptosis.

Why do some oncogenes promote apoptosis? Studies on c-Myc have been unable to separate its proliferative functions from those which promote apoptosis, implying that the processes are coupled (Amati *et al.*,1993). In this study, We also show that overexpressing proto-oncogene ,c-Src, can induce apoptosis in 3Y1 rat fibroblasts under serum starvation condition. The details are going to be discussed in the latter charpter.

Apoptosis in cancer and cancer therapy

Most anticancer agents now in use were developed using empirical screens designed to identify agents that selectively kill tumor cells. Until recently, most research into drug action focused on their intracellular targets, the nature of the cellular damage produced by the drug–target interaction, or resistance mechanisms that prevent the drug target interaction. However, in the 1970s pathologists noticed that radiation and chemotherapy can induce cell death with morphological features of apoptosis (Searle *et al.*,1975) although the significance of these observations was not widely appreciated. In particular, the premise that anticancer agents induce apoptotic cell death implies that cellular

responses occurring after the drug–target interaction can have impact on drug-induced cell death (Dive *et al.*, 1991).

It is now well-established that anticancer agents induce apoptosis, and that disruption of apoptotic programs can reduce treatment sensitivity (Schmitt *et al.*, 1999). Since agents with distinct primary targets can induce apoptosis through similar mechanisms, mutations in apoptotic programs produce multi-drug resistance (Dive, *et al.*, 1991). For example, many agents activate p53, and that p53 loss can attenuate drug-induced cell death (Wallace-Brodeur *et al.*, 1999). Moreover, *p53* mutations reduce therapy-induced apoptosis and tumor regression in experimentally generated and spontaneous murine tumors (Lowe *et al.* 1994), whereas re-introduction of normal p53 to *p53* mutant tumor lines and xenographs cooperates with chemotherapy to induce apoptosis and tumor regression (Fujiwara, *et al.*, 1994). p53 is not strictly required for drug-induced cell death; indeed, at sufficient doses virtually all anticancer agents induce apoptosis (and other types of death) independently of p53. In fact, the contribution of p53 to drug-induced apoptosis is determined by a variety of factors, including agent, dose, tissue and mutational background of the tumor (Wallace-Brodeur *et al.*, 1999). In short-term assays, Bcl-2 can promote resistance to a wide range of anticancer agents (Miyashita *et al.*, 1993) and can even prevent p53-independent deaths (Strasser *et al.*, 1994). Because Bcl-2 is considered as a general apoptosis inhibitor, these results argue for the broad importance of apoptosis in treatment sensitivity. Additionally, death receptor pathways may also contribute to therapy-induced apoptosis (Friesen *et al.*, 1996), although the relative contribution of these effects is controversial (Villunger *et al.*, 1998).

The strategy that disruption of apoptosis can promote tumor initiation, progression and treatment resistance have been widely used in the cancer therapies. Recently, it has been reported that PLD activity is significantly elevated in human breast cancer (Uchida *et al.*, 1997), human renal cancer (Zhao *et al.*,2000), human gastric cancer tissue (Uchida,) and experimental colon cancer (Yoshida *et al.*, 1998), suggesting that PLD might be implicated in tumorigenesis. Taken together, these reports suggest that PLD may play a pivotal role in the signal transduction pathways of cellular proliferation and carcinogenesis.

Protein Kinase C Family (PKC)

The molecular hetereogeneity of the PKC superfamily, involvement in transformation, and their functional divergence make them attractive targets for anticancer drug development in the future.

The protein kinase C superfamily of lipid dependent and diacylglycerol-activated serine-threonine kinases is important as cytosolic intracellular signal tranducers involved in numerous signaling pathways. PKCs are 80 kDa phosphoproteins that play key roles in cellular processes like proliferation and differentiation (Nishizuka *et al.*,1984) as well as being implicated in receptor desensitization, neurotransmitter release, regulation of gene expression, hormone release, ion channels, mediating immune response, modulating membrane structure, development, tumirogenesis, apoptosis, and neural plasticity (Mellor *et al.*, 1998). PKC has been implicated recently in cell cycle control at two sites, G1/S

progression and G2/M transition (Fishman *et al.*,1998). PKC functions as the transducer of the second messenger, diacylglycerol (DG), and is considered the major receptor for the tumor promoting phorbol esters. DG and phorbol esters recruit PKC to the membrane, a process referred to as translocation, by acting as hydrophobic anchors at the membrane.

There exist multiple PKC isozymes within cells mediating isozyme-specific functions. There are at least 11 known mammalian PKC isoforms (Baron-Delage & Cherqui,1997) to date that have been categorized by structure and cofactor requirements into three distinct groups: Conventional [alpha (α), beta I, II (β1,β2), and gamma (γ)] are calcium/DG/phosphatidylserine-dependent; Novel [delta (δ), epsilon (ε), theta (θ), mu (μ), and eta (η)] are calcium independent and DG/phosphatidylserine-dependent; Atypical [zeta (ζ), lambda (λ), and iota (ι)] are calcium/DG-independent and phospholipid responsive (Nishizuka *et al.*, 1995).

Most cells express more than one type of PKC with each potentially possessing different subcellular localization and cofactor requirements (Ohno *et al.*,1991) as well as different levels of expression and availability of target substrates that can vary by cell type. As a consequence, it has been difficult to ascribe a specific role to individual PKCs in cells. However, the recent advent of specific PKC isoform chemical inhibitors and the availability of PKC constructs that code for isozyme-specific dominant negative inhibitors for the first time provide a means of resolving this critical issue in cell culture models.

Structure:

Comparing their primary structures as inferred from cDNA sequences revealed the structure of the PKC family (Ohno *et al.*,1991). PKC has five variable regions and

four conserved domains. Two major functional stretches exist coding for the regulatory domain in the amino terminus and a catalytic domain in the carboxyl terminus separated by a hinge region that is cleaved after PKC is membrane bound releasing the short-lived constitutively active kinase domain (PKM or PKC catalitical fragment) . The regulatory domain (V1-C2) possesses a pseudosubstrate, a C1 phorbol-ester/DG-binding-site, and a C2 phosphatidylserine-calcium-binding site. Within the C1 domain there are two cysteine-rich zinc-finger-like-motif regions. The catalytic domain possesses one ATP and one substrate-binding site.

Schematic structure of PKC Isoforms

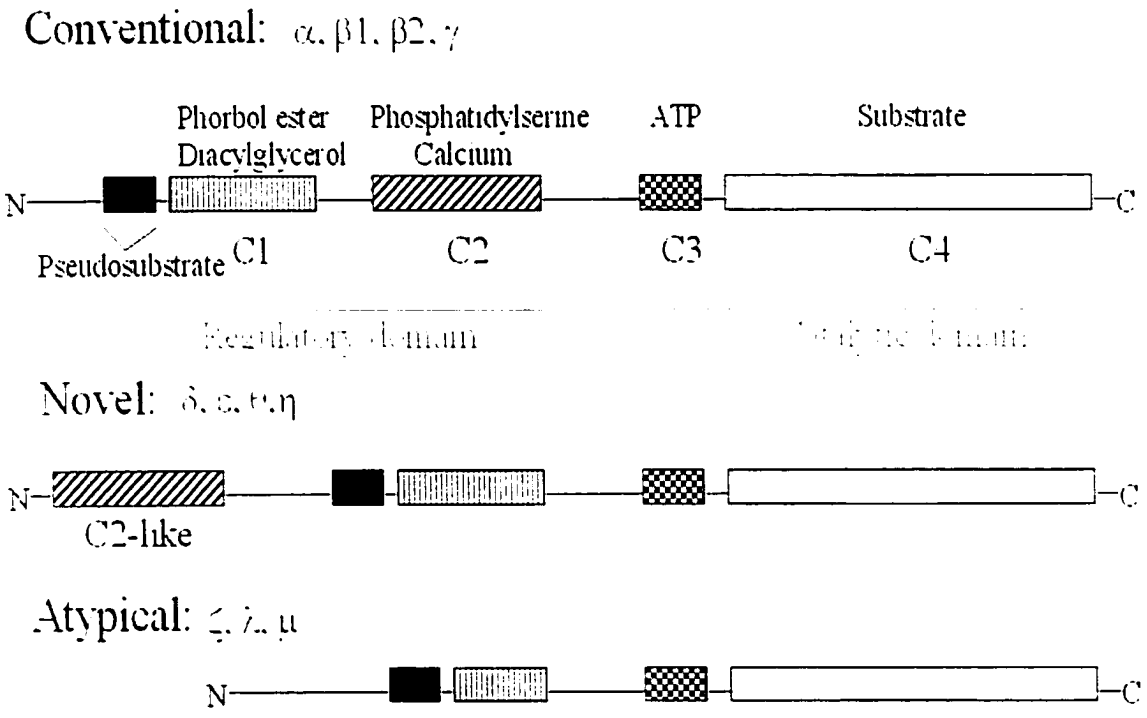
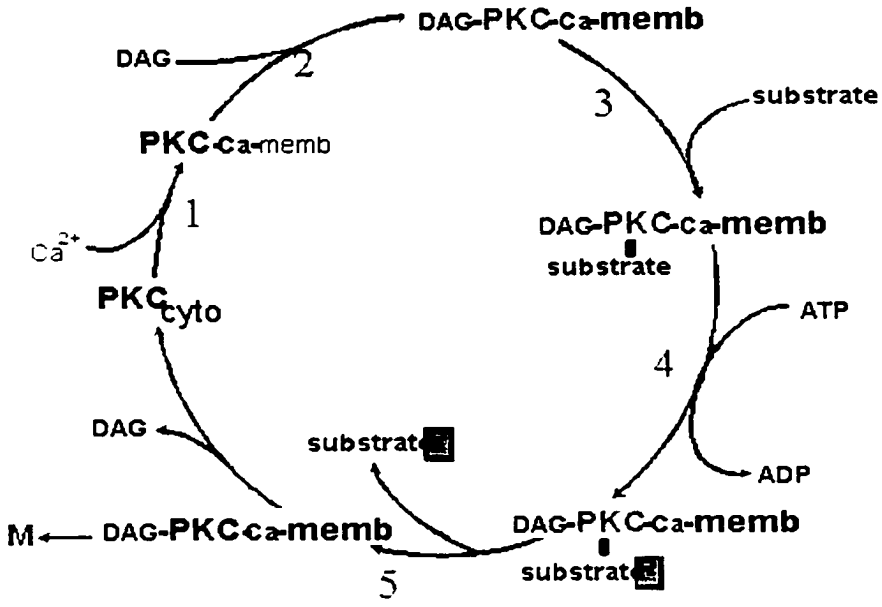


Fig 1.3



1. Translocation 2. Membrane interaction 3. Activation 4. Phoshorylation 5. Downregulation

Fig 1. 4 Activation cycle of PKC. (DAG- Diacylglycerol)

Common to all members is the presence of the pseudosubstrate in the aminoterminal regulatory domain, which renders the kinase inactive by interacting with the carboxyterminal substrate-binding site of the catalytic domain. Activation of PKC requires the release of this conformational autoinhibitory state leading to its translocation from the cytosol to the membrane. Extracellular stimuli trigger an increase in the level of DG that binds to and activates PKC by changing its conformation so that the

pseudosubstrate no longer is bound to the substrate binding site. DG can be produced rapidly by the action of Phospholipase C on phosphatidylinositol or slowly by the action of PLD on phosphatidylcholine producing phosphatidic acid (PA) which is further metabolized to DG by PA phosphohydrolase (Murayama *et al.*, 1989).

Function:

PKC has a multitude of substrates like MARCKS (myristoylated alanine-rich C kinase substrate), which binds actin, calmodulin, phosphatidylinositol 4,5-bisphosphate (PIP_2), and is implicated in playing a role in phagocytosis, membrane traffic, and cell motility. PKC also has nuclear proteins as substrates, including DNA methyltransferase, CREB, DNA polymerease α, RNA polymerase II, and DNA topoisomerases (Sahyoun et al.,1986).

The exact role of each isoform is unclear; however, the elucidation of the roles of PKC δ, in the activation of PLD and transformation of cells overexpressing a protooncogene will be made evident in this work. Furthermore a correlation between PKC/PLD activity and transformation gains greater support from this work.

PKC and apoptosis:

Besides the involvement of PKC in the regulation of cell growth and differentition, PKC might play a role in apoptosis .. In certain cell type , apoptotic stimuli were associated with proteolytic activation of PKC δ, ε and θ by executed caspases. Whereas PKC α,β and ζ were not affecte . In this study, cleaved PKC δ has been used as one of apoptosis parameters.

Overexpression of catalytic kinase fragment of PKC δ, but not of full-length PKC δ or a kinase-inactive fragment, caused phenotypic change associated with apoptosis(Ghayur *et al.*, 1996). This study strongly suggest that PKC δ is not only of caspases substrate but also of an upsteam regulator of caspases. The following two evidence may give part of mechanisms that PKC δ is an upsteam regulator of caspase. Bharti reported on an interaction of catalytic fragment of PKC δ with and phosphorylation of the DNA-dependent protein kinase(DNA-PK) resulting in the inactivation of this kinase. DNA-PK is essential in the repair of DNA double-strand breaks(Bharti *et al.*,1998). Thus, interaction of PKC δ and DNA-PK may contribute to DNA damage-induced apoptosis. Cross T(Cross *et al.*,2000)show that protein kinase C-delta co-localized with lamin B during apoptosis and activation of PKC-delta by caspase 3 was concomitant with lamin B phosphorylation and proteolysis. Inhibition of PKC-delta delayed lamin proteolysis, even in the presence of active caspase 6, whilst inhibitors of mitotic lamin kinases were without effect. In addition recombinant human PKC-delta was able to phosphorylate lamin B in vitro suggesting that its actions are direct and not via an intermediary kinase. They propose that PKC-delta is an apoptotic lamin kinase and that efficient lamina disassembly at apoptosis requires both lamin hyperphosphorylation and caspase mediated proteolysis. There are many reports on the effect of PKC on apoptosis . however, the results are very controversial.

PHOSPHOLIPASE D

Phospholipase D(EC 3.1.4.4; PLD) which was first discovered in plant is an ubiquitous enzyme that hydrolyzes phosphatidylcholine to phosphatidic acid and

choline.(Hanahan *et al.*,1947). However, widespread interest in this enzyme began once experiments in cultured animal cells revealed its rapid and dramatic activation to extrcellular stimuli. (Cockcroft *et al.*, 1984, Exton *et al.*,1990).

Phospholipase D (PLD) is a widely distributed enzyme that is under the elaborate control of hormones, neurotransmitters, growth factors and cytokines in mammalian cells. The activation of PLD is believed to play an important role in the regulation of cell function and cell fate. Multiple PLD activities were characterized in eukaryotic cells, and more recently, several PLD genes have been cloned. A PLD gene superfamily, defined by a number of structural domains and sequence motifs, also includes phosphatidyltransferases, and certain phosphodiesterases.

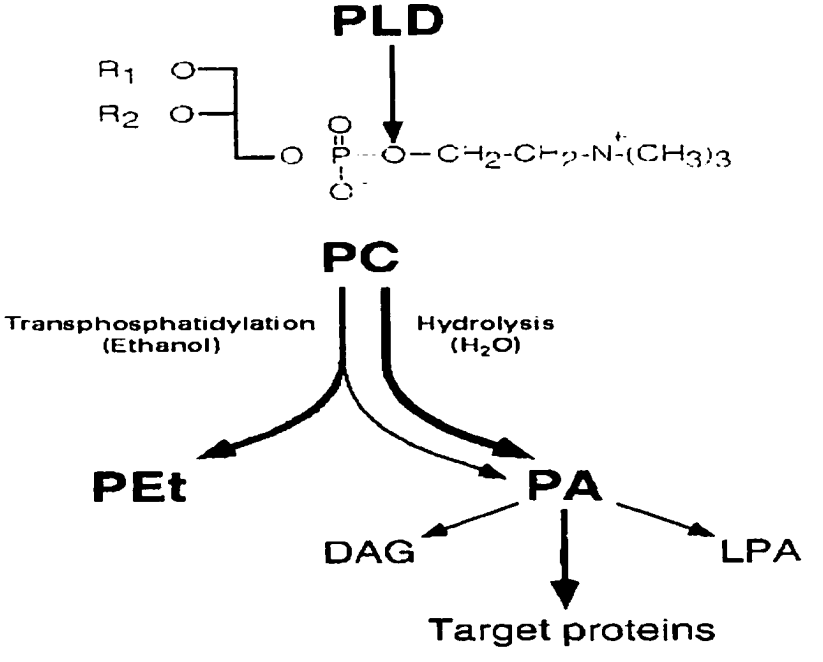


Fig 1.5 PLD specific transphosphatidyl reaction

PLD hydrolyses the distal phosphodiester bond in phospholipids such as PC (the structure shown above in red). A phosphatidyl-enzyme intermediate is believed to form transiently which normally is hydrolysed by water, generating PA. Primary short-chain alcohols (e.g. ethanol) can substitute for water in a competing, transphosphatidylation, reaction. In the presence of ethanol the product of PLD-catalysed transphosphatidylation is PA ethyl ester or phosphatidylethanol (PEt). This reaction (thick red arrow pointing to the left) occurs at the expense of the hydrolytic reaction (thin black arrow pointing to the right), decreasing PA formation. PA can also be produced by diacylglycerol kinase and by acylation of glycerol 3-phosphate. In contrast, phosphatidylalcohols are uniquely formed by PLD. PA can be further metabolized (thin black arrows) to diacylglycerol (DAG) and lyso-PA (LPA). In contrast, phosphatidylalcohols are metabolically stable and would accumulate in cells upon PLD activation. Because cellular phosphatidylalcohol levels are normally extremely low, their accumulation upon PLD activation is readily detectable. These properties have made phosphatidylalcohols useful markers of PLD activation *in vitro* and *in vivo*. Attenuation of PLD-catalysed formation of PA by trapping the phosphatidyl moiety in a biologically inactive phosphatidylalcohol has enabled 'alcohol trap' experiments designed to establish the role of PLD in various physiological responses. It should be noted that although phosphatidylalcohols are formed only by PLDs, not all PLDs can catalyse this reaction.(Mayr *et al.*, 1996; Waksman *et al.*, 1997; madesh *et al.*, 1997 & Juneja *et al.*, 1988)

PLD STRUCTURE AND LOCALIZATION

PLD has recently been cloned from yeast, bacteria, plant, and mammalian sources (Morris *et al.*, 1996). Two separate mammalian PLD genes approximately 50% identical have been reported (hPLD1 and hPLD2;), (Colley *et al.*, 1997), with hPLD1 having two splice variants hPLD1a and hPLD1b. hPLD1a has 1072 amino acids and a molecular mass of 124 kDa (Hammond *et al.*, 1995). It is specific for PC and was obtained by using the yeast PLD gene (SPO14) (Rose *et al.*,1995) to identify a human expressed sequence tag for screening a HeLa cDNA library. A shorter splice variant of hPLD1a with 1034 amino acids (hPLD1b) (Figure1.6), which has similar regulatory properties, has been identified (Hammond *et al.*, 1997), and another PLD (PLD2) (Figure 1.6), which has 932 amino acids and 51% amino acid sequence identity to hPLD1a, has been cloned from a mouse embryonic library (Colley *et al.*,1997). The amino acid sequence of PLD1 contains regions that are conserved with PLD2 as well as other nonmammalian species. In addition it contains a "loop region" that is unique to PLD1. Possible functions that have been proposed or demonstrated for these regions are shown in (Figure 1.7).

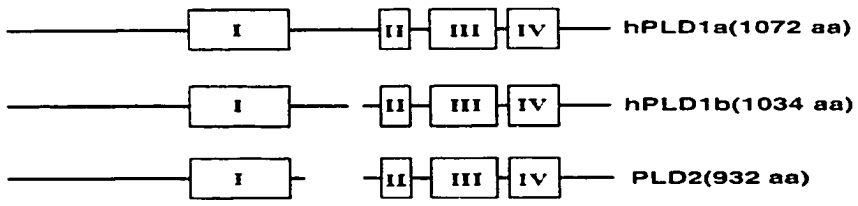


Figure 1.6 Alignment of conserved regions common to hPLD1a, hPLD1b, and PLD2.

(Borrowed from Exton, J.H. 1997)

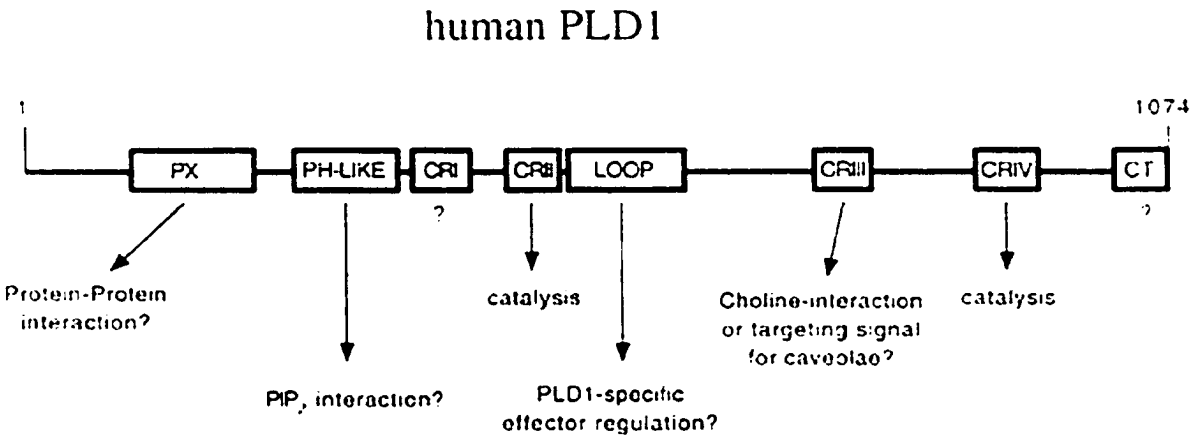


Figure 1.7 Conserved and unique features for human PLD1.

The PLD1 amino acid sequence encodes regions of sequence that either is unique to PLD1 (loop region) or is conserved with mammalian PLD2 and some or all PLD's from nonmammalian species (other boxed regions). Possible functions that have been proposed or demonstrated for these regions are listed underneath each box. CT, carboxyl terminus; LOOP, loop region. (Borrowed from Sung, *et al.*, 1999).

The subcellular localization of PLD is still unclear at this time. Some groups have reported that in fibroblasts PLD2 localizes predominantly in the caveolin-rich membrane domains of the plasma membrane, whereas PLD1 is perinuclear, i.e. in endoplasmic reticulum, Golgi, and late endosomes (Colley *et al.*, 1997; Czarny *et al.*, 2000). While other groups, including our own, have found PLD1 and PLD2 to be localized in the caveolin enriched membrane fraction (Kim *et al.*, 1999; unpublished results) (Figure1.8).

PKC/ARF/Rho Responsive	Yes	No
PIP2 Dependence	Yes	Yes
Molecular Weight kDa	~120	~106
Basal activity	Low	High
Substrate Specificity	PC	PC
Transphosphatidylation	Yes	Yes
Subcellular localization	PM,CEM,ES	PM,CEM

Figure 1.8 Biochemical properties of Phospholipase D 1 and 2

Presented are various characteristics of PLD 1 and 2, corresponding references are presented in the text. PM-plasma membrane; CEM-caveolae enriched membrane; ES-endosomes; PIP2-phosphatidylinositol-4, 5-bisphosphate.

Regulation of PLD

PLD has been shown to be responsive to several signaling proteins including, Protein Kinase C alpha (PKCa), and the small GTPases RhoA, and ADP ribosylation Factor (ARF). In addition, we have recently shown that a PLD activity was found to be associated with the Ras-family GTPase RalA, which is required for the activation of PLD by both v-Src and v-Ras (Jiang *et al.*, 1995) and the active complex includes PLD1, RalA and Arf (Luo *et al.*, 1997; 1998).

The ADP ribosylation factor (ARF) was first discovered as a factor that stimulated cholera toxin-induced ADP-ribosylation of Gs (Lopez *et al.*, 1995). It is now recognized to play a role in vesicle trafficking in Golgi and has been implicated in the fusion of microsomal vesicles and endosomes, the assembly of nuclear membranes, and the formation of clathrin-coated vesicles (Moss *et al.*, 1995; Springer *et al.*,1999 & Roth *et al.*, 1999). The activation of PLD by ARF was first recognized by the groups of Sternweis and Cockcroft (Brown. *et al.*, 1993; Cockcroft *et al.*,1994) and has now been shown using PLD from many sources (Exton *et al.*,1997). Studies with cloned PLD purified from Sf9 cells indicate that ARF interacts directly with the enzyme (Hammond *et al.*, 1997). Some reports have indicated that cytosolic factors greatly enhance the effect of ARF on PLD (Singer *et al.*, 1996). Two of these factors are PKC (Singer *et al.*, 1996) and calmodulin (Takahashi *et al.*, 1996). It is interesting to note that RalA has been shown to contain a calmodulin binding site (Wang *et al.*, 1997) and in this way may act to stabilize a RalA/PLD1/Arf complex.

The activation of PLD by the oncogenic tyrosine kinase v-Src is mediated by a GTPase cascade of Ras and RalA (Jiang *et al.*, 1995). An active PLD can be precipitated

from cell lysates with immobilized GST-RalA fusion protein (Jiang *et al.*, 1995). Subsequent studies demonstrated that the PLD associated with RalA is PLD1 and that this interaction is direct (Luo *et al.*, 1997). Further investigation of the mechanism of PLD activation in RalA-PLD1 complexes revealed that Arf is associated with an active RalA-PLD1 complex (Luo *et al.*, 1998). The level of Arf protein associated with RalA correlated well with the level of PLD activity in the RalA-PLD1 complexes, and the levels of Arf associated with RalA were substantially elevated in the presence of a non-hydrolyzeable analogue of GTP (GTPγS) that stimulated Arf membrane association. In addition, Brefeldin A (BFA), which inhibits GDP to GTP exchange on Arf (Chardin *et al.*, 1999), blocked the v-Src and v-Ras induced PLD activity.

The interaction between RalA and Arf is likely an indirect one. Immobilized RalA was unable to precipitate significant levels of Arf from a partially purified preparation of Arf, suggesting that the association between RalA and Arf is facilitated by another factor. Thus, there is a GTP-dependent association between Arf and a RalA-PLD1 complex that is involved in the activation PLD1 in response to the oncogenic signals generated by v-Src and v-Ras. In addition, one group has found that RalA interacts directly with PLD1 but unlike our results they find that RalA synergistically enhances Arf dependent PLD1 activity (Kim *et al.*, 1998). While another group has found that RalA is able to restore PMA induced PLD activity blocked by treatment with bacterial toxins, TcsL and TcdB-1470, which glucosylates and inactivates Rac, Rap, and Ral GTPases. Indicating a role for Ral in PKC dependent PLD activation (Schmidt *et al.*, 1998).

Rho family proteins regulate many cellular activities including those involving the actin cytoskeleton. The proteins include Rho, which controls the formation of focal

adhesions and actin stress fibers, Rac, which regulates lamellipodia formation and membrane ruffling, and Cdc42, which controls the formation of filopodia (Machesky *et al.*, 1996). The first evidence that PLD could be regulated by Rho proteins came from a study by (Bowman *et al.*,1993). They showed that the stimulatory effect of GTPγS on PLD in neutrophil plasma membranes was inhibited by RhoGDI, a protein that inhibits GDP dissociation from Rho proteins and thereby blocks their activation. In subsequent studies using plasma membranes from rat liver, HL-60 cells, and neutrophils, it was found that RhoA was the most effective Rho protein to activate PLD, but Rac1 or Cdc42Hs showed some activity (Malcolm *et al.*, 1994; Siddiqi *et al.*, 1995; Kwak *et al.*, 1995). Studies with cloned PLD purified from Sf9 cells indicate that RhoA interacts directly with the enzyme and that Rac1 and Cdc42 are also active (Hammond *et al.*, 1997). Interestingly, like the case for RalA and Arf-1 (Kim *et al.*, 1998) a combination of RhoA and ARF results in synergistic activation of homogeneous or partially purified PLD (Hammond *et al.*, 1997; Singer *et al.*, 1996). This suggests the presence of separate but interacting sites for Rho and ARF on PLD. In agreement it has recently been shown that RhoA interacts with a unique c-terminus site of hPLD1 (Yamazaki *et al.*, 1999). As in the case of ARF and RalA, there is evidence that RhoA action on PLD is enhanced by other as yet unidentified cytosolic proteins (Kwak *et al.*, 1995).

There is abundant evidence that PLD is regulated by PKC in most mammalian cells. This comes from studies of the effects of phorbol esters, PKC inhibitors, down-regulation of the enzyme, and overexpression and deletion of specific PKC isozymes (Lu, *et al.*, 2000; Hornia, *et al.*, 1999). Although a role for PKC in the actions of many agonists on PLD in many cells has been indicated, there are also instances where the enzyme does not

seem to be involved, as in the case of v-Src and v-Ras (Exton *et al.*, 1997; Song *et al.*, 1993).

CHAPTER II

MATERIALS AND METHODS

Cells, Cell Culture, and Transfection Conditions

Rat 3Y1 fibroblasts, v-Src-transformed 3Y1 cells, and 3Y1 cells overexpressing c-Src were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% bovine calf serum (HyClone) as described previously (Lu *et al.*, 1997). Rat 3Y1 fibroblasts and 3Y1 cells overexpressing c-Src that conditionally express PLD1 and PLD2 were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% bovine calf serum (HyClone) as described previously(Joseph, 2001 &2002) Anchorage independent growth was assayed by suspending cells in soft agar as follows: 1 x 10^3 cells were suspended in top agar (DMEM, 20% calf serum, 0.38% agar) and overlaid onto hardened bottom agar (DMEM, 20% calf serum, 0.7% agar) as described previously . Colonies were counted 14 days later.

Transient transfection of the $3Y1^{c\text{-}Src}$ cells with the catalytically-inactive PKC δ was performed as described previously (Lu et al., 2000) using lipofectamine reagent (GIBCO) according to the vendors instructions.

Materials

Monoclonal antibody to PARP was obtained from Pharmgen; polyclonal antibody to protein kinase C δ (PKC δ) was obtained from Santa Cruz Biotechnology; polyclonal antibodies for Akt and phosphorylated Akt were from New England Biolabs. Antibodies to cytochrome C and Cox-4 were from CloneTech as part of the ApoAlert cell fractionation kit. Caspase inhibitors were obtained from BioRad. DAPI (4',6-diamidino-2-phenylindole, dihydrochloride) was obtained from Molecular Probes. LY-294002 was from Calbiochem.

Western Analysis

Extraction of proteins from cultured cells was performed as previously described (Lu *et al.*, 1997;1998; 2000). Equal amounts of protein were subjected to SDS-PAGE using a 8% to 15% acrylamide separating gels, transferred to nitrocellulose and blocked overnight at 4°C with 5% non-fat dry milk isotonic phosphate buffered saline (PBS: 136 mM NaCl, 2.6 mM KCl, 1.4 mM KH_2PO_4, 4.2 mM Na_2HPO_4). The nitrocellulose filters were washed three times for five minutes in PBS and then incubated with antibodies as described in the text. Depending upon the origin of the primary antibodies, either anti-mouse or anti-rabbit IgG was used for detection using the ECL system (Amersham).

Apoptosis Assays

Trypan blue exclusion was used to quantify apoptosis. After various treatments, cells were harvested with trypsin and washed in PBS. Trypan blue (Sigma) was added to suspended cells at a concentration of 0.4% w/v. After 10 min, trypan blue uptake (dead cells) was determined by counting on a hemocytometer.

DNA fragmentation: DNAzol regent (Gibco BRL) was used to extract DNA from 100 mm plates, according to manufacturer's instructions. After RNase treatment, isolated DNA was subjected to 2% agrose gel electrophoresis. The DNA was visualized by ethidium bromide staining under UV light.

DAPI staining: Cells were plated into 8-well chamber slides. After treatments indicated in the text, cells were fixed in 4% formaldehyde for 15 min at room temperature and permeabilized for 2 min with ice cold methanol. The cells were then blocked with

DMEM/10% fetal calf seum for 30 min at room temperature, followed by incubation with DAPI stain (Molecular probe) at 1 μg/ml in PBS for 5 min at room temperature. Nuclei were visualized by fluorescent microscope.

Caspase Assays

Both adherent and suspended cells from cultures were collected and washed with PBS. 100 μl of lysis buffer per 10^7 cells was added with gentle vortex. Samples were subjected to three rounds of freeze and thaw by transferring from an iso-propanol-dry ice bath to 37°C water bath. The resultant cell lysates were centrifuged at 4°C for 30 min at full speed. Equal amounts of protein were then incubated with caspase-specific oligopeptide substrates tagged with the fluorescent 7-amino-4-trifluoromethyl coumarin (AFC) substrate (BIORAD) at 37°C for 1 hour. Caspase activation was measured by AFC release as detected spectrotometrically according to the vendors instructions. Caspase inhibitors were the same peptide substrates tagged with the inhibitory fluoromethyl ketone (FMK) tag that prevents cleavage by the recognizing caspase.

Subcellular fractionation

Separation of mitochondrial and and cytosolic fractions was performed by using *ApoAlert Cell Fractionation Kit* (CLONTECH) according to the manufacturer's specifications. Cox 4 antibody (included in the kit) was used to confirm successful separation of mitochondrial and cytosolic fractions.

DNA Synthesis Assays

Cell cultures were made quiescent by growing to confluence and then placing in fresh media containing 0.5% bovine calf serum for 36 hr in 24 well tissue culture dishes. DNA synthesis was measured by a one hr pulse with [^{3}H]-thymidine (1 μCi/ml; 20 Ci/mmole). Cells were then collected and trichloroacetic acid precipitable counts were determined by scintilation counting as described previously (Lu *et al.*, 1997).

PKCδ Kinase Assays

PKCδ activity was determined using a protein kinase assay kit from Calbiochem. Cells were prepared by washing with cold phosphate buffered saline (PBS: 136 mM NaCl, 2.6 mM KCl, 1.4 mM KH_2PO_4, 4.2 mM Na_2HPO_4, pH 7.4), scraping with a rubber policeman, suspendsuspension in kinase sample buffer (50 mM Tris-HCl pH 7.5, 5 mM EDTA, 5 mM EGTA, 50 mM 2-mercaptoethanol, 100 μM Na-VO_4, protease inhibitor cocktail), and then sonicating for 30 to 60 sec to disrupt cell membranes. The cell extracts were then clarified by centrifugation at 100,000 X g for 1 hr at 4^0 C. Equal amounts of protein were then subject to immunoprecipitation with an anti-PKCδ antibody (Santa Cruz Biotechnology). Immune complexes were recovered with protein A agarose beads and subjected to the kinase assay according to the manufacturers instructions.

PLD assays (transphosphatidylation reactions).

PLD activity was measured by the ability of cells to convert the metabolically labeled [^{3}H]-PC into PBt in the presence of exogenously provided 1-BtOH. Cells were grown in 60 mm culture dishes to confluency and made quiescent as described above. These

quiescent cells were labeled for 4-6 hr with [^{3}H]-myristate (40 Ci/mmol) at final concentration of 1 μCi/ml, and followed by 15 min of incubation with 1% (v/v) 1-BtOH. Afterwards, cells were placed on ice, washed twice with cold PBS and collected in 0.5 ml of methanol / 6 M HCl (50:1, v/v). Lipids were extracted by adding 0.5 ml of chloroform. Phase separation was achieved by the addition of 155 μl of 1 M NaCl and the organic phase was recovered after centrifugation. This was followed by reextraction through the addition of 350 μl H_2O, 115 μl 1 M NaCl and 115 μl methanol. An aliquot of the thus obtained organic phase was counted in a liquid scintillation counter and the volume of each sample that had the same intensity of radioactivity was calculated according to the readings. Lipids containing an equal amount of radioactivity were then dried under a stream of nitrogen and redissolved in 50 μl of chloroform / methanol (9:1, v/v). Samples were then spotted on a precoated silica (60A) plates and separated by thin layer chromatography (TLC) with a solvent system of ethylacetate / trimethylpentane / acetic acid / H_2O (9:5:2:10, v/v, upper layer). The transphosphatidylation product PBt was visualized by autoradiography of the TLC plates and the films were scanned in a densitometer for quantification.

Morphological studies

Cells were grown in 6 well plates to 50% confluency, made quiescent, and then treated as described in the figure legends. The cells were photographed under an Olympus OMT-2 inverted microscope with a Dage MTI CCD 72 video camera and images were obtained using Oncor Image analysis software. .

Immunofluorescence microscopy.

Cells were seeded onto chamber slide. The different treatment cells indicated in the text were then washed twice with PBS, fixed in 4% (w/v) paraformaldehyde in PBS for 10 min at room temperature, washed with PBS, and then permeablized by incubation with ice-cold methanol for 2 minon ice. Cells were then again washed with PBS, incubated with 10% (w/v) bovine serum albumin / DMEM for 5 min, and subjected to successive incubation with primary and fluorophore-conjugated secondary antibodies or DAPI. Each antibody incubation was in 2% (w/v) bovine serum albumin / PBS at room temperature for 1 hr and followed by rinse with PBS. After final rinse, the top part of chamber slide was removed and coverslips were mounted with 50% (w/v) glycerol in PBS and photographed under a Nikon Optiphot 2 upright microscope with a Sony 'Cats Eye' high resolution color video camera and images were obtained using Compix Simple 32 image analysis software.

CHAPTER III

Novel Tumor-promoting Property of Tamoxifen

INTRODUCTION

Tamoxifen, a non-steroidal mixed estrogen agonist / antagonist, is widely employed as a first line of therapy for patients suffering from metastatic as well as primary breast carcinoma (Fisher *et al.*, 1987). Tamoxifen has also been shown to reduce the incidence of primary breast carcinoma in women who are at high risk for developing this disease (Fisher *et al.*, 1998) and prophylactic administration of tamoxifen has been suggested for these high-risk patients (Vogel *et al.*, 2000). Five year tamoxifen treatment substantially improves prognosis for breast cancer patients, however ten year treatment showed no significant improvement and substantially increased the risk for endometrial cancer and possibly gastrointestinal cancers as well (van Leeuwen *et al.*,1994;Fisher *et al.*,1994; Rutqvist *et al.*,1995 & Stearns *et al.*,1998). The increase in endometrial cancer has been widely attributed to an estrogen mimetic effect of tamoxifen in endometrial tissues where both estrogen and tamoxifen induce hyperplasia (Deligdisch *et al.*, 2000). However, an increase in cancer incidence within 10 years of exposure to tamoxifen suggests that tamoxifen possesses a tumor-promoting capability beyond the estrogen-mimetic properties of tamoxifen.

We demonstrated previously that the tumor-promoting phorbol ester TPA (12-O-tetradecanoylphorbol-13-acetate) cooperates with c-Src to transform 3Y1 rat fibroblasts (Lu *et al.*, 1997). In this cell culture model system, 3Y1 rat fibroblasts overexpressing c-Src ($3Y1^{c\text{-}Src}$ cells) became transformed when treated with TPA. The effect of TPA could be mimicked by inhibitors of the δ isoform of protein kinase C (PKCδ), indicating that the effects of TPA were due its ability to downregulate PKCδ (Lu *et al.*, 1997; Honia *et al.*, 1999). Consistent with these results, transgenic mice overexpressing PKCδ in their

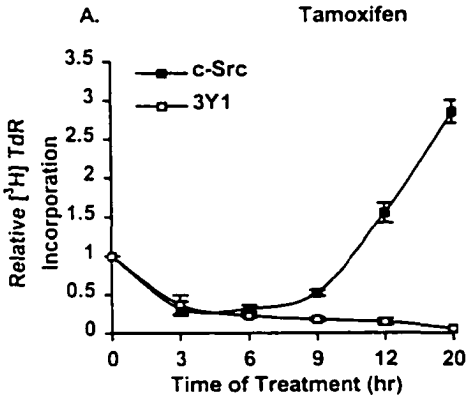
epidermis were recently reported to be resistant to skin tumor promotion by TPA (Redding *et al.*, 1999). Thus, the $3Y1^{c\text{-}Src}$ cells appear to accurately reflect the tumor-promoting effects of TPA with c-Src being the initiating mutation and PKCδ downregulation the promoting effect in a two-stage tumorogenisis model. The $3Y1^{c\text{-}Src}$ cells are therefore capable of detecting tumor-promoting compounds able to cooperate with a signaling oncogene such as an overexpressed tyrosine kinase - a common genetic defect in human cancers (Biscardi *et al.*, 1998) - to transform cells.

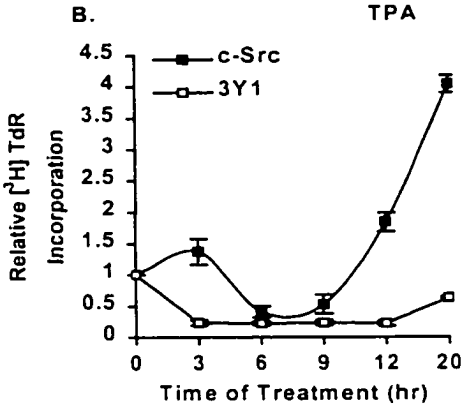
Because of the likelihood that tamoxifen has tumor-promoting properties beyond its estrogen-mimetic capability, we examined the effect of tamoxifen on the $3Y1^{c\text{-}Src}$ cells. We report here that tamoxifen, like TPA, causes the down regulation of PKCδ and has tumor promoting effects that cooperate with an overexpressed tyrosine kinase to transform rat fibroblasts.

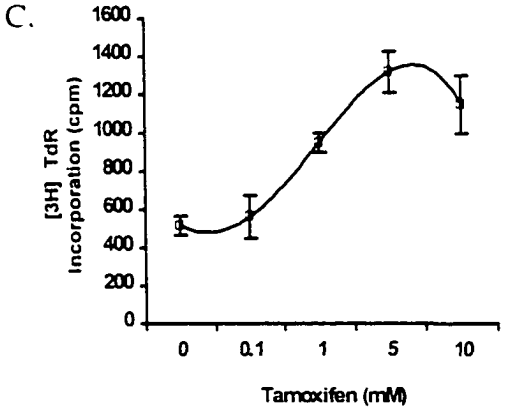
Results

Tamoxifen and TPA induce DNA synthesis in 3Y1$^{c\text{-}Src}$, but not parental 3Y1 cells. We first examined the ability of tamoxifen to stimulate DNA synthesis in both 3Y1$^{c\text{-}Src}$ and parental 3Y1 cells after serum withdrawal. Sub-confluent cultures of 3Y1 or 3Y1$^{c\text{-}Src}$ cells maintained in medium containing 10% serum were transferred to medium containing 0.5% serum for 36 hours. At this point, both the 3Y1 and 3Y1$^{c\text{-}Src}$ cells exhibited minimal uptake of [^{3}H]-thymidine. Tamoxifen was then added and DNA synthesis was monitored by [^{3}H]-thymidine uptake. As shown in Fig.3.1A, tamoxifen stimulated an increase in DNA synthesis in the 3Y1$^{c\text{-}Src}$, but not in the parental 3Y1 cells. The increase was observed between 12 and 20 hr after treatment. For comparison, we also examined the effect of TPA on DNA synthesis in these cells. As shown in Fig. 1B, TPA stimulated a biphasic increase in DNA synthesis in the 3Y1$^{c\text{-}Src}$ cells with an increase in [^{3}H]-thymidine uptake between 2 and 4 hr and another between 12 and 20 hr. The biphasic increase in DNA synthesis suggested that serum deprivation of 3Y1$^{c\text{-}Src}$ cells results in two populations of cells that are arrested in different places in the cell cycle. We did not observe the short term increase in DNA synthesis using tamoxifen, indicating that TPA may have effects beyond those of tamoxifen. The tamoxifen-induced increase in DNA Synthesis was dose dependent and could be detected at concentrations as low as 0.1 μM tamoxifen, with maximal levels seen between 2 and 5 μM (Fig. 3.1C). Importantly, this level of tamoxifen is achieved in the serum of individuals on standard 20 mg/day protocols (El-Yazigi *et al.*, 1997). Estrogen was unable to stimulate DNA synthesis in either the 3Y1 or 3Y1$^{c\text{-}Src}$ cells (data not shown). As shown in Fig. 3.2, the 3Y1$^{c\text{-}Src}$ cells express detectable levels of the β, but not the α estrogen receptor. Thus,

while the $3Y1^{c\text{-}Src}$ cells do contain estrogen receptor β, the effects of tamoxifen go beyond estrogen mimetic effects.







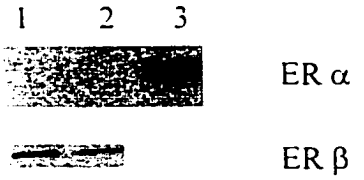


Fig 3.1. Tamoxifen and TPA induce DNA synthesis in $3Y1^{c\text{-}Src}$, but not parental 3Y1 cells. Sub-confluent 3Y1 and $3Y1^{c\text{-}Src}$ cells were placed in media containing 0.5% serum for 36 h and then treated with either tamoxifen (5 μM) (A) or TPA (400 nM) (B) for the indicated times. The cells were then pulsed with [^{3}H]-thymidine for one hr and the incorporation of [^{3}H]-thymidine (TdR) into TCA-insoluble fractions was determined as reported previously (Lu *et al.*, 1997). Values were normalized to the cpm/cell for the untreated time zero controls, which were given a value of 1. Actual cpm/cell values were 8.8 X 10^{-3} for the 3Y1 cells and 4.0 X 10^{-3} for the $3Y1^{c\text{-}Src}$ cells. Relative [^{3}H]-TdR incorporation values were obtained from three independent experiments. (C) The dose response to tamoxifen for induction of DNA synthesis was examined by doing a one hr [^{3}H]-thymidine pulse at 20 hr after addition of tamoxifen at the concentrations shown. Error bars represent the standard deviation for the fold effect of tamoxifen and TPA treatments as shown. All experiments were performed at least two times.

Fig.3.2. Estrogen receptor levels in $3Y1^{c\text{-}Src}$ cells. Lysates from 3Y1 (Lane 1) and $3Y1^{c\text{-}Src}$ (Lane 2) cells were analyzed for estrogen receptor α and β levels as shown by Western blot analysis as described in Materials and Methods. As a positive control for estrogen receptor α, rat uterus tissue was used (Lane 3).

Tamoxifen induces anchorage-independent growth in c-Src-overexpressing, but not parental 3Y1 cells.

We next examined the effect of tamoxifen upon anchorage-independent growth as measured by the ability to form colonies in agar suspension. 3Y1 and $3Y1^{c\text{-}Src}$ cells were seeded into agarose and colony-forming efficiency was determined in the presence or absence of tamoxifen. We previously reported that TPA causes a 6-fold increase in the efficiency of colony formation in the $3Y1^{c\text{-}Src}$ cells (Lu *et al.*, 1997). Tamoxifen similarly induced a 5-fold increase in colony-forming efficiency (Fig. 3.3A). Tamoxifen had no effect upon the colony forming efficiency in the parental 3Y1 cells (Fig. 3.3A). The dose dependence for colony formation was similar to that observed for DNA synthesis (Fig. 3.3B). These data further support the hypothesis that tamoxifen has tumor-promoting properties similar to those of TPA.

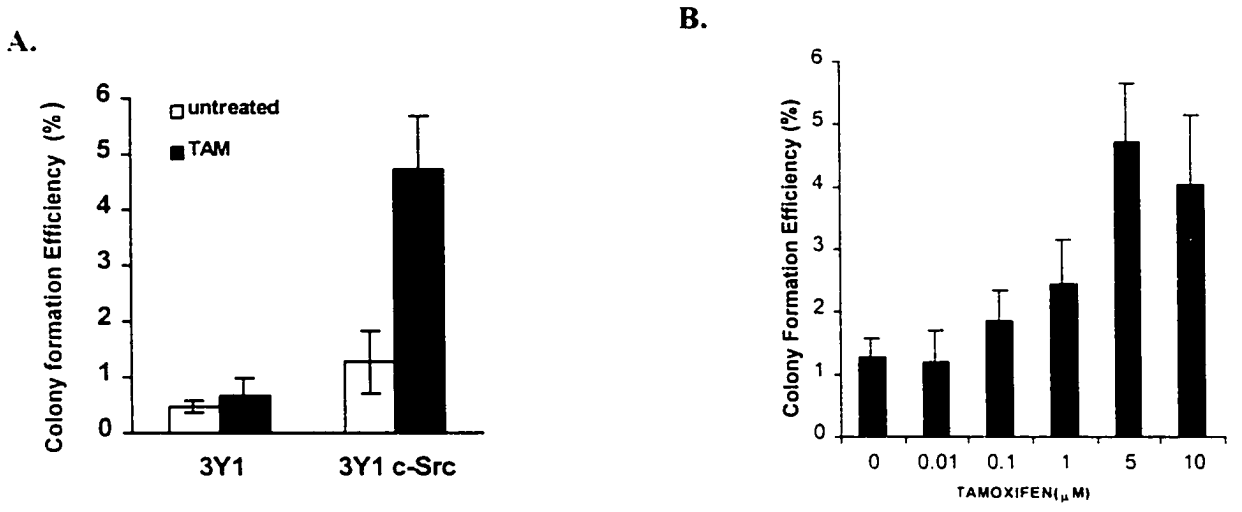


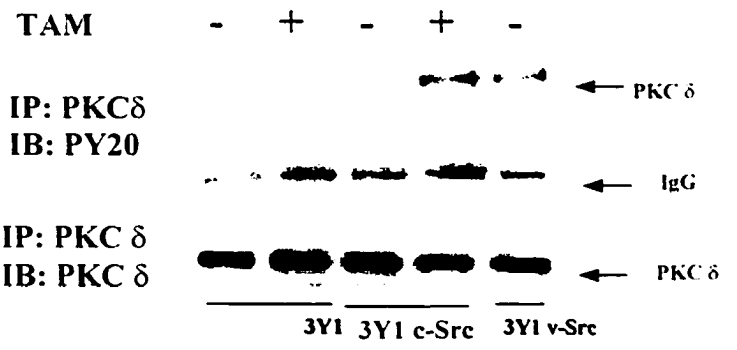
Fig 3.3 Tamoxifen induces anchorage-independent growth in c-Src-overexpressing, but not parental 3Y1 cells. (A) Anchorage-independent growth of the 3Y1 and $3Y1^{c\text{-}Src}$ cells was examined in the absence and presence tamoxifen (5 μM). (B) Colony formation was determined in the presence of increasing concentrations of tamoxifen as shown. 10^3 cells were suspended in soft agar and the percentage of cells that formed colonies was determined two weeks later. Tamoxifen was replenished every four days. Error bars represent the standard deviation for 3 independent experiments performed in duplicate.

Tamoxifen induces tyrosine phosphorylation and downregulation of PKCδ in cells overexpressing c-Src.

As described above, the tumor promoting effect of TPA was the result of depleting cells of PKCδ (Lu *et al.*, 1997). PKCδ has been implicated as a tumor suppressor gene in a variety of cell and animal systems (Lu *et al.*, 1997; Hornia *et al.*, 1999; Redding *et al.*, 1999; Mischak *et al.*, 1993; Blake *et al.*, 1999; Perletti *et al.*, 1999 & Ashton *et al.*, 1999). And importantly, in cells transformed by v-Src, PKCδ was shown to associate with v-Src and become phosphorylated on tyrosine (Zang *et al.*, 1997). The tyrosine phosphorylation of PKCδ by v-Src leads to reduced PKCδ levels (Blake *et al.*, 1999), further indicating that reduced levels of PKCδ are critical for transformation. As shown in Fig. 4A, tamoxifen stimulated tyrosine phosphorylation of PKCδ in the $3Y1^{c\text{-}Src}$, but not the parental 3Y1 cells. The level of tyrosine phosphorylation was comparable to that seen in v-Src-transformed cells (Fig. 3.4A). The effect was dose dependent with a dose response similar to that observed for DNA synthesis and colony formation with induction occurring at concentrations as low as 0.1 μM tamoxifen (Fig. 3.4B). As observed for DNA synthesis, estrogen did not stimulate tyrosine phosphorylation of PKCδ in the $3Y1^{c\text{-}Src}$ cells (not shown). We next examined whether we could detect c-Src in PKCδ immunoprecipitates, and as shown in Fig. 3.4C, tamoxifen treatment caused PKCδ to co-immunoprecipitate with c-Src. These data indicate that tamoxifen stimulates association between overexpressed c-Src and PKCδ, leading to the phosphorylation of PKCδ on tyrosine. Since tyrosine phosphorylation of PKCδ by v-Src causes degradation of PKCδ (Blake *et al.*, 1999), we examined the effect

of tamoxifen upon level of PKCδ. As shown in Fig. 3.4D, tamoxifen treatment reduced PKCδ levels to those seen in v-Src-transformed cells. Tamoxifen treatment did not reduce the level of PKCδ comparably in the v-Src-transformed cells (Fig. 3.4D), indicating that only a sub-population of PKCδ is involved. Tamoxifen had little or no effect upon the level of PKCδ in the parental 3Y1 cells (Fig. 3.4D). We next examined the effect of tamoxifen upon PKCδ activity, and as shown in Fig. 4E, tamoxifen reduced the PKCδ activity (Gallo *et al.*, 1997) in the $3Y1^{c\text{-}Src}$, but not the parental cells. The level of reduced PKCδ activity seen in Fig. 4E was quantitatively similar to the reduced level of PKCδ protein seen in Fig. 3.4D. Thus, in response to tamoxifen, as with TPA (Lu *et al.*, 1997; Hornia *et al.*, 1999), there are reduced levels of both PKCδ protein and activity. Although PKCδ levels are not reduced to the levels achieved by TPA treatment, this is likely due to an effect that restricted to a sub-population of PKCδ that associates with c-Src. However, this subpopulation is likely to be a critical sub-population of PKCδ.

A.



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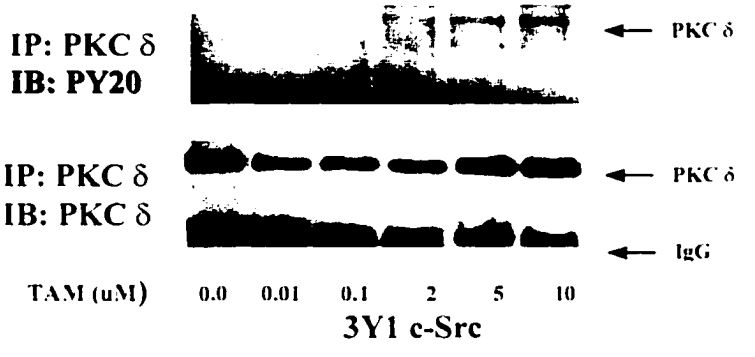

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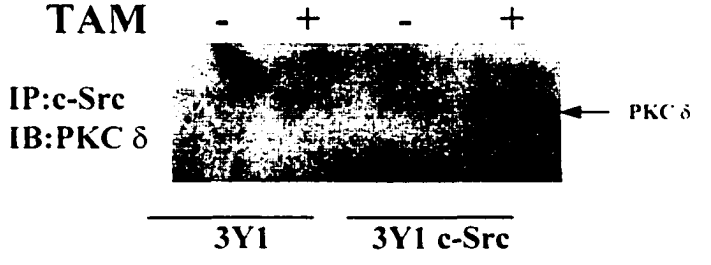
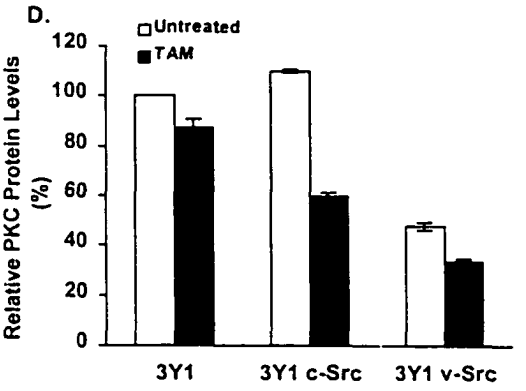




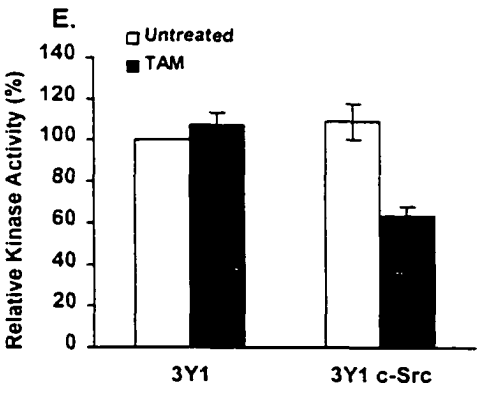


Fig 3.4 Tamoxifen induces tyrosine phosphorylation and downregulation of PKCδ in cells overexpressing c-Src. (A) 3Y1 and 3Y1$^{c\text{-}Src}$ cells were treated with tamoxifen (TAM) (5 μM) as shown. The cells were harvested after 14 hr and lysed. Lysates were immunoprecipitated with an anti PKCδ antibody and subjected to Western blot analysis with either anti-phosphotyrosine (upper panel) or anti-PKCδ (lower panel) antibodies as shown. 3Y1 cells transformed v-Src were also analyzed for tyrosine-phosphorylated PKCδ as shown. (B). A dose response for stimulation of PKCδ tyrosine phosphorylation by tamoxifen was performed on the 3Y1$^{c\text{-}Src}$ cells as described in (A). (C) 3Y1 and 3Y1$^{c\text{-}Src}$ cells were treated with tamoxifen (5 μM, 14 hr) as shown. Lysates of 3Y1 and 3Y1$^{c\text{-}Src}$ cells were immunoprecipitated with anti-c-Src antibody and the Immunoprecipitates were subject to Western blot analysis using an antibody raised against PKCδ. (D) PKCδ protein levels in lysates from tamoxifen-treated (5 μM, 14 hr) and untreated 3Y1, 3Y1$^{c\text{-}Src}$, and v-Src-transformed 3Y1 cells was determined by Western blot analysis. PKCδ levels were quantified by densitometer tracing of the Western blots. Values were normalized to the level of PKCδ in the untreated parental 3Y1 cells, which was given a value of 100%. Error bars represent the standard deviation for 3 independent experiments. (E) PKCδ kinase activity in lysates from 3Y1 and 3Y1$^{c\text{-}Src}$ cells treated with tamoxifen where indicated (5 μM, 14 hr) was determined as described in Materials and Methods. As in (D), values were normalized to kinase activity in the untreated parental 3Y1 cells, which were given a value of 100%. Error bars represent the standard deviation for 3 independent experiments.

Tamoxifen induces PLD activity 3Y1 cells overexpressing c-Src. We previously reported that inhibition or downregulation of PKCδ led to an elevation of PLD activity in the $3Y1^{c\text{-}Src}$ cells (Lu *et al.*, 1997) and in 3Y1 cells overexpressing the epidermal growth factor (EGF) receptor (Hornia *et al.*, 1999). Moreover, we went on to demonstrate that PLD1 overexpression could cooperate with the EGF receptor to transform 3Y1 cells (Land *et al.*, 1983). We have also found that stimulation of PLD activity transforms the $3Y1^{c\text{-}Src}$ cells (*our unpublished results*). Since tamoxifen reduced the level of PKCδ in the $3Y1^{c\text{-}Src}$ cells, we examined whether tamoxifen treatment elevated PLD activity in the $3Y1^{c\text{-}Src}$ cells. As shown in Fig. 4, tamoxifen treatment led to an increase in PLD activity in $3Y1^{c\text{-}Src}$, but not parental 3Y1 cells. Thus tamoxifen, like TPA and other negative regulators of PKCδ (Hornia *et al.*,1997), elevates PLD activity. And since PLD activity is sufficient to transform 3Y1 cells overexpressing a tyrosine kinase (Land *et al.*, 1983), it is possible that the ability of tamoxifen to elevate PLD activity in the $3Y1^{c\text{-}Src}$ cells is important for the transformed phenotypes observed.

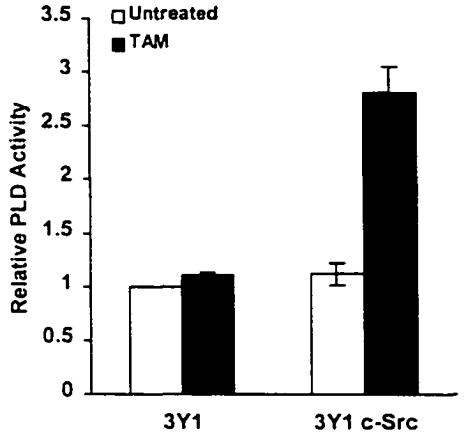


Fig 3.5 Tamoxifen induces PLD activity 3Y1 cells overexpressing c-Src. 3Y1 and 3Y1[c-Src] cells were placed in media containing 0.5% serum for 24 hr and then treated with tamoxifen (5 μM, 14 hr). Cells were prelabeled with [^{3}H]-myristate for 4 hr prior to harvesting; butanol (1.0%) was added for 1 hr prior to harvesting. The transphosphatidylation of phosphatidylcholine to phosphatidylbutanol (PLD activity) was determined as described previously (Hoffmann *et al.*, 1983). The relative PLD activity was normalized to the PLD activity in the untreated 3Y1 cells. Error bars for represent the standard deviation for two independent experiments performed in duplicate, where duplicates varied by less that 10%.

Discussion

How tamoxifen stimulates the association between c-Src and PKCδ is not known. We demonstrated previously that association between v-Src and PKCδ depended upon an active Src kinase. Thus, it is likely that tamoxifen treatment leads to the activation of c-Src through a mechanism yet to be determined. Tamoxifen had no effect upon c-Src protein levels (data not shown). The effect may be specific for c-Src or non-receptor class tyrosine kinases since tamoxifen does not have the tumor promoting effects seen in the $3Y1^{c\text{-}Src}$ cells in 3Y1 cells that overexpress the epidermal growth factor receptor (our unpublished results). Tamoxifen is known to have estrogen-mimetic effects in endometrial cells (Gallo *et al.*, 1997). However the effects of tamoxifen observed here go beyond estrogen-mimetic effects, since estrogen did not cause the transformation-related phenotypes caused by tamoxifen.

Weinberg and colleagues described two oncogene complementation groups for the transformation of primary rodent cells (Dotto *et al.*, 1985). The essential features of this model have been verified in an updated version, which integrates the role of telomerase in the malignant transformation of human cells (Nevins *et al.*, 1993). According to this model, a cytoplasmic signaling oncogene such as Ras or Src cooperates with a nuclear oncogene, such as Myc or SV-40 large T antigen to cause transformation (Nevins *et al.*, 1993). Interestingly, TPA was able to cooperate with the signaling, but not the nuclear oncogenes to transform primary cells (Dotto *et al.*, 1985), indicating that TPA could accomplish the equivalent of Myc or SV-40 large T. This would indicate that

tamoxifen facilitates passage through the G_1/S cell cycle checkpoint, since this is where large T-antigen exerts its effects by sequestering Rb and p53 (Nevins *et al.*, 1983).

The relatively rapid increase in endometrial cancers seen with tamoxifen treatment suggests that tamoxifen may have multiple carcinogenic effects. The estrogen mimetic effects of tamoxifen, which causes endometrial hyperplasia, most likely explains why endometrial cancers are seen first with prolonged tamoxifen treatment (Gallo *et al.*, 1997 & Cano *et al.*, 2000). The tamoxifen metabolite 4-OH-tamoxifen, which has DNA-damaging capability (Beland *et al.*, 1999) is present in the serum of patients taking tamoxifen (Langan-Fahey *et al.*, 1990) and could therefore also contribute to tumor progression. Stimulating cell proliferation in the presence of DNA-damaging agents significantly increases mutation rates and tumor progression is accelerated (Ames *et al.*, 1995). Thus, the combination of the cell proliferation-inducing estrogen-mimetic effects of tamoxifen and DNA damage brought about by 4-OH-tamoxifen should make tamoxifen a potent carcinogen. However cigarette smoke, which also contains both tumor-promoting and DNA-damaging agents (Hoffmann *et al.*, 1983) takes more than 20 years before significant increases in lung cancer are observed. Therefore, it is likely that there are other effects of tamoxifen that make it an even more potent carcinogen that induces endometrial cancers within 10 years. The TPA-mimetic properties reported here could be the additional carcinogenic effect responsible the rapid tumor producing ability of tamoxifen. Thus, tamoxifen may be a three-pronged carcinogen that produces estrogen-mimetic hyerplasia, DNA damage, and a TPA-mimetic downregulation of the tumor-suppressing PKCδ. While the effects of tamoxifen in this cell culture model may not reflect exactly the effects tamoxifen in human endometrial cells, they do provide

evidence of a tumor promoting effect for tamoxifen that is consistent with its apparent tumor-promoting effects in patients taking tamoxifen for extended periods. And if true, even longer term tamoxifen treatment, which has been proposed as a preventative step for women deemed at risk for breast cancer, could ultimately result in a much higher incidence of endometrial cancers and perhaps other cancers later on. While the benefits of tamoxifen treatment for 5 years clearly outweigh the risks of a small increase in endometrial tumors, longer-term prophylactic treatment needs to be carefully considered.

CHAPTER IV

Downregulating PKC δ provides a PI3K/Akt-independent survival signal that overcomes apoptotic signals generated by c-Src overexpression

Introduction

Overexpression of a tyrosine kinase is a common genetic defect in a variety of human tumors (Biscardi *et al.*, 1999). We demonstrated previously that 3Y1 rat fibroblasts overexpressing c-Src become transformed when treated with tumor-promoting phorbal esters (Lu *et al.*1997; 1998). This effect is similar to a model for cooperating oncogenes proposed by Weinberg and colleagues where the transformation of primary cells required two cooperating oncogenes such as Ras and Myc (Land *et al.*1983; Hahn *et al.*, 1999). Ras also cooperated with phorbol esters to transform primary cells (Dotto *et al.*, 1985). However, expression of Myc alone resulted in apoptosis when cells were subjected to serum deprivation (Evan *et al.*, 1992). Thus, while Myc expression provides a mitogenic stimulus, in the absence of Ras or another cooperating oncogene, Myc sensitizes the cell to the apoptotic stress of serum deprivation (Hueber and Evan , 1998). The effect of Ras and other oncogenes of the signal transduction family is not as clear. Activated Ras leads to cell senescence in the absence of a cooperating oncogene (Serano *et al.*, 1997). However, high intensity Ras signaling has been reported to induce apoptosis (Joneson and Bar-Sagi, 1999), indicating that extreme mitogenic Ras signaling can also activate apoptotic pathways. It has been suggested that Ras and Myc are able to generate a complete mitogenic signal because Myc overrides the cytostatic action of Ras, and Ras generates the survival signals that overcomes the apoptotic signals of Myc (Hueber and Evan, 1998).

A model for controlling cell proliferation is emerging whereby a mitogenic signal such as Myc or Ras simultaneously activates both mitogenic and apoptotic or senescence pathways. For cell division to proceed, a survival signal(s) must also be generated that suppresses apoptotic signals (Hueber and Evan, 1998). Signaling through phosphatidylinositol-3-kinase (PI3K) and the downstream kinase Akt has been widely implicated as an important survival signal in cell proliferation (Datta *et al.*, 1997; 1998; Kandel and Hay, 1998; Kaufmann-Zeh *et al.*, 1997; Kennedy *et al.*, 1997). Interestingly, the activated Src kinase (v-Src) does not induce apoptosis upon serum deprivation (Johnson *et al.*, 2000), suggesting that v-Src generates both the mitogenic and survival signals necessary for proliferation. Consistent with this hypothesis, apoptosis has been reported in v-Src-transformed cells where downstream signaling components such as Ras and PI3K were blocked (Johnson *et al.*, 2000; Hakak *et al.*, 2000; Webb *et al.*, 2000). Activating mutations within the Src gene have been reported in late stage colon cancer (Irby *et al.*, 1999; Irby and Yeatman, 2000). However, two subsequent studies did not find a correlation between Src mutation and colon cancer (Daigo *et al.*, 1999; Wang *et al.*, 2000). Thus, activating mutations to Src are apparently rare in human cancer, in spite of the fact that single base pair changes in the carboxy terminus are able to generate an activated Src kinase. This is not true for activating mutations to Ras, which like Src, requires only a single mutation to generate an activated Ras protein. However, activated Ras is not sufficient to transform primary cells (Land *et al.*, 1983) and needs another genetic alteration to transform cells. Unlike Src, activating mutations in Ras are seen in approximately 25% of human cancer (Hanahan and Weinberg, 2000). More common for tyrosine kinases are mutations that lead to increased expression rather than increased

activity (Biscardi *et al.*, 1999). It is not clear why activating mutations for tyrosine kinases are not commonly observed in cancer, but it is likely that cells acquiring a mutation that simultaneously provides both mitogenic and survival signals are eliminated before they can contribute to progression to cancer. Overexpression of a tyrosine is not sufficient to transform cells, however cells overexpressing c-Src become transformed when treated with tumor-promoting phorbol esters (Lu *et al.*, 1997). The effect of the phorbol esters was due to the down regulation of protein kinase C δ (PKC δ) and consistent with conclusion, the PKC δ inhibitor rottlerin similarly transformed 3Y1 cells overexpressing either c-Src (Lu *et al.*, 1997) or the EGF receptor (Hornia *et al.*, 1999). Thus, an initiating mutation, such as an overexpressed tyrosine kinase, in combination with downregulation of PKC δ may be a critical aspect of tumor progression. We report here that while cells partially-transformed by c-Src overexpression become sensitive to apoptotic stress, downregulating PKC δ provides an Akt-independent survival signal that overrides apoptotic signals generated in cells overexpressing this tyrosine kinase.

RESULTS

Serum starvation triggers cell death in c-Src-overexpressing 3Y1 cells, but not in parental or v-Src-transformed 3Y1 cells

We demonstrated previously that 3Y1 cells overexpressing c-Src ($3Y1^{c\text{-}Src}$) became transformed when depleted of PKC δ (Lu *et al.*, 1997). However, we noticed that if the $3Y1^{c\text{-}Src}$ cells were deprived of serum, many of the cells died. We wished to determine whether this was a property of 3Y1 cells or due to overexpressed c-Src. As shown in Figure 4.1A, upon serum withdrawal, many of the $3Y1^{c\text{-}Src}$ began to round up and ultimately detached. This effect was not observed in either the parental or v-Src-transformed 3Y1 cells. Quantification of cell death in the $3Y1^{c\text{-}Src}$ cells using trypan blue exclusion revealed that approximately 50% of the cells died after 14 h of serum deprivation (Figure 4.1B). This This level of cell death is similar to the level of apoptotic cell death reported in rat fibroblasts expressing the Myc oncogene (Evan *et al.*, 1992). These data indicate that overexpression of c-Src renders cells sensitive to serum withdrawal and that this effect is overcome by the active kinase of v-Src. The lack of cell death observed in the v-Src-transformed cells is consistent with observations by Webb *et al.*, (2000) and Johnson *et al.*, (2000), who reported that v-Src-transformed cells do not die under conditions of low serum.

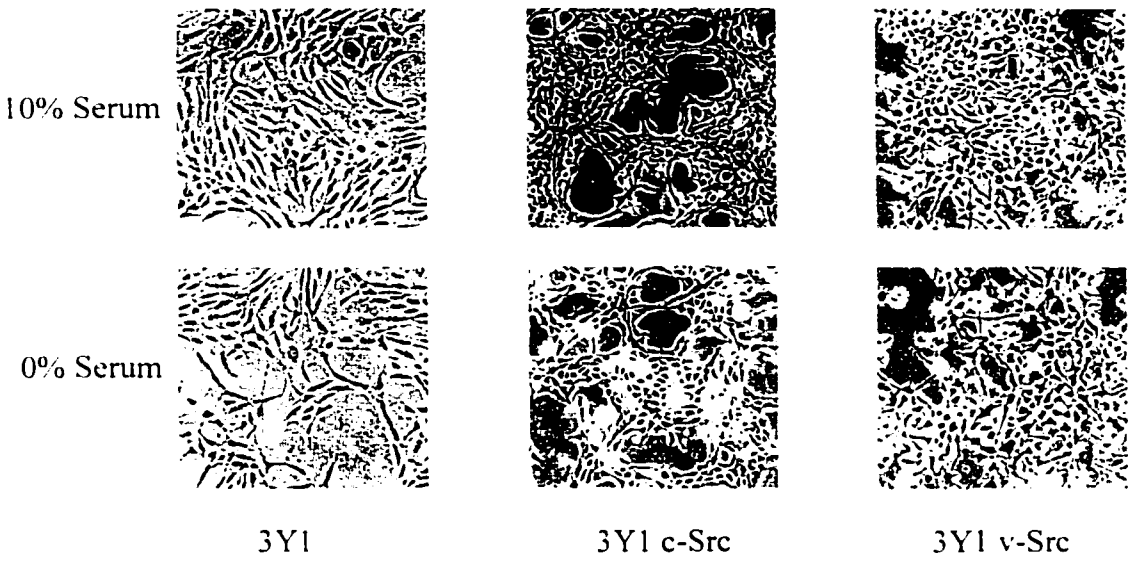
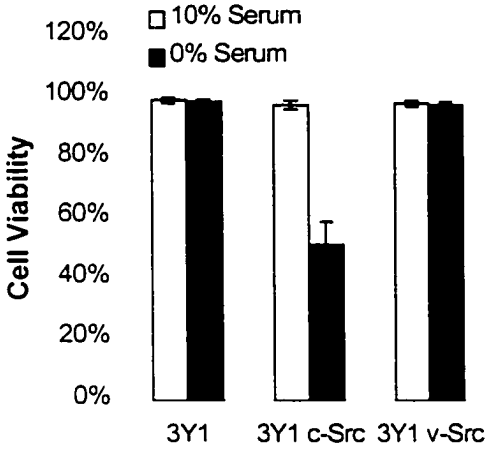


Fig 4.1(A) Serum starvation triggers cell death in $3Y1^{c\text{-}Src}$, but not in parental or v-Src-transformed 3Y1 cells. Parental, $3Y1^{c\text{-}Src}$, and v-Src-transformed 3Y1 cells were maintained in DMEM supplemented with 10% bovine calf serum or in DMEM lacking serum for 14 h. At this point cells were observed microscopically (A).



(B) Cell viability was then quantified by trypan blue exclusion. 400 cells from the respective cell cultures were counted. The data represent the % of cells that excluded trypan blue. Error bars represent the standard error for three independent experiments.

Cell death in 3Y1$^{c\text{-}Src}$ cells is due to apoptosis

Cell death in response to serum deprivation is commonly due to apoptosis (Evan and Littlewood, 1998). We therefore examined whether the 3Y1$^{c\text{-}Src}$ cells displayed characteristic markers for apoptosis upon serum withdrawal. As shown in Figure 4.2A, serum withdrawal resulted in DNA laddering in the 3Y1$^{c\text{-}Src}$, but not the parental or v-Src-transformed 3Y1 cells. Proteolytic cleavage of poly-(ADP-ribose) polymerase (PARP) and PKC δ has also been used to indicate apoptosis (Pongracz *et al.*, 1999; Schuler *et al.*2000). Upon serum withdrawal proteolytic breakdown of both PARP and PKC δ (Figure 4.2B) was observed in the 3Y1$^{c\text{-}Src}$, but not the parental or v-Src-transformed 3Y1 cells. DAPI nucleic acid staining, which highlights nuclear fragmentation and condensation (Finucane *et al.*, 1999), revealed apoptotic nuclei in the 3Y1$^{c\text{-}Src}$ cells deprived of serum (Figure 4.2C). Thus, serum deprivation results in the appearance of several apoptotic phenotypes in 3Y1$^{c\text{-}Src}$ cells where cell death was observed. These data indicate that the cell death induced in 3Y1$^{c\text{-}Src}$ cells by serum deprivation was due to apoptosis.

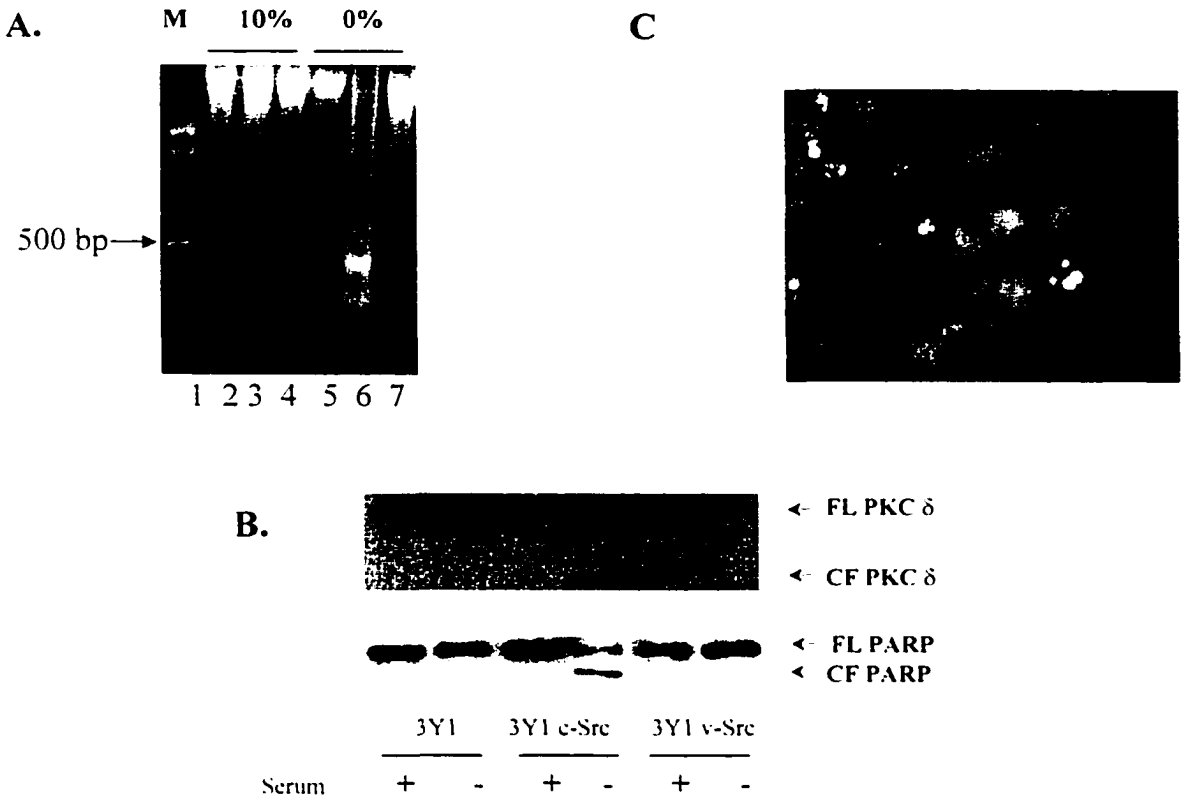


Figure 4.2 The cell death induced by c-Src upon serum starvation is apoptosis. A. DNA fragmentation: cells were placed in DMEM containing 10% bovine calf serum or in DMEM lacking serum for 14 h. At this point, DNA was isolated and subjected to 2% agarose gel electrophoresis. The DNA was visualized by ethidium bromide staining. Lane 1 contains marker (M) DNA. Lanes 2 and 5 are 3Y1 cells; lanes 3 and 6 are $3Y1^{c\text{-}Src}$ cells; lanes 4 and 7 are v-Src-transformed 3Y1 cells. Lanes 5, 6, and 7 were serum starved for 14 h. (B) Cleavage of death substrates PARP and PKC δ was monitored by Western blot analysis. FL: Full length. CF: catalytic fragment. (C) DAPI staining: the arrows indicate the typical morphology of apoptotic nuclei, which were only observed in $3Y1^{c\text{-}Src}$ cells upon serum starvation.

Serum deprivation leads to cytochrome C release from mitochondria in $3Y1^{c\text{-}Src}$ cells

Two apoptotic pathways have been described. These two pathways have been characterized as death by design and death by neglect (Green, 1998; Strasser *et al.*, 2000). Cyochrome C release is always seen in the neglect pathway and frequently in the design pathway (Green, 1998). Cytochrome C initiates the activation of proteases that begin the apoptotic process (Green, 1998). We therefore examined the presence of cytochrome C in cytosolic and mitochondrial fractions from the $3Y1^{c\text{-}Src}$ cells before and after serum deprivation. As shown in Figure 4.3, upon serum withdrawal, there was a large increase in cytochrome C in the cytosolic fraction and a concomitant reduction of cytochrome C in the mitochondrial fraction. These data further indicate that the cell death in the $3Y1^{c\text{-}Src}$ cells is apoptotic and that the apoptosis is mediated by cytochrome C release.

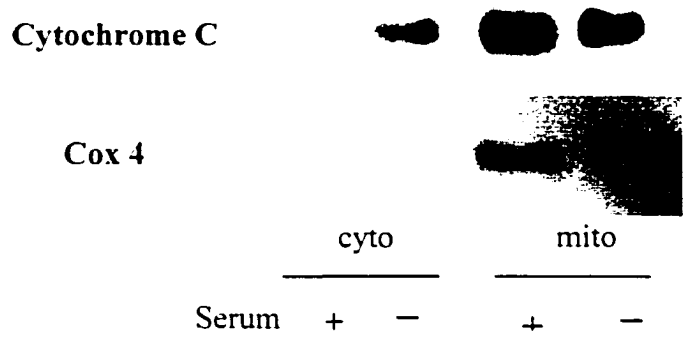
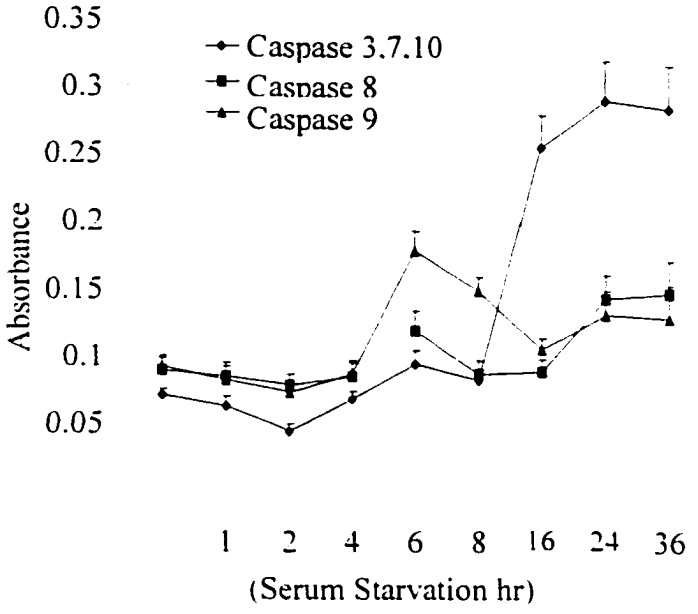


Figure 4.3 Cytochrome C released from mitochondria in 3Y1 cells overexpressing c-Src upon serum starvation 3Y1 cells overexpressing c-Src were placed in the medium either containing (+) or lacking (-) serum for 14 h. The cytosolic (cyto) and mitochondrial (mito) fractions were isolated using ApoAlert Cell Fractionation Kit (CloneTech). The subcellular distribution of cytochrome C (cyto c) was determined by Western blot analysis on cytosolic and mitochondrial fractions. Cytochrome C oxidase subunit IV (Cox 4), a mitochondrial protein that is not released to the cytosol during apoptosis, was also monitored by Western blot analysis to confirm the mitochondrial fractionation procedure.

Caspase activation in response to serum deprivation in 3Y1$^{c\text{-}Src}$ cells

Apoptosis begins with the activation of initiator caspases - most notably caspases 8 and 9. These caspases then proteolytically activate executioner caspases such as caspase 3 (Budihardjo *et al.*, 1999; Strasser *et al.*, 2000). We examined the activity of caspases 3, 8 and 9 in 3Y1$^{c\text{-}Src}$ cells upon serum withdrawal. As shown in Figure 4A, a large increase in caspase 3 activity can be detected between 10 and 16 hr. This was preceded by an increase in caspase 9 activity. A small increase in caspase 8 activity was detected, but it was after the increase in caspase 3 (Figure 4.4A). Consistent with these data, inhibition of either caspase 3 or 9, but not caspase 8, prevented cell death caused by serum deprivation in the 3Y1$^{c\text{-}Src}$ cells (Figure 4.4B). These data suggest that the apoptosis induced in 3Y1$^{c\text{-}Src}$ cells by serum withdrawal is initiated by caspase 9 and executed by caspase 3.

A



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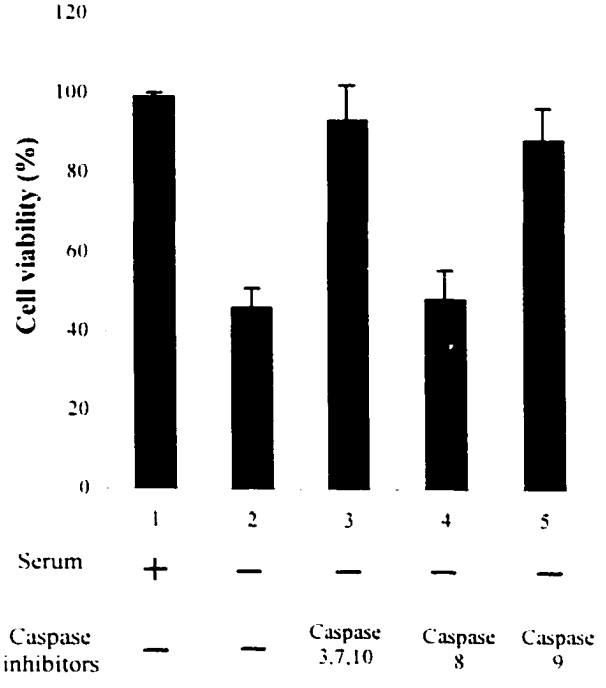

Figure 4.4 Caspase activity in $3Y1^{c\text{-}Src}$ cells subjected to serum withdrawal. (A) $3Y1^{c\text{-}Src}$ cells were placed in medium lacking serum for the times indicated. Both adherent and floating cells were then collected and subjected to measurement of caspase activity as described in Materials and Methods. (B) 3Y1 cells overexpressing c-Src were placed in DMEM containing 10% serum (+) or lacking serum (-) and treated with specific caspase inhibitors for 14 h. At this time point, cell death was quantified as in Figure 4.1.

Downregulating PKC δ provides a survival signal that overcomes apoptosis in $3Y1^{c\text{-}Src}$ cells subjected to serum withdrawal

We demonstrated previously that downregulation of PKC δ in response to TPA (12-O-tetradecanoylphorbol-13-acetate) transformed the $3Y1^{c\text{-}Src}$ cells (Lu *et al.*, 1997). We therefore examined the effect of PKC δ downregulation on cell viability in the $3Y1^{c\text{-}Src}$ cells subjected to serum withdrawal. As shown in Figure 4.3, TPA increased cell viability in the $3Y1^{c\text{-}Src}$ cells subjected to serum withdrawal. Consistent with an involvement of PKC δ, rottlerin, which functions as a specific inhibitor of PKC δ (Lu *et al.*, 1998; Hornia *et al.*, 1999) also increased cell viability in serum starved $3Y1^{c\text{-}Src}$ cells. In contrast, $G_0$6976, which functions as a specific inhibitor of PKC α (Hornia *et al.*, 1999), had no effect upon cell viability (Fig.4.3).

We next examined the effect of PKC δ downregulation upon apoptotic phenotypes induced by serum withdrawal in the $3Y1^{c\text{-}Src}$ and v-Src-transformed cells. As shown in Figure 4.4A, withdrawal of serum from the $3Y1^{c\text{-}Src}$ cells resulted in the characteristic 45 kDa PKC δ fragment sometimes referred to as PKM (protein kinase M)

(Cressman *et al.*, 1995). As expected, TPA treatment led to the disappearance of PKC δ as described previously (Lu *et al.*, 1997; 1998). However, treatment with the PKC δ inhibitor rottlerin inhibited the cleavage of PKC δ to PKM in the $3Y1^{c\text{-}Src}$ cells subjected to serum withdrawal (Figure 4.4A). Both TPA and rottlerin also inhibited PARP cleavage induced by serum withdrawal in the $3Y1^{c\text{-}Src}$ cells (Figure 4.4A). The PKC α inhibitor $G_0$6976 had no effect upon the cleavage of either PKC δ or PARP. Cytochrome C release from the mitochondria stimulated by serum withdrawal in the $3Y1^{c\text{-}Src}$ cells was also inhibited by TPA and rottlerin, but not by $G_0$6976 (Figure 4.4B). Similarly, TPA treatment prevented the increase in the activity of the executioner caspase 3, 7 and 10 seen in the $3Y1^{c\text{-}Src}$ cells (Figure 4.4C). The data shown in Figures 3 and 4 indicate that downregulating PKC δ provides a survival signal that prevents apoptosis in $3Y1^{c\text{-}Src}$ cells subjected to serum withdrawal.

The specificity of the PKC inhibitors used above has been questioned recently in a systematic study using a series of purified kinases and variety of kinase inhibitors (Davies *et al.*, 2000). However, we reported previously that activation of PKC was required for ubiquitination and degradation, and that PKC inhibitors prevented ubiquitination and degradation of PKC (Lu *et al.*, 1998). Importantly, $G_0$6976 prevented ubiquitination and degradation of PKC α, but not PKC δ. And reciprocally, rottlerin inhibited ubiquitination and degradation of PKC δ, but not PKC α. Moreover, since rottlerin was able to transform 3Y1 cells overexpressing either the EGF receptor or c-Src, it is likely the reported lack of specificity for rottlerin and $G_0$6976 on purified protein kinases *in vitro* (Davies et al., 2000) does not correspond with the *in vivo* specificity, where specificity of rottlerin and $G_0$6976 for PKC α and δ respectively seems to hold up.

To further establish that the survival signals reported here were in fact due to downregulating PKC δ, we examined the effect of a catalytically-inactive PKC δ (Hirai *et al.*, 1994), which we have used previously as a dominant-negative PKC δ mutant (Lu *et al.*, 1997; 1998; Hornia *et al.*, 1999). In Figure 4.5A, it is shown that transient transfection with the kinase-dead PKC δ inhibited the proteolytic degradation of both PKC δ and PARP in $3Y1^{c\text{-}Src}$ cells deprived of serum. Note also that as reported previously (Lu et al., 1998), the kinase dead PKC δ is not degraded in response to treatment with TPA (compare the third and fifth lanes). This observation also confirms expression of the kinase dead PKC δ mutant. The kinase dead PKC δ mutant also prevented the loss of cell viability induced in the $3Y1^{c\text{-}Src}$ cells by serum deprivation (Figure 4.5B). These data further establish that downregulation of PKC δ provides a survival signal that prevents apoptosis induced by serum deprivation in $3Y1^{c\text{-}Src}$ cells and also support the *in vivo* specificity of rottlerin for PKC δ.

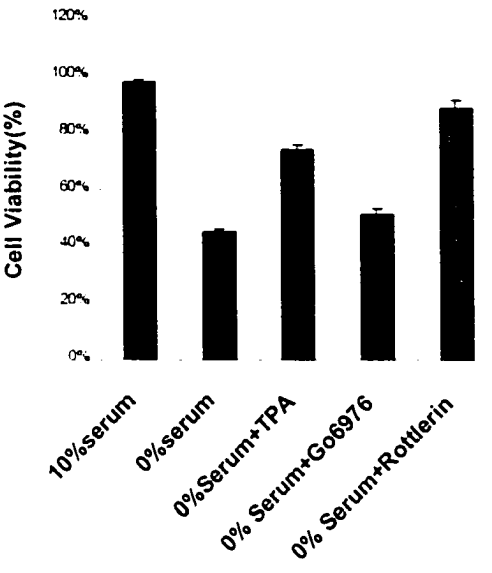

Figure 4.3 Downregulation of PKC δ increases cell viability in $3Y1^{c\text{-}Src}$ cells subjected to serum withdrawal. $3Y1^{c\text{-}Src}$ cells were maintained in DMEM supplemented with 10% bovine calf serum or in DMEM lacking serum for 14 h as shown. TPA (400 nM), rottlerin (15 μM) and G_06976 (0.5 μM) were included as indicated and were added at the same time as serum withdrawal. Cell viability was determined by trypan blue exclusion as in Figure 1. The data represent the % of cells that excluded trypan blue. Error bars represent the standard error for three independent experiments.

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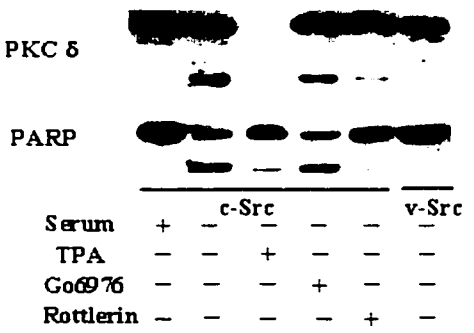

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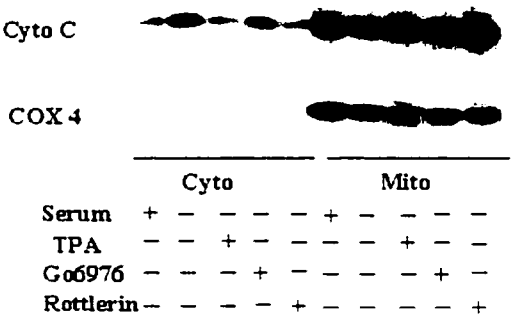

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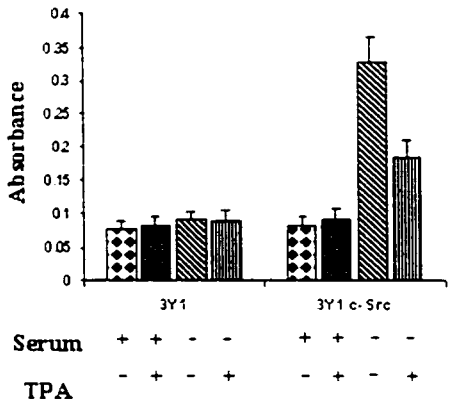

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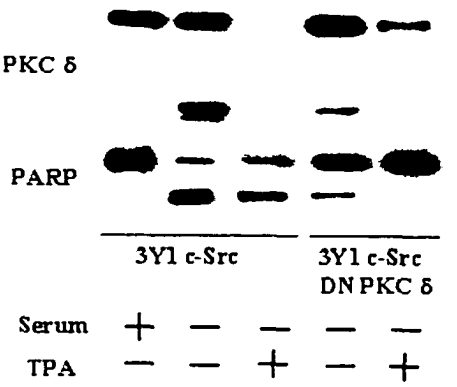

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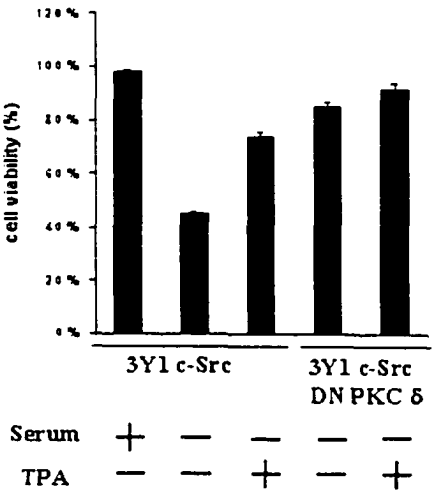


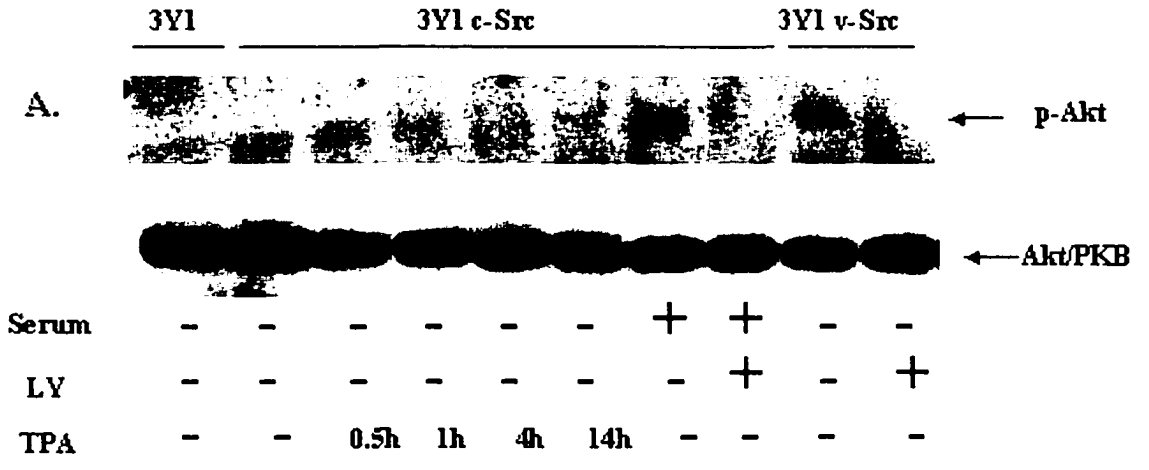
Figure 4.4 Downregulation of PKC δ inhibits apoptotic phenotypes in $3Y1^{c\text{-}Src}$ cells subjected to serum withdrawal. (A) $3Y1^{c\text{-}Src}$ cells and v-Src-transformed cells were subjected to serum withdrawal as indicated and Western blot analysis of PKC δ and PARP was performed. TPA (400nM), $G_0$6976 (0.5 μM), or rottlerin (15 μM) were included as shown at the same time as serum withdrawal. (B) Cytochrome C release from the mitochondria was assayed as in Figure 2 in the presence and absence TPA, rottlerin, $G_0$6976, and serum as shown. The time course was the sama as in (A). (C) The effect of TPA upon the activity of caspases 3, 7, & 10 in 3Y1 and $3Y1^{c\text{-}Src}$ cells was determined as in Figure 4.3. Error bars in (C) represent the standard error for three independent experiments. The data presented in (A) and (B) are representative of experiments repeated three times.

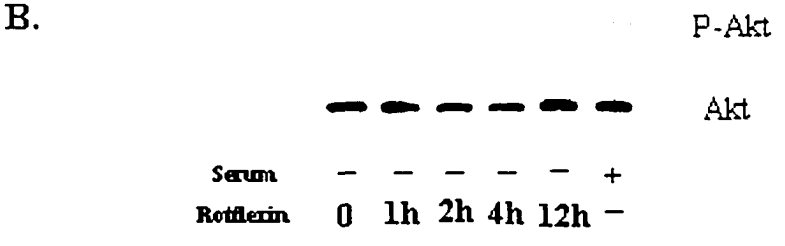
Figure 4.5 A catalytically-inactive mutant of PKC δ inhibits apoptotic phenotypes in $3Y1^{c\text{-}Src}$ cells subjected to serum withdrawal. (A) $3Y1^{c\text{-}Src}$ cells and $3Y1^{c\text{-}Src}$ cells transiently transfected with catalytically inactive PKC δ were subjected to serum withdrawal as indicated and Western blot analysis of PKC δ and PARP was performed as in Figure 4. Cells were split 24 hr after transfection and serum was withdrawn 24 hr later. TPA (400nM) was included as shown at the same time as serum withdrawal. The inability of TPA to lead to the downregulation of PKC δ (lane 5) serves as a positive control for the expression of catalytically-inactive PKC δ which is not downregulated like the wild type protein (Lu et al., 1998). The control $3Y1^{c\text{-}Src}$ cells were subjected to mock transfection with empty vector DNA. (B) The effect of the catalytically-inactive PKC δ and TPA upon cell viability was determined as in Figure 4. 3.

Survival signals provided by TPA are independent of the PI3K/Akt pathway

As described above, the v-Src-transformed cells did not undergo apoptosis upon serum withdrawal. This is consistent with reports that cells overexpressing v-Src only undergo apoptosis when part of the signal(s) induced by v-Src are blocked (Johnson *et al.*, 2000; Webb *et al.*, 2000). In both of these reports, inhibition of PI3K led to apoptosis in v-Src-transformed cells. PI3K mediated survival signals are mediated by the recruitment of Akt, which gets phosphorylated in response to activation (Kandel and Hay, 1999). We therefore examined whether Akt is phosphorylated in response to the survival signals generated in response to PKC δ downregulation. As shown in Figure 4.6A, TPA treatment did not result in increased Akt phosphorylation. In fact, TPA actually inhibited background Akt phosphorylation in the $3Y1^{c\text{-}Src}$ cells. Akt phosphorylation could be detected in v-Src-transformed 3Y1 cells and be induced in $3Y1^{c\text{-}Src}$ cells by a 30 min treatment with serum (Figure 4.6A), indicating that the pathway was viable in the $3Y1^{c\text{-}Src}$ cells. The elevated Akt phosphorylation observed in response to serum and in v-Src-transformed cells was prevented by the PI3K inhibitor LY-294002, indicating that Akt phosphorylation was dependent upon PI3K. We also examined the effect of rottlerin upon Akt phosphorylation and, as shown in Figure 4.6B, rottlerin did not stimulate Akt phosphorylation. We next examined the effect of LY294002 upon the ability of TPA and rottlerin to prevent PKC δ and PARP cleavage. As shown in Figure 4.6C, LY294002, did not significantly affect the ability of TPA to inhibit PARP cleavage and rottlerin to prevent either PKC δ or PARP cleavage. Lastly, we examined the effect of TPA and rottlerin on cell viability in serum starved $3Y1^{c\text{-}Src}$ cells in the presence of LY-294002. While LY-294002 reduced cell viability in serum-starved $3Y1^{c\text{-}Src}$ cells, TPA increased

cell survival in the absence or presence of the PI3K inhibitor to approximately the same extent. The effect of rottlerin was even more pronounced, further establishing that the effect of downregulating PKC δ goes above and beyond any effects of PI3K. These data indicate that the survival signal generated by PKC δ downregulation in the $3Y1^{c\text{-}Src}$ cells is independent of the PI3K/Akt survival pathway.





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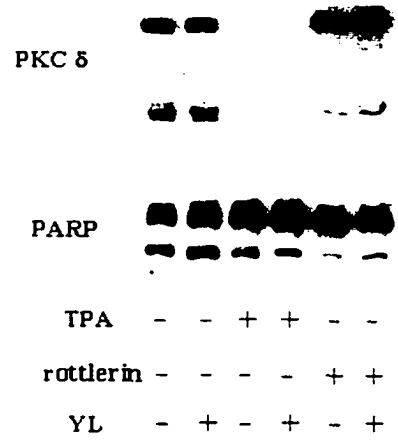

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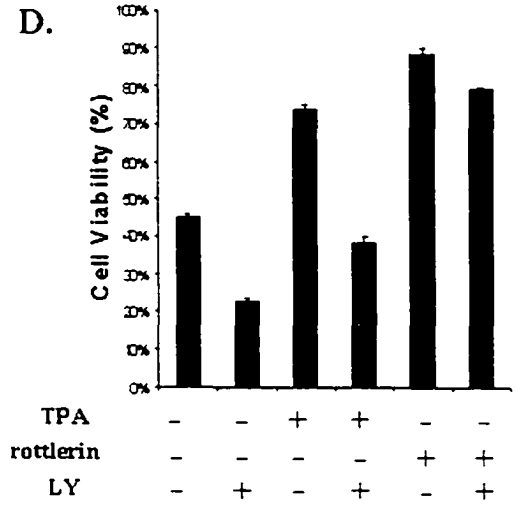


Figure 4.6 Survival signal generated by downregulating PKC δ is independent of the PI3K/Akt pathway. (A) 3Y1, $3Y1^{c\text{-}Src}$ and v-Src-transformed cells were all subjected to serum withdrawal for 14 h. TPA treatment (400 nM) for the times indicated was prior to harvest at 14 hr after serum withdrawal. The PI3K inhibitor LY-294002 (LY) (50 μM) was added to the $3Y1^{c\text{-}Src}$ cells along with serum for 30 min as shown. LY-294002 (50 μM) was added to the v-Src-transformed cells for 30 min. The cells were then subjected to Western blot analysis using antibodies raised against Akt (lower panel) and phosphorylated Akt (upper panel). The experiment presented is a representative that was performed twice. (B) The effect of rottlerin (15 μM) on Akt phosphorylation was examined as in (A). (C) The effect of LY294002 upon the inhibition of PKC δ (upper panel) and PARP (lower panel) cleavage by TPA (400 nM) and rottlerin (15 μM) was examined as in Figure 4 and 5. (D) $3Y1^{c\text{-}Src}$ cells were subjected to serum withdrawal for 14 h in the absence of LY-294002 (50 μM), TPA (400 nM), and rottlerin (15 μM) as shown. Cell viability was determined by trypan blue exclusion as in Figure 1. The data represent the % of cells that excluded trypan blue. Error bars represent the standard error for three samples from a single experiment that was repeated twice.

Discussion

c-Src overexpression cooperates with tumor promoting phorbol esters (Lu *et al.*, 1997) to transform rat fibroblasts. Thus, c-Src overexpression provides a growth signal that is not sufficient to give a fully transformed phenotype. Emerging models for protection against cancer suggest that mitogenic signals also induce apoptotic signals that must be overcome by survival signals in order for cell proliferation to proceed (Hueber and Evan, 1998). Data presented here indicate that c-Src overexpression sensitizes 3Y1 rat fibroblasts to apoptotic stress and that tumor-promoting phorbol esters provide a survival signal that overrides apoptotic signals generated by c-Src overexpression. Interestingly, v-Src did not sensitize cells to apoptotic stress, indicating that v-Src is able to provide both mitogenic and survival signals.

It was reported recently that inhibition of both PI3K and Ras resulted in apoptosis in v-Src-transformed rat fibroblasts in the presence of serum (Webb *et al.*, 2000). In the absence of serum, inhibition of PI3K alone was sufficient to cause apoptosis in v-Src-transformed rat fibroblasts (Johnson *et al.*, 2000). Similarly, in the absence of IL-3, inhibiting Ras signaling induced apoptosis in IL-3-dependent Ba/F3 cells expressing v-Src (Odajima *et al.*, 2000). Collectively, these data indicate that v-Src activates survival signals mediated by Ras and PI3K. In the absence of survival signals provided by serum and growth factors, inhibition of either the PI3K or Ras alone leads to apoptosis. The observation that cells overexpressing c-Src undergo apoptosis in the absence of serum suggests that they are missing the survival signals activated by v-Src. However,

downregulation of PKC δ by either prolonged TPA treatment or inhibition of PKC δ by rottlerin was able to activate survival signal(s) sufficient to overcome the apoptotic signals primed by c-Src overexpression.

We demonstrated previously that downregulating PKC δ transformed cells overexpressing either c-Src or the EGF receptor (Lu *et al.*, 1997; Hornia *et al.*, 1999). In cells transformed by v-Src, PKC δ is phosphorylated on tyrosine, which results in reduced levels of PKC δ (Zang *et al.*,1997; Blake *et al.*, 1999). Overexpression of c-Src did not cause any tyrosine phosphorylation of PKC δ (Zang *et al.*, 1997). Thus, the downregulation of PKC δ by v-Src may contribute to the survival of v-Src-transformed cells. Consistent with this notion, the level of PKC δ in the v-Src-transformed cells seen in Figure 1C was substantially reduced relative to that seen in the $3Y1^{c\text{-}Src}$ cells. The emerging paradigm suggests that PKC δ may be part of a priming mechanism for apoptotic signaling. In this regard, it is of interest that in response to apoptotic signaling, PKC δ is proteolytically cleaved to release the catalytic domain of PKC δ. Release of the active catalytic fragment of PKC δ may be critical for apoptosis to proceed and therefore downregulating PKC δ could be critical for mitogenic signals to be complete.

A pathway for survival signals stimulated by TPA and PKC δ downregulation is not yet clear. However since TPA actually inhibited basal Akt phosphorylation, and the PI3K inhibitor LY-294002 had no effect upon the ability of TPA or rottlerin to induce apoptosis, the mechanism does not likely involve the well-established PI3K/Akt survival pathway. Therefore, it is likely that downregulation of PKC δ represents a novel survival pathway that is able to overcome the apoptotic phenotype generated in cells with an overexpressed tyrosine kinase.

CHAPTER V

Phospholipase D Generates either Survival or Apoptotic Signals Depending on Cellular Context

INTRODUCTION

Overexpression of a tyrosine kinase is a common genetic defect in many human cancers (Biscardi et al., 1998). However, elevated expression of a tyrosine kinase is not sufficient to transform cells. We reported previously that in 3Y1 rat fibroblasts overexpressing c-Src ($3Y1^{c\text{-}Src}$ cells) could be transformed by the downreglation of protein kinase C δ (PKC δ) (Lu et al., 1997). Moreover, overexpression of c-Src rendered 3Y1 cells sensitive to the apoptotic stimulus of serum withdrawal (Zhong et al., 2002). Consistent with a tumor promoting effect of PKC δ downregulation, inhibiting PKC δ provided a survival signal that overcame the apoptotic effect of serum withdrawal (Zhong et al., 2002). We also reported that in cells overexpressing either c-Src or the EGF receptor, that inhibiting PKC δ led to an increase in phospholipase D (PLD) activity (Hornia et al., 1999). In addition, tamoxifen, which reduces PKC δ levels, elevated PLD activity (Zhong et al., 2001). Elevated expression of either PLD1 or PLD2 could transform cells overexpressing either c-Src or the EGF receptor (Lu et al., 2000; Joseph et al., 2001). These studies revealed an inverse relationship on cell proliferation and survival between PKC δ and PLD, whereby PKC δ suppresses and PLD can stimulate proliferation in a cellular context where there is elevated expression of a tyrosine kinase. In this regard it is significant that 3Y1 cells transformed by v-Src are not sensitive to the apoptotic stimulus of serum withdrawal (Zhong et al., 2002). v-Src, with an activated kinase, stimulates PLD activity, whereas c-Src and its less active kinase does not. This observation suggested that PLD activity, like PKC δ downregulation might also provide a survival signal that overcomes apoptotic signals. In this report, we describe the effect of PLD activity on cell survival in different cellular contexts. We present evidence

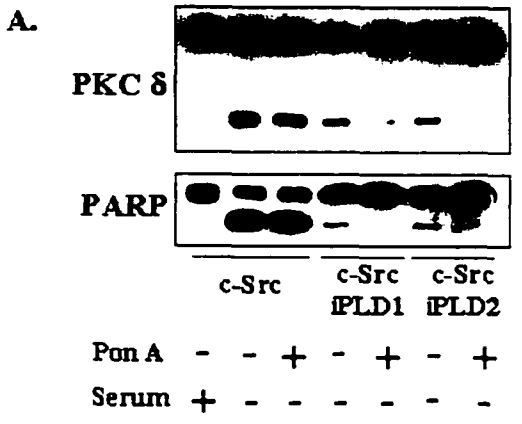
indicating different effects of PLD on cell survival in parental and transformed 3Y1 rat fibroblasts.

RESULTS

Overexpression of either PLD1 or PLD2 provides a survival signal in 3Y1$^{c\text{-}Src}$ cells subjected to serum withdrawal. We reported previously that 3Y1$^{c\text{-}Src}$ cells underwent apoptosis when subjected to serum withdrawal (Zhong et al., 2002). This could be overcome by downregulation of PKC δ. Downregulating PKC δ in the 3Y1$^{c\text{-}Src}$ cells results in an increase in PLD activity (Hornia et al., 1999). We therefore wanted to determine whether elevated PLD activity could provide a survival signal that could overcome the apoptosis induced by serum withdrawal in the 3Y1$^{c\text{-}Src}$ cells. To address this question we utilized cells that had been stably transfected with vectors that conditionally express either PLD1 or PLD2 (Joseph et al., 2001). These cells express elevated levels of PLD1 or PLD2 and upon induction with ponasterone A (PonA) PLD levels are elevated further (Joseph et al., 2002). As shown in Fig. 5.1A, there is substantial cleavage of PARP, a commonly used marker for apoptosis in the 3Y1$^{c\text{-}Src}$ cells subjected to serum withdrawal. Treatment of these cells had no effect upon PARP cleavage in these cells. In the 3Y1$^{c\text{-}Src}$ cells with the PLD1 or PLD2 vectors, PARP cleavage was substantially reduced and treatment with PonA led to a further reduction in PARP cleavage. These data indicate that elevated expression of either PLD1 or PLD2 can prevent PARP cleavage.

We next examined what effect PLD1 and PLD2 expression had upon cell viability. As shown in Fig. 5.1B, cell viability was reduced to about 50% when the 3Y1$^{c\text{-}Src}$ cells were subjected to serum withdrawal. The addition of PonA had no effect on the cell viability of the 3Y1$^{c\text{-}Src}$ cells subjected to serum withdrawal. In the cells expressing PLD1 or PLD2 however, viability was substantially increased and the addition of PonA

brought viability close to 100%. These data are consistent with the PARP cleavage data and indicate that elevated expression of either PLD1 or PLD2 provides a survival signal that overcomes the apoptotic effects of serum withdrawal on the $3Y1^{c\text{-}Src}$ cells.



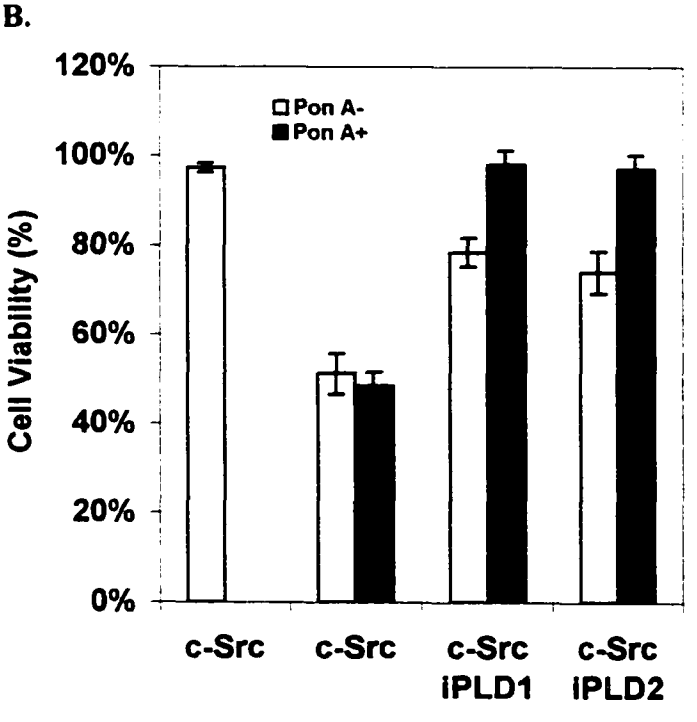
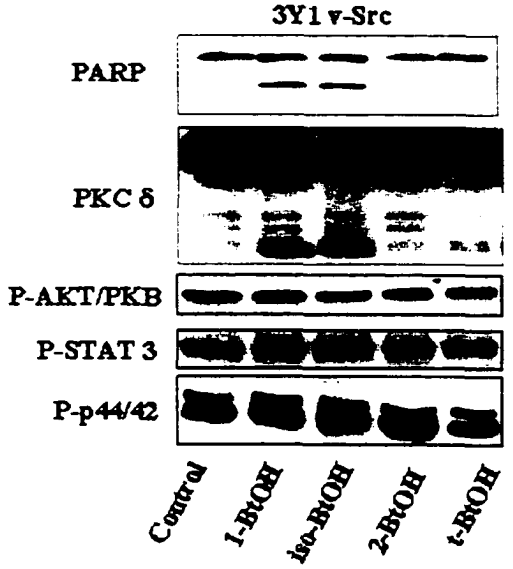


Fig. 5.1. Overexpression of either PLD1 or PLD2 provides a survival signal in $3Y1^{c\text{-}Src}$ cells subjected to serum withdrawal. $3Y1^{c\text{-}Src}$ cells and $3Y1^{c\text{-}Src}$ cells containing inducible expression vectors for either PLD1 or PLD2 were maintained in DMEM supplemented with either 10% or 0.5% bovine calf serum serum for 14 h as indicated. The inducer of PLD expression PonA was provided (10 μM) as shown 20 hr prior to serum withdrawal. After 14h in the absence of serum, cells were harvested and proteolytic cleavage of death substrates PARP and PKC δ was monitored by Western blot analysis (A), and cell viability was quantified by trypan blue exclusion (B). The data represent the % of cells that excluded trypan blue. Error bars represent the standard error for three independent experiments.

Blocking PLD induced increases in PA induces apoptosis in $3Y1^{v\text{-}Src}$ cells. In $3Y1^{c\text{-}Src}$ cells, which express elevated levels of c-Src, there is no significant increase in PLD activity relative to the parental cells (Lu et al., 1997). In contrast, the activated Src protein v-Src stimulates an increase in PLD activity (Song et al., 1991; Wyke et al., 1992; Jiang et al., 1995). Since the v-Src-transformed 3Y1 cells do not undergo apoptosis upon serum withdrawal (Zhong et al., 2002), it is possible that the elevated PLD activity in these cells is providing a survival that allows them to overcome serum withdrawal. If this is true, then inhibiting PLD activity should render the v-Src-transformed cells sensitive to serum withdrawal. To test this hypothesis, we used the "alcohol trap" assay (Chalifa-Caspi et al., 1998), which prevents the production of PA because of the preferential utilization of primary alcohols over H_2O in the hydrolysis of phosphatidylcholine. This assay has become widely used to demonstrate PLD activity and to indicate a PLD requirement by blocking the production of PA, the product of PLD. As negative controls, secondary and tertiary alcohols can be used because they have a bulky structure that prevents them from being utilized in the reaction. In Fig. 5.2A, it shown that both PARP and PKC δ cleavage is induced in the v-Src-transformed cells subjected to serum withdrawal by the primary alcohols 1-BtOH and iso-BtOH. In contrast the secondary (2-BtOH) and tertiary (t-BtOH) alcohols did not have this effect. Similarly, the primary, but not the seconday or tertiary alcohols reduced cell viability. These data further support the hypothesis that PLD provides a survival signal necessary for v-Src-transformed cells to withstand serum withdrawal.

A.



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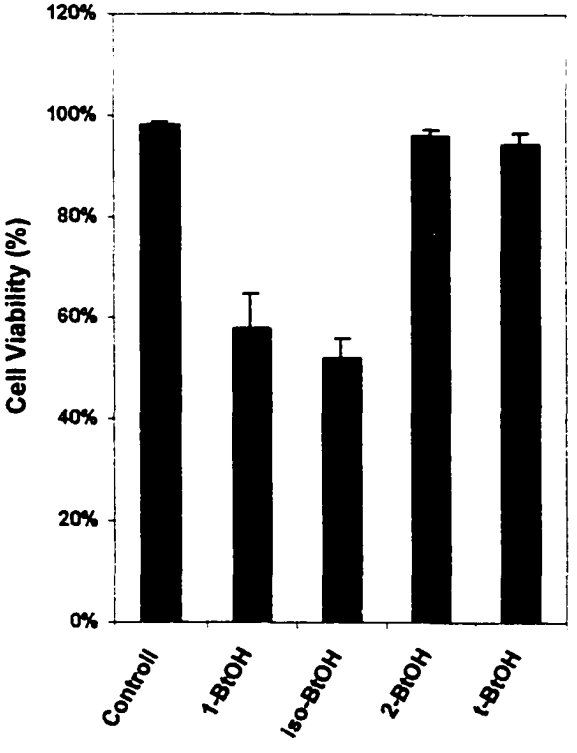
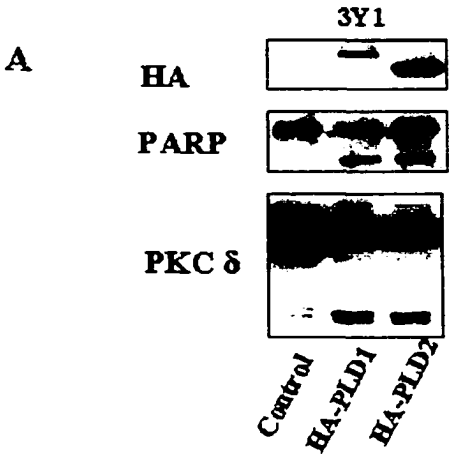


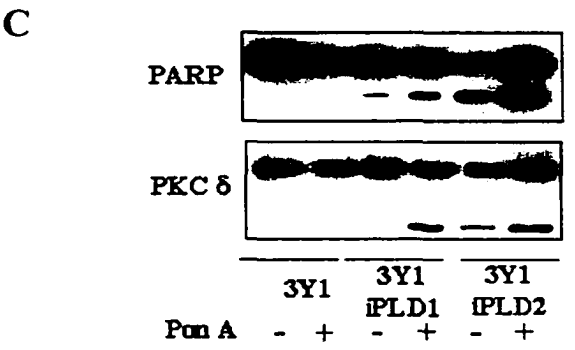
Fig. 5.2. Blocking PLD induced increases in PA induces apoptosis in $3Y1^{v\text{-}Src}$ cells. v-Src-transformed 3Y1 cells were placed in DMEM with 0.5% serum for 14 h in the absence and the presence of the indicated alcohols (1%). At this point, PARP and PKC δ cleavage (A) and cell viability was examined as in Fig. 5.1.

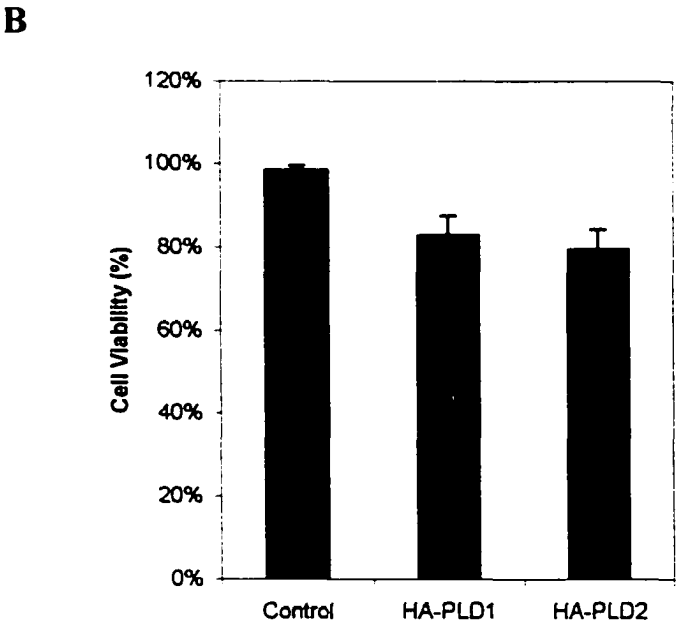
Elevated expression of either PLD1 or PLD2 induces apoptosis in 3Y1 cells. We reported previously that while $3Y1^{c\text{-}Src}$ cells transfected with a PLD expression vectors become transformed, the parental 3Y1 cells, similarly transfected with PLD expression vectors do not survive (Lu et al., 2000; Joseph et al., 2001). This suggested the possibility that elevated expression in the parental cells caused cell cycle arrest or apoptosis. To test this hypothesis, we transiently tranfected vectors expressing flu-tagged PLD1 or PLD2 into the 3Y1 cells. As shown in Fig. 5.3A, transient transfection resulted in the expression of flu-tagged PLD1 and PLD2 proteins. Also shown in Fig. 5.3A is the appearance of the cleavage products for PARP and PKC δ. In Fig. 5.1B, it is shown that cell viability was reduced when PLD1 and PLD2 was expressed. The relatively small reduction in cell viability is likely due to the efficiency of transfection. The 75 – 80% viability would be consistent with about a 50% transfection efficiency if we were to achieve the 50% viability levels seen in the $3Y1^{c\text{-}Src}$ cells subjected to serum withdrawal or the v-Src-transformed cells treated with primary alcohols in the absence of serum.

To further establish this effect, we generated 3Y1 cell lines with the inducible PLD1 and PLD2 expression vectors characterized previously (Joseph t al. 2001) and utilized above (Fig. 5.1). The ability to increase both PLD and PLD activity with PonA is shown in Figs.5. 3C and 5.3D respectively. As with other cell lines we have

developed with these vectors, the ability to induce PLD2 was better than we could obtain with PLD1. As shown in Fig. 5.3C, The addition of PonA had little or no effect upon PARP cleavage. In the cells with inducible PLD1, there was detectable PARP cleavage in the absence of PonA, consistent with the elevated PLD1 expression seen in Fig. 5.3A. The addition of PonA led to an increase in PARP cleavage. The induction of PLD2 led to a more pronounced increase in PARP cleavage (Fig. 5.3C), which is consistent with the greater increase in PLD2 protein and activity observed in Figs 5.3A and B. The increased PARP cleavage correlated well with reduced cell viability (Fig. 5.3D). These data further indicate that elevated PLD activity renders 3Y1 cells sensitive to the apoptotic stimulus of serum withdrawal.







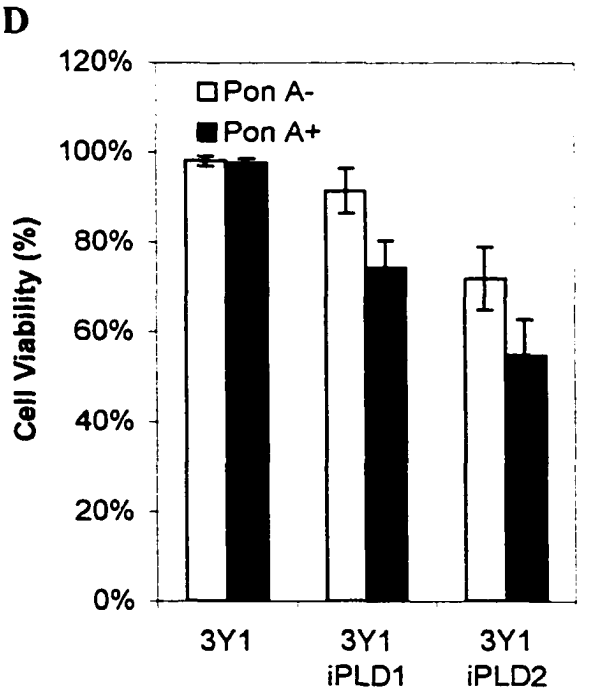


Fig. 5.3 Increased expression of PLD in 3Y1 cells induces apoptosis. Transient expression of either PLD1 or PLD2 induces apoptosis in 3Y1 cells. Flu-tagged PLD1 and PLD2 expression vectors were transfected into 3Y1 cells. After 24 h, the cells were placed in DMEM with 0.5% serum and the cells ware analyzed for PLD expression, PARP and PKC δ cleavage and cell viability by Western blot analysis (A). An antibody raised against the Flu epitope was used for PLD expression. (B) Cell viability was assayed 14h after placing in low serum as in Figs 5.1 and 5.2. Inducible expression vectors for PLD1 and PLD2 were tranfected into 3Y1 cells in the absence of the inducer PonA. Colonies were selected and amplified as described previously (Joseph et al., 2001). We then examined the effect of PonA upon PARP cleavage (C) and cell viability (D) 20h after treatment as in Fig 5.1.

DISCUSSION

In this report, we have provided evidence that PLD activity provides a survival signal in cells where there is elevated tyrosine kinase expression or activity. Elevated PLD activity overcame apoptotic signal generated by serum withdrawal in $3Y1^{c\text{-}Src}$ cells and inhibiting PLD. Interestingly, elevated expression of either PLD1 or PLD2 rendered the parental 3Y1 cells to the apoptotic stimulus of serum withdrawal, which may explain why it has been difficult to generate cell lines expressing elevated levels of PLD (Lu et al., 2000). The data presented here indicate that PLD provides a critical component for tyrosine kinase signaling and that when activated in the absence of tyrosine kinase signaling, PLD primes the cell for apoptosis.

The cooperation between c-Src expression and PLD resembles the model for cooperating oncogenes proposed by Weinberg and colleagues where the transformation of primary cells required two cooperating oncogenes such as Ras and Myc, or Ras and T-antigen (Land *et al.*1983; Dotto et al., 1985; Hahn *et al.*, 1999). Elevated expression of Myc, like PLD, in the absence of Ras signals resulted in apoptosis when cells were subjected to serum deprivation (Evan *et al.*, 1992). A model for the control of cell proliferation has emerged whereby mitogenic signals simultaneously activate both mitogenic and apoptotic pathways. For cell division to proceed, a survival signal(s) must also be generated that suppresses apoptotic signals (Hueber and Evan, 1998; Evan and Littlewood, 1998). v-Src can apparently activate both mitogenic and survival signals and the activation of PLD is apparently critical since inhibiting PLD activity renders these cells sensitive to the apoptotic signals generated by serum withdrawal. However, additional survival signals are likely generated since v-Src-transformed cells can be

primed for apoptosis by inhibition of Ras or phosphatidylinositol-3-kinase (Johnson *et al.*, 2000; Hakak *et al.*, 2000; Webb *et al.*, 2000; Odajima et al., 2000).

It is not clear how PLD activity and the generation of PA provides survival signals. PA has been reported to be critical for the activity of phosphatidyinoditol-4-phosphate-5-kinase (Jenkins et al., 1994), which generates phosphatidylinositol-4,5-bis-phosphate, a critical co-factor for PLD and a substrate for phospholipase C. Raf has a PA binding site for PA (Ghosh et al., 1996; Rizzo et al., 1999) and it has been proposed that the generation of PA facilitates the recruitment of Raf to appropriate sites on the plasma membrane. PA has been reported to be required fro activation of mTOR (Fang et al., 2001), the target of the immunosuppressant rapamycin and a regulator of S6 kinase, which regulates protein synthesis and is activated in response to mitogens. PLD activity has also been reported to mediate receptor endocytosis (Shen et al., 2001), and may enhance signaling through growth factor receptors by increasing internalization of receptors, which has been shown to be important for receptor signaling (Vieira et al., 1996; Kranenberg et al., 1999; Shen et al., 2001). Thus, while at present the exact mechanism for survival signaling through PLD is not known, there are several attractive candidates for this important property of PLD signaling.

CHAPTER VI

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