A Low Molecular Weight Factor from Dividing Cells Activates Phospholipase D in Caveolae-Related Membrane Microdomains

by

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Abstract

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Sergey Bychenok

Advisor: Dr. David A. Foster

Lipids play an important role as precursors for a number of mitogenic signaling molecules. Phosphatidylcholine (PC), a major phospholipid of cellular membranes, is a significant source for these molecules. The principal reaction of phosphatidylcholine hydrolysis is catalyzed by PC-specific phospholipase D (PLD). Phosphatidic acid, a primary metabolite of this reaction, has been shown to have mitogenic properties by itself as well as serving as a precursor for other lipid second messengers.

PLD activity is elevated in cells transformed by a number of oncogenes including v-Ras. It is believed that increase in the PLD activity contributes directly to acquisition of a transformed phenotype in these cells. Molecular mechanisms regulating PLD activity in v-Ras-transformed cells are not yet well understood. In this

report we present evidence that a small molecular weight cytosolic factor is responsible for increased PLD activity in v-Ras-transformed cells.

The large difference in PLD activity between the Ras-transformed and non-transformed parental cells disappeared in membranes isolated from these two cell lines. In reconstitution experiments, heat-denatured cytosolic fractions from Ras-transformed, but not from parental NIH 3T3 cells, elevated PLD activity in isolated membranes. This heat-resistant PLD-stimulating activity from the Ras-transformed cells was sensitive to proteases and passed through a 1 kDa MW cut-off membrane, suggesting that the factor is a peptide of less than 10 amino acids. The ability of this PLD-stimulating factor, designated PLD-SF, to elevate PLD activity in isolated membranes was restricted to the caveolae-enriched light membranes, where many signaling molecules, including Ras, are localized. PLD-SF was also elevated in v-Src- and v-Raf-transformed cells and in serum-stimulated NIH 3T3 cells. PLD-SF was detected in a variety of rat tissues, but was highest in testes, where a large percentage of cells are dividing. A similar low molecular weight PLD stimulating activity was found in actively dividing, but not stationary yeast cells. The data here provide evidence for a ubiquitous PLD-stimulating peptide that is elevated in dividing cells.

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Spasibo to all my Russian friends who loved me and who fed me all this years.

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List of Abbreviations

Arf - ADP ribosylation factor.

DAG - Diacylglycerol.

EGF - Epidermal growth factor.

FGF - Fibroblast growth factor.

GTγS - Guanosine 5'-O-(3-thiotriphosphate)

LPA - Lysophosphatidic acid.

PA - Phosphatidic acid.

PDGF - Platelet-derived growth factor.

PIP2 – Phosphatidylinositol 3,4-bisphosphate

PKC - Protein kinase C. (EC 2.7.1.)

PLA - Phospholipase A (EC 3.1.1.)

PLC - Phospholipase C (EC 3.1.4.3)

PLD - Phospholipase D (EC 3.1.4.4)

PLD-SF – Phospholipase D stimulating factor

PPro - Phosphatidylpropanol

TLC - Thin layer chromatography.

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Chapter I

Introduction

In recent years we came to understand that phospholipids not only play a role as structural components of cellular membranes, but are also important mediators of mitogenic signal transduction. Phosphatidylcholine is a major phospholipid of mammalian cell membranes. There are profound changes in the phosphatidylcholine turnover rate in membranes of cells transformed by activated Ras oncogene. Some of these changes are believed to contribute directly to acquisition of a transformed phenotype. Most notably there is an increase in activity of phosphatidylcholine-specific phospholipase D. There is a strong possibility that this activation of phospholipase D is an essential component of mitogenic signaling initiated by Ras oncogene since it is also activated by many mitogenic stimuli and growth factors. Therefore there is a great interest in understanding the molecular mechanisms responsible for phospholipase D activation in Ras-transformed cells.

1. Ras oncogene

Ras genes are among the most frequently activated oncogenes in human cancers. Initially they were identified as the transforming genes of the Harvey (Ha-Ras) and Kirsten (K-Ras) murine sarcoma viruses. (Harvey, 1964; Kirsten and Mayer,

1967). It was later shown that these genes were derived from the mouse genome and acquired their transforming potential due to a single base pair substitution (Tabin et al., 1982).

The proteins encoded by Ras protooncogenes are ubiquitously expressed in mammalian tissues and play a central role as mediators of mitogenic signaling for many growth factors. Ras proteins belong to a large family of small molecular weight monomeric GTPases. These proteins bind guanine nucleotides (GDP and GTP) with high affinity and possess intrinsic GTPase activity. The ability to alternate between two forms - GDP-bound and GTP-bound relates to their function as molecular switches in signal transduction. All small GTPases examined to date are active in their GTP-bound form and interact with a number of downstream effector molecules. The GDP-bound form is a resting state for small GTPases. The GDP/GTP exchange is tightly regulated by different external signals through a number of exchange factors (Malumbres and Pellicer, 1998). The return to an inactive GDP-bound form is also regulated by specialized molecules – GAPs (GTPase Activating Proteins) which stimulate the rate of GTP hydrolysis.

The oncogenic mutants of Ras proteins have impaired GTPase activity as well as resistance to activation by GAPs. Therefore these mutant proteins constitutively remain in the active GTP-bound form and produce mitogenic signals independently of cellular environment. The presence of such mutated alleles of Ras in up to 30 % of

human tumors (Bos, 1989) emphasizes the importance of Ras signaling for oncogenic transformation.

There are a number of proteins that have been identified as downstream effector molecules of Ras either by virtue of their interaction with GTP-bound form of Ras *in vivo* and *in vitro* or by their ability to be activated by growth factors in a Ras-dependent manner. Some of these proteins are also able to suppress Ras-induced transformation when overexpressed in their dominant-negative form. Most notably among these proteins are the serine/threonine kinase Raf (Vojitek et al., 1993; Stokoe et al., 1994), phosphatidylinositol 3-kinase (Rodriguez-Viciana et al., 1994), small GTPases of the Rho family, Rho, Rac and CDC42 (Qui et al., 1995; Joneson et al., 1996) and RalGDS, a guanine nucleotide exchange factor for Ral, which is a member of the Ras family of small GTPases (Spaargaren and Bischoff, 1994; Feig et al., 1996). It is still not clear which of these molecules are indispensable for Ras-induced transformation (Malumbres and Pellicer, 1998).

A great amount of information was collected supporting the hypothesis that activation of Ras leads to oncogenic transformation. Recently, however, this point of view came under scrutiny. It was demonstrated that in primary cell cultures activation of Ras leads to cellular senescence (Serrano et al., 1997). Activated Ras has also been found to be an effective promoter of apoptosis through the Raf pathway. (Kauffmann-Zeh et al., 1997). Therefore we need to continue investigation of the molecular

mechanisms of Ras-initiated signaling in order to understand how its activation leads to oncogenic transformation.

2. Changes in phosphatidylcholine metabolism in cells transformed by activated Ras

In animal tissues phosphatidylcholine is synthesized from CDP-choline and diacylglycerol in a reaction catalyzed by the enzyme phosphocholine transferase. CDP-choline is formed from free choline in two sequential enzymatic reactions catalyzed by choline kinase and phosphocholine citidyltransferase (Figure 1) (Lehninger, 1975).

The major metabolic pathway of phosphatidylcholine degradation is the hydrolysis by phospholipase D with the production of free choline and phosphatidic acid (Figure 2) (Exton, 1994). Phosphatidylcholine can also be hydrolized by phospholipase A_2 resulting in a free fatty acid (typically arachidonate) and lysophosphatidylcholine (Exton, 1994; Divecha and Irvine, 1995). The existence of a PC-specific phospholipase C activity have never been convincingly demonstrated (Carnero et al., 1994; Exton, 1997).

It has been shown that phospholipase D (PLD) activity and the product of its reaction phosphatidic acid (PA) are significantly elevated in cells transformed by an activated Ras oncogene (Teegarden et al., 1989; Carnero et al., 1994; Jiang et al., 1995; Martinet al., 1997). There is also a significant increase in activity of

Scheme of Phosphatidylcholine Biosynthesis

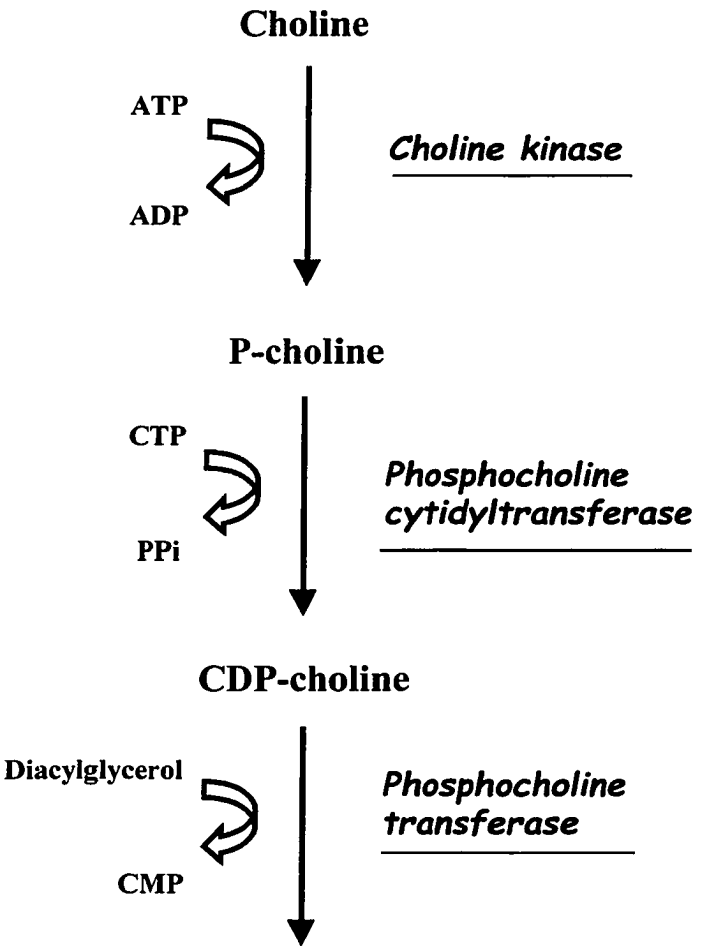

Figure 1. (Lehninger 1975)

Hydrolysis of Phosphatidylcholine by Phospholipases

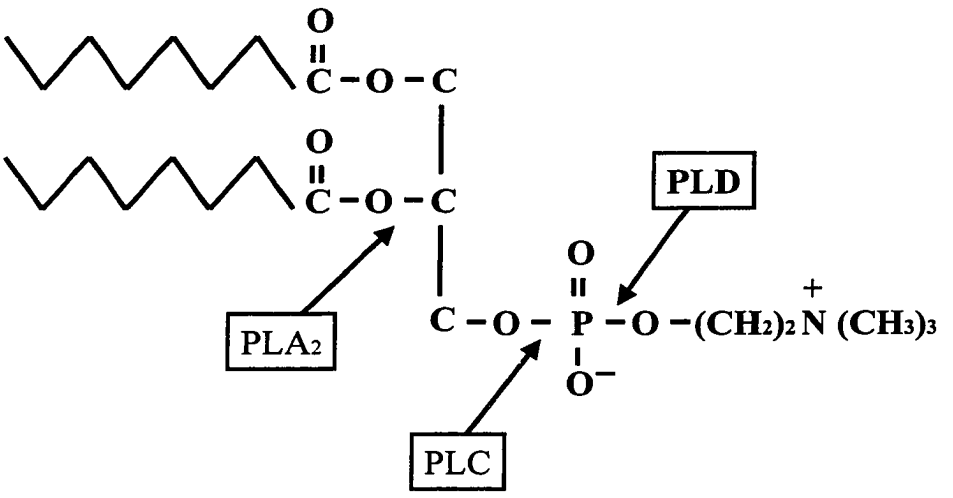

Figure 2.

choline kinase (Lacal, 1990; Ratnam and Kent, 1995), a rate limiting reaction of phosphatidylcholine biosynthesis. This increase is apparently a compensatory mechanism for elevated rate of phosphatidylcholine degradation in cells transformed by Ras oncogene.

3. Enzymology of phospholipase D

Phospholipase D activity has been detected in bacteria, fungi, plants and all mammalian tissues examined (Exton, 1997). Its major substrate in animal cells is phosphatidylcholine, which is hydrolyzed to phosphatidic acid and choline. Mammalian PLD also catalyzes a transphosphatidylation reaction in which a primary alcohol such as ethanol, propanol, or butanol acts as nucleophilic acceptor in place of H_2O (Kanfer, 1980). The resulting production of a relatively stable product phosphatidylalcohol is now regarded as unequivocal evidence of PLD activity. Formation of phosphatidylalcohol occurs at the expense of a natural product of PLD-catalyzed reaction - phosphatidic acid. Therefore, primary alcohols are often used as surrogate inhibitors of PLD. (Bonser et al., 1989). Secondary and tertiary alcohols can not be utilized by PLD for transphosphatidylation. Therefore they are employed as a controls for nonspecific alcohol action.

Two mammalian PLD genes have recently been cloned. Both enzymes are specific for phosphatidylcholine and have an absolute requirement for PIP2 as a cofactor (Hammond et al., 1997; Colley et al., 1997). Purified PLD1 has very low

enzymatic activity but can be significantly activated by a number of soluble cytosolic factors. In contrast, PLD2 has high basal activity and can not be further activated (Colley et al., 1997).

These two cloned genes, however, are not the only ones present in mammalian genome (Exton, 1998). Biochemical and subcellular fractionation studies strongly suggest the existence of PLD isoforms in addition to PLD1 and PLD2. The isoform that is stimulated by oleate (Massenburg et al., 1994; Siddiqui and Exton, 1992) has not been cloned and there is also evidence for the existence of PLD isoforms that are unresponsive to PIP2 (Massenburg et al., 1994; Nakamura et al., 1996).

4. Regulation of phospholipase D activity

In mammalian cells PLD activity is rapidly elevated in response to a variety of hormones, growth factors, and other extracellular signals (Exton, 1997). Several proteins have been identified in recent years as regulators of PLD activity. The major regulators belong to three different families of proteins. They are the PKC, ARF and Rho families.

There is abundant evidence that PLD is regulated by PKC in most mammalian cells. Tumor-promoting phorbol esters such as phorbol 12-myristate 13-acetate (PMA) are very specific regulators of PKC activity (Nishizuka, 1992). Addition of PMA to cell culture results in rapid activation of PLD activity in all cells examined (Billah and Anthes, 1990). On the other hand, downregulation of PKC with prolonged

PMA treatment has been found to completely or partially inhibit the activation of PLD by growth factors (Kaszkin et al., 1992; Yeo and Exton, 1995) and G protein-linked agonists (Ben-Av et al., 1993). Work conducted in our laboratory has demonstrated that EGF-induced PLD activation was dependent on PKCα (Hornia et al., 1999). Direct effects of PKC on purified preparations of PLD1 have been recently demonstrated. It was shown that conventional Ca^{2+}-dependent PKC isoenzymes, PKCα and PKCβ can activate PLD1 in cell free system (Hammond et al., 1997; Singer et al., 1995). Surprisingly, this activation does not require PKC catalytic activity (Conricord et al., 1992; Singer et al., 1996). Furthermore, a regulatory fragment of PKCα alone was shown to be sufficient for PLD1 activation (Singer et al., 1996). The significance of these findings is not yet understood. Although a role for PKC in mediating the action of several agonists on PLD in many cells has been established, there are also instances where it does not seem to be involved (Exton, 1997). PKC is also not required for PLD activation in cells transformed by v-Src (Song and Foster, 1993), v-Ras (del Paso et al., 1996) and v-Raf oncogenes (Frankel et al., 1999).

Several groups previously demonstrated that cytosolic fractions contain a soluble GTP-dependent activator of membrane PLD activity. Later, this factor was purified to homogeneity and has been shown to be Arf protein (Brown et al., 1993; Cockcroft et al., 1994). Arf (ADP-ribosylation factor) was originally discovered as a factor required for ADP-ribosylation of $G_s\alpha$ by cholera toxin (Kahn and Gilman,

1984; Kahn and Gilman, 1986) and later shown to be involved in protein trafficking in the Golgi (Balch et al., 1992; Boman and Kahn, 1995). Studies with recombinant purified PLD and Arf indicated that all members of the Arf family can activate PLD *in vitro* (Brown et al., 1995; Exton 1997) and that this activation is mediated by direct interaction of two proteins (Hammond et al., 1995). The majority of the investigations of PLD regulation by ARF have been conducted *in vitro*. The evidence for Arf in agonist stimulated PLD activation in intact cells is limited. It was shown that brefeldinA, an inhibitor of Arf activation, interferes with carbachol stimulation of PLD in cells overexpressing M3 muscarinic receptor (Rumenap et al., 1995). The evidence was also presented that Arf mediates PLD activation by fMetLeuPhe in neutrophils (Morgan et al., 1997) and PDGF and insulin in fibroblasts (Shome et al., 1997). Other authors, however, were unable to see any effects of brefeldin A on the activation of PLD by fMetLeuPhe in HL-60 cells under conditions where the Arf was completely inactivated (Guillemain and Exton, 1997). The evidence that function of Arf proteins is under agonist control is also very limited (Exton, 1998). More work is required to substantiate the role of Arf in PLD activation *in vivo*.

Another small GTPase, RhoA has been identified as an activator of PLD in a study of GTPγS effects on PLD activity in purified neutrophil membranes (Bowman et al., 1993). Rac1 and CDC42, two other members of the Rho family, were later shown to activate PLD to a similar extend (Malcolm et al., 1994; Siddiqi et al., 1995). Studies with highly purified or cloned proteins have demonstrated direct activation of

PLD1 by these small GTPases (Hammond et al., 1997; Min et al., 1998; Singer et al., 1995). The involvement of Rho family proteins in the regulation of PLD by agonists has also been demonstrated through the use of toxin from *Clostridium botulinum*. The C3 exoenzyme from this organism ADP-ribosylates Rho proteins *in vivo* and *in vitro* and renders them inactive (Sekine et al., 1989). Treatment of different cell types with this C3 exoenzyme greatly attenuates PLD responses to a variety of agonists and growth factors (Malcolm et al., 1996; Ohguchi et al., 1997; Hess et al., 1997). This data is further supported by experiments where expression of constitutively active or dominant negative forms of these proteins was shown to affect the activation of PLD by growth factors (Hess et al., 1997; Plonk et al., 1998).

In addition to the well-characterized activators of PLD described above, a number of other potential PLD regulators have been identified in studies of intact or permeabelized cells. Examples of these are ceramides (Abousalham et al., 1997), calmodulin (Takahashi et al., 1996) and the GM2 ganglioside activator of β-hexaminidase A (Nakamura et al., 1998). The physiological significance of these findings is unclear.

It has been noted that there is a synergistic effect on PLD activation by proteins from different families. PKCα has been shown to amplify the effect of Arf (Hammond et al., 1997; Singer et al., 1996) and RhoA (Ohguchi et al., 1996) on PLD activation. Similarly, Arf has been shown to potentiate the effect of RhoA (Singer et al., 1995; Shimooku et al., 1996; Hammond et al., 1997). The 'cross-talk' between

Arf, Rho and PKC in the regulation of PLD provides an opportunity for the complex control of the enzyme activity in intact cells.

5. Physiological role of phospholipase D

Despite its ubiquitous distribution and regulation by a wide variety of extracellular signals, the physiological role of PLD remains unclear. The activation of PLD leads to increased concentration of phosphatidic acid (PA) and intracellular free choline. It is believed that only increase in PA has physiological significance, since the resting concentration of free choline is high (0.1-0.5 mM) (Pelech and Vance, 1984). The decrease in local concentration of PC and subsequent increase in PA can have a profound effect on physical properties of cellular membranes. First, it can create a net increase in local negative charge and second, it will affect the curvature and fluidity of the membrane. Many proteins have been shown to change their activity upon binding to a negatively charged lipid interface made of PA (Ha and Exton, 1993; Limatola et al., 1994). It was demonstrated that PA plays an important role in the regulation of Raf-1 kinase, which is involved in signal transduction from several growth factor receptors (Ghosh et al., 1996). Raf-1 kinase has a high affinity binding site for PA and it is translocated to membranes and subsequently activated under the conditions where PLD activity is increased (Ghosh et al., 1996; Stokoe et al., 1994).

Cells display profound morphological changes upon stimulation with mitogens and growth factors. Members of the Rho family of small GTPases are major regulators of these changes. Rho proteins control the assembly of actin stress fibers and focal adhesion complexes, Rac regulates actin filament accumulation at the plasma membrane to produce lamelopodia and membrane ruffles, and Cdc42 stimulates the formation of filopodia (Olson et al., 1995; Nobes and Hall, 1995). As described above, proteins of the Rho family are also potent physiological regulators of PLD activity. Therefore, it is likely that changes in physical properties of cellular membranes produced by PLD action directly contribute to membrane remodeling and cell shape alteration induced by extracellular stimuli.

Arf proteins are major regulators of vesicle trafficking in both endoplasmic reticulum and Golgi membranes. Recently identified interaction between Arf and PLD suggest PLD involvement in secretion and vesicular trafficking. Arf is a cytosolic protein which upon binding GTP redistributes to membranes where it stimulates formation of coated vesicles (Kahn, 1993). High levels of Arf-responsive PLD have been found in Golgi-enriched membranes from a variety of cell lines (Ktistakis et al., 1995) and stimulation of PLD by Arf was inhibited by brefeldin A, a fungal metabolite that prevents binding of Arf to Golgi membranes and inhibits secretion *in vivo* (Ktistakis et al., 1995). Further evidence was presented that PLD mediates Arf-dependent formation of coated vesicles in Golgi (Ktistakis et al., 1996) and stimulates release of secretory vesicles from the trans-Golgi network (Chen et al.,

1997). It was also shown that primary alcohols, which reduce PA formation due to the production of phosphatidylalcohols, inhibited secretion in intact CHO cells (Kun et al., 1997). This data demonstrates an important role of PLD in regulation of intracellular vesicle trafficking.

The potential function of PLD in regulation of cell proliferation and transformation is not yet clear. However, PLD activity is rapidly elevated in response to most, if not all, mitogenic stimuli suggesting an important role in cell proliferation. Phosphatidic acid, a primary metabolite of PLD-mediated hydrolysis, has been shown to have mitogenic properties by itself (Moolenaar et al., 1986; Siegmann, 1987). In addition, PA can contribute to mitogenic signaling by serving as a physiological precursor to other lipid second messengers. In many cells PA is rapidly converted to DAG by the action of phosphatidate phosphohydrolase. DAG activates multiple mitogenic intracellular signaling pathways through the stimulation of PKC activity (Nishizuka, 1992; Foster, 1993; Spiegel et al., 1996). A phospholipase A_2-mediated hydrolysis of PA generates lysophosphatidic acid (LPA) which is recognized as a major extracellular mitogen present in animal serum (Moolenaar, 1995). LPA produce its diverse biological effects through the activation of G-protein coupled receptors (van Corven et al., 92).

Several distinct functions were proposed recently for PLD activity. As described above, these include membrane remodeling, regulation of vesicle formation and generation of mitogenic lipid messengers. It is possible that this list will grow further

as we continue to investigate effects of PLD activity on cellular mechanisms. It is not common for a single enzyme to have such a vast array of distinct intracellular functions. One possible explanation for these findings is that PLD function is determined by its intracellular localization. Thus, PLD is involved in formation of intracellular vesicles when it is associated with Golgi membranes and at the same time it can regulate membrane remodeling and cell shape change when associated with the plasma membrane. Of particular interest for this work are recent findings that a significant part of the cellular PLD activity is associated with caveolae (Czarny et al., 1999; Kim et al., 1999a). Caveolae, are membrane organelles formed by invaginations at the cell surface. Their physiological functions are not well understood. Caveolae can be distinguished biochemicaly from other cellular membranes due to a particular molecular composition. They are rich in glycosphingolipids, cholesterol, phospholipids with saturated fatty acids and lipid-anchored proteins (Anderson, 1998). Recent advances in caveolae purification techniques led to a discovery that many proteins involved in mitogenic signaling are highly enriched in this membrane fraction (Ocamoto et al., 1998; Anderson, 1998). This data suggests an intriguing hypothesis that PLD activity associated with caveolae is the PLD activity responsible for generation of mitogenic lipid messengers. This idea is currently under intense investigation.

6. Regulation of PLD activity in transformed cells

Phospholipase D activity is elevated in cells transformed by several transforming oncogenes including v-Src (Song et al., 1991; Wyke et al., 1992), v-Ras (Carnero et al., 1994; Jiang et al., 1995a; Martin et al., 1997), v-Fps (Jiang et al., 1994) and v-Raf (Frankel et al., 1999). It is believed that this activity contributes directly to acquisition of a transformed phenotype in many cells. Work conducted in our laboratory has significantly advanced our understanding of the molecular mechanisms responsible for this activation. It has been shown that Ras protein plays a central role in PLD activation, as it mediates PLD activation by v-Src and probably v-Fps (Jiang et al., 1994; Jiang et al., 1995a). It was also demonstrated that the PLD activated in response to v-Src and v-Ras had an apparent substrate specificity for PC that had been prelabeled with saturated fatty acids (Song et al., 1991; Song and Foster, 1993). Elevated PLD could be detected in cells prelabeled with [^{3}H]-saturated fatty acids, but not when prelabeled with the polyunsaturated fatty acid arachidonate. The differential utilization of the PC prelabeled with saturated and unsaturated fatty acids in v-Src-transformed cells was lost when examined *in vitro* (our unpublished results), suggesting that there was no inherent substrate specificity for the v-Src-induced PLD. The apparent *in vivo* substrate specificity could be explained by a PLD that acted on PC localized to regions of the membrane that were enriched for saturated fatty acids.

Recently we have also demonstrated that activation of PLD by Ras is mediated by newly identified Ras/Ral-GDS/Ral signaling cascade (Jiang et al., 1995b). Overexpression of RalA potentiated PLD activation by Src, and expression of dominant negative RalA mutant inhibited both v-Src and v-Ras-induced PLD activity (Jiang et al., 1995b). The PLD activated in response to v-Src and v-Ras is apparently PLD1 (Luo et al., 1997). This PLD isoform could be precipitated from transformed cell lysates with immobilized RalA protein or with an anti-Ral specific antibody (Jiang et al., 1995b; Luo et al., 1997). Subsequent studies from our laboratory have further defined the molecular mechanisms of PLD1 activation by Src and Ras by showing that RalA stimulates the formation of an active RalA/PLD1/Arf complex (Figure 3) (Luo et al., 1998). Arf protein was found in the RalA precipitates from v-Src and v-Ras-transformed cells. The formation of a complex between RalA and Arf was dependent on GTPγS, suggesting a physiologically relevant association rather then a nonspecific binding. The Arf involvement in v-Src and v-Ras-induced PLD activity has been further validated using brefeldin A, an inhibitor of Arf GDP-GTP exchange. Brefeldin A inhibited PLD activity in the transformed cells, but not in the normal fibroblasts (Luo et al., 1998). The interaction between RalA and Arf, however, is likely an indirect one, since immobilized RalA was unable to precipitate Arf from a partially purified preparation (Luo et al., 1998). It is also unlikely that Arf is bound to PLD1 in RalA/PLD1/Arf complexes precipitated from cell lysates. The amount of PLD1 protein was very low in these complexes, far from sufficient to be in

stoichiometric interaction with the amount of Arf present in the precipitates (Luo et al., 1998). These data implicated existence of additional factors present in transformed cells that contribute to PLD activation. In this report, we describe the characterization of a low molecular weight PLD stimulating factor (designated PLD-SF) that is elevated in the cytosol of transformed and dividing cells. Evidence is presented suggesting that PLD-SF is specific for a subset of PLD localized in caveolae and caveolae-related membrane microdomains.

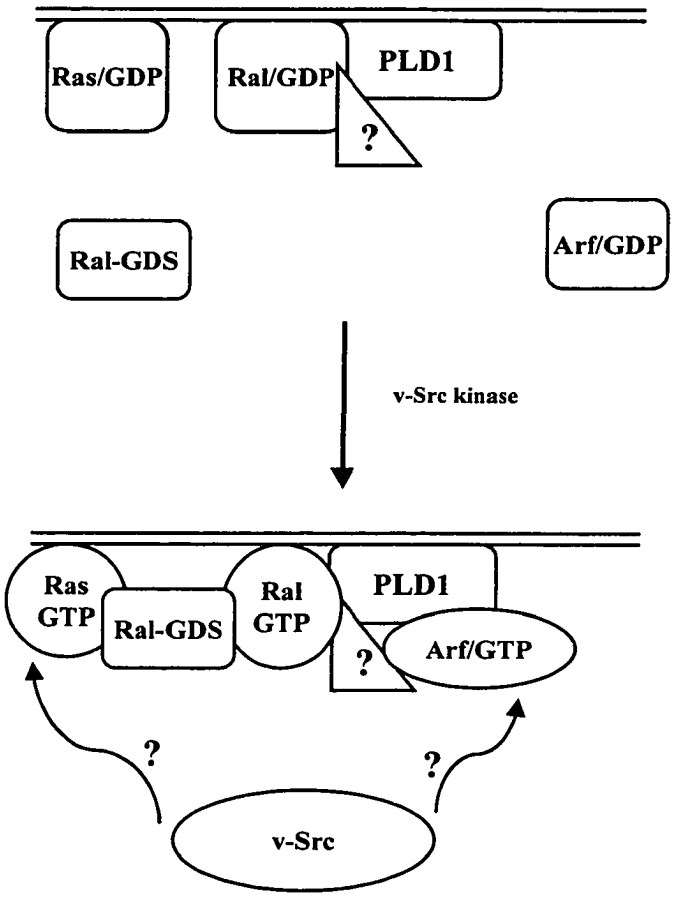

Figure 3. Model for activation of PLD in v-Src and v-Ras-transformed cells.

In response to tyrosine kinase activity of v-Src, Ras undergoes transformation from inactive (Ras-GDP) to the active (Ras-GTP) form. The activated Ras associates with Ral-GDS, resulting in the recruitment of a Ral-PLD1 complex and GDP-GTP exchange on Ral. Unknown factors are postulated to facilitate the interaction between Ral/PLD1 and an active Arf resulting in activation of PLD1. (From Luo et al., 1998).

Chapter II

Materials and Methods

1. Materials

L-α-dipalmitoyl phosphatidylcholine (PC) ([methyl- ^{3}H]-choline), [methyl- ^{3}H]-choline chloride, and [^{3}H]-myristate were from New England Nuclear. All tissue culture reagents were from Gibco BRL. All enzymes were from Boehringer Mannheim. All other chemicals and organic solvents (ACS grade) were purchased from Sigma. Precoated silica gel (60A) thin layer chromatography (TLC) plates were from Whatman. Reagents for SDS-polyacrylamide gel electrophoresis were from Bio-Rad. 1 kDa molecular weight cut-off membranes were from Amicon (YM-1). Antibodies raised against Ha-Ras (F235) and fibroblast growth factor receptor-1/*flg* (C-15) were obtained from Santa Cruz Biotechnology; an antibody raised against caveolin 2 (C57820) was from Transduction Laboratories.

2. Cells and cell culture conditions

Normal and v-Ras-transformed NIH 3T3 cells were maintained in Dulbecco's modified Eagle medium supplemented with 10% bovine calf serum (HyClone). Cell cultures were made quiescent by growing to confluence without medium change for 4

days. *Saccharomyces cerevisiae* strain W 303 1A was a generous gift of Peter Lipke (Hunter College, New York, NY).

3. Generation of Ras-transformed cells

Parental NIH 3T3 fibroblasts were stably transfected with v-Ras mammalian expression vector (White et al., 1995). Several neomycin-resistant colonies were pooled together in order to avoid clonal variants and propagated for several passages, after which they were stored in the liquid nitrogen.

4. Labeling of cellular lipids

For metabolic labeling of cellular lipids, cells were treated with either 2 μCi/ml of [^{3}H]-myristate (50 Ci/mmole) for 24 hours or with 2 μCi/ml of [^{3}H]-choline chloride (80 Ci/mmole) for 48 hours. At these times, the lipids in both normal and v-Ras-transformed NIH 3T3 cells were labeled to equilibrium.

5. Subcellular fractionations

Cells were washed with cold phosphate buffered saline (PBS: 136 mM NaCl, 2.6 mM KCl, 1.4 mM KH_2PO4, 4.2 mM Na_2HPO4), aspirated and then scraped with a Teflon policeman in hypotonic homogenization buffer [20 mM HEPES (pH = 7.5), 25 mM KCl, 5 mM choline chloride, 1 mM EDTA, 1mM EGTA, 1 mM DTT, and protease inhibitors (1 mM AEBSF, 1 μM E-64 and 1 μM leupeptin)]. Cells were then

homogenized with a 'Polytron' homogenizer (2 x 30 seconds at 20,000 rpm). Nuclei and cellular debris were removed by centrifugation for 10 min at 1000 g. The resulting supernatant was then centrifuged at 150,000g for 45 minutes. This supernatant was collected and stored at -80 ^{0}C (cytosolic fraction). The pellet was washed by resuspension in homogenization buffer and centrifugation. The final pellet was homogenized in homogenization buffer with a glass/Teflon homogenizer and stored at -80 ^{0}C (membrane fraction).

To examine PLD activity in different membrane fractions, the crude membrane fraction obtained as described above was broken into small fragments by sonication on ice (6 pulses, 10 seconds each) with a probe sonicator (Sonics and Materials). 2 ml of this preparation was then layered on a top of a discontinuous sucrose gradient consisting of 0.25, 0.5, 0.75, 1.0 and 1.25 M sucrose (2 ml each) prepared in the homogenization buffer and centrifuged for 4 hours (Beckman SW 41 rotor) at 200,000 x g. Opaque bands on a top of each sucrose cushion were aspirated and diluted with 4 volumes of homogenization buffer. The pellet at the bottom of the tube was recovered and resuspended in homogenization buffer. Membranes from each fraction were then pelleted by centrifugation at 150,000g for 45 minutes and resuspended in homogenization buffer. The different membrane fractions were then analyzed for PLD activity and membrane proteins as described below.

6. In vitro choline release assay

Reactions were conducted in a total volume of 50 µl containing reaction buffer [20 mM HEPES (pH = 7.5), 125 mM KCl, 10 mM $MgCl_2$, 5 mM choline chloride] and 125,000 cpm of substrate in the form of [^{3}H]-choline-labeled membranes (15-20 µg of membrane protein). Reactions were allowed to proceed for 1 hour at 37 ^{0}C and then were stopped by the addition of 400 µl of solution containing 1% Triton X-100, 0.1% sodium deoxycholate, 5 mM EDTA and 5 mM choline chloride. Control samples were kept on ice. Lipids were precipitated after addition of 50 µl of 100% trichloroacetic acid and centrifugation for 5 minutes in microcentrifuge. 400 µl aliquots of the supernatants containing released [^{3}H]-choline were removed and counted in a liquid scintilation counter.

For the analysis of aqueous metabolites released from the *in vitro* assays reactions were terminated by addition of 1 ml of chloroform/methanol mixture (1:1). After phase separation, 500 µl of upper methanolic phase was removed, dryed in SpeedVac and analyzed by TLC with methanol/0.5% sodium chloride/ammonia (70:50:5) as a mobile phase (Teegarden et al., 1989). TLC plates were dried, spayed with [^{3}H]-Enhancer (DuPont) and exposed to X-Ray film (RX, Fuji) at –80 ^{0}C. Standards of choline, phosphocholine and GDP-choline were separated as described above and their position on TLC plate was determined after exposure to iodine vapor.

7. Transphosphatidylation reactions

Cells were metabolically labeled with [^{3}H]-myristate as described above, and 1-propanol was added to cell culture medium to give a final concentration of 1%.

After incubation for 1 hour cells were washed with PBS and scraped in 500 µl of ice cold methanol/6 N HCl (50:1) mixture. Phase separation was obtained by adding 500 µl of chloroform. The organic phase was recovered and dried under stream of N_2. 1 x 10^6 cpm of total radioactivity was analyzed by TLC using the upper phase of ethylacetate/isooctane/acetic acid/water (90:50:20:100) as mobile phase (Song 91). TLC plates were dried, sprayed with [^{3}H]-Enhancer (DuPont) and exposed to X-Ray film (RX, Fuji) at –80 ^{0}C.

8. Partial purification of PLD-SF

Partial purification of PLD-SF was done by method described previously (Kai et al., 1989) with modifications. Briefly, 4-5x10^7 cells were homogenized in 10 ml of ice cold 0.1 N HCl with a 'Polytron' homogenizer. Debris were removed by centrifugation for 10 min. at 10,000 g. Supernatant was deproteinized by addition of 0.1 volume of 2 M $HClO_4$ and centrifugation as above. An equal volume of 1 N $NaHCO_3$ was added to the supernatant and pH was adjusted to 7.5 with 1 N NaOH. This solution was then filtered through a 1 kDa molecular weight cut-off membrane (Amicon, YM-1). The filtrate was applied to a Cyclohexyl solid phase extraction cartridge (500 mg, Bond Elut, Varian) which was conditioned according to

manufacturer specifications. Each cartridge was washed with 2.5 ml of H_2O. Bound material was then eluted with 1 ml of aqueous 90% methanol, dried by SpeedVac centrifugation (Savant) and stored at –20 ^{0}C.

Where indicated, PLD-SF was also purified from rat testes. In this case 10 grams of frozen rat testes (Pell Freez Biologicals) was used for standard purification as described above.

9. Apoptosis assays

Cells were grown in 12-well tissue culture plates in medium containing DMEM and 10 % bovine calf serum. When 50 % confluent, cells were made quiescent by changing the medium to DMEM with 0.5 % bovine calf serum and growing overnight. After incubation with partially purified PLD-SF from rat testes, plates were washed with PBS and cells that were still attached to plates were lysed in 0.5 N NaOH (0.5 ml per well). Lysates were collected, centrifuged for 5 minutes at 10,000g, and protein concentration in supernatant was determined.

For analysis of DNA fragmentation, cells were treated as above and DNA was isolated with DNAzol reagent (Gibco) according to manufacturer's protocol. Isolated DNA was then separated by electrophoresis in 2 % agarose gel in TBE buffer.

10. Protein concentration determination

Protein concentrations were determined using the Micro BCA method (Pierce) with bovine serum albumin as a standard.

11. Western blot analysis

Proteins from membrane fractions were precipitated with 20% trichloroacetic acid on ice, washed with acetone and subjected to SDS-polyacrylamide gel electrophoresis using 10% acrylamide separating gel. After transferring to nitrocellulose, the filters were incubated with appropriate antibodies as described (Luo et al., 1998). The position of specific protein bands was determined using the ECL system (Amersham).

Chapter III

A Heat-Resistant PLD-Activating Factor is Present in the Cytosol of Ras-Transformed Cells

Introduction

Theoretically, there could be two reasons for the increase in PLD activity observed in transformed cells. It is possible that transformed cells upregulate transcription of some of the PLD genes resulting in the increased amount of PLD enzymes. Alternatively, higher levels of PLD activity in these cells is the result of an increased amount or increased activity of PLD-regulating factors. Unfortunately, lack of good isotype-specific antibodies for PLD makes it difficult to address the former hypothesis. It is more likely, however, that postranslational events are the major mode of regulation of PLD activity. PLD is rapidly activated in response to a number of mitogenic and growth factors (Exton, 1998). In many cells this activation could be detected within 5 – 10 minutes after addition of a stimulus, much earlier then required for transcripional activation to take place. Work conducted in our laboratory has demonstrated that cells expressing a temperature-sensitive mutant of v-Src show elevated PLD activity within 15 minutes after a temperature shift (Song et al., 1991) before substantial protein synthesis can occur. This activation is mediated by Ras protein (Jiang et al., 1994; Jiang et al., 1995a). Therefore, it is likely that activation of

PLD in Ras-transformed cells utilizes a similar mechanism that does not alter the transcription of PLD genes. In this chapter we describe experiments performed to prove this hypothesis.

Results

1. Soluble cytosolic factor is responsible for increased PLD activity in Ras-transformed cells.

We demonstrated previously that PLD activity is elevated in Ras-transformed NIH 3T3 cells relative to the parental cells (Jiang et al., 1995a). This was determined by examining the PLD-catalyzed transphosphatidylation of PC to the corresponding phosphatidyl-alcohol in the presence of exogenously provided alcohol. We prelabeled cellular lipids with [^{3}H]-myristate which is incorporated exclusively into PC (Song et al., 1991; Song and Foster, 1993). In the presence of 1-propanol, PC is converted to PPro, and as reported previously, there was approximately 3-fold more PPro generated in the Ras-transformed cells relative to the parental NIH 3T3 cells (Fig.4*a* and *b*). However, if membranes were isolated from these cells and PLD activity determined in the absence of cytosolic components, the difference in the PLD activity between the Ras-transformed and parental cells is lost (Fig. 4*c*). This suggests that a soluble cytosolic factor is responsible for increased PLD activity in Ras-transformed cells.

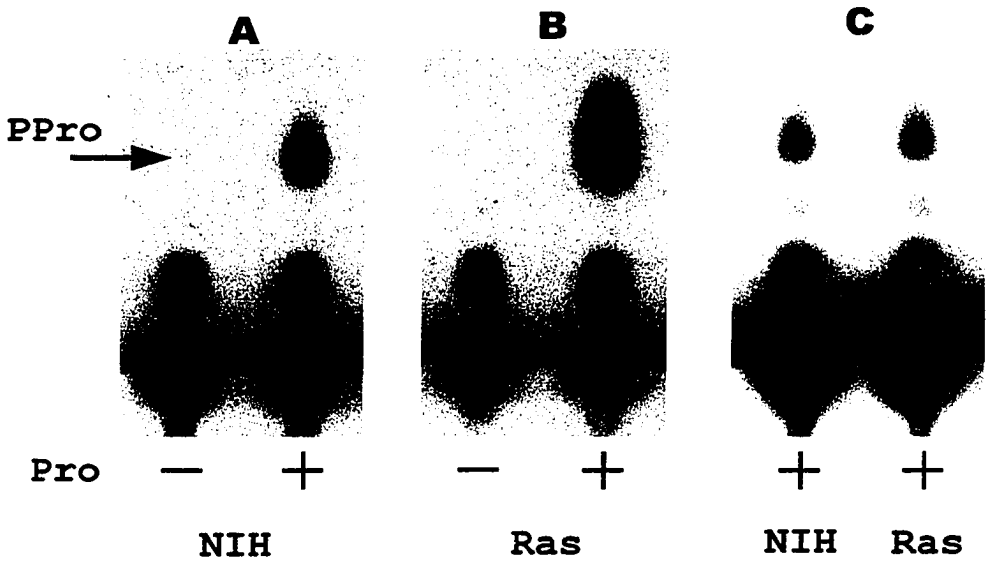

Figure 4. *In vivo* PLD activity is regulated by cytosolic factors.

Cells were labeled with [^{3}H]-myristate and *in vivo* transphosphatidylation reactions were performed with NIH 3T3 (Panel A) and Ras-transformed cells (Panel B) as described in Materials and Methods. Membranes from [^{3}H]-myristate-labeled cells were isolated and *in vitro* transphosphatidylation reactions were performed under conditions described for choline release assay (Panel C). Where indicated, 1-propanol (Pro) was added to a final concentration of 1%. After incubation, cellular lipids were extracted and analyzed by TLC. The position of the PLD-catalyzed transphosphatidylation product PPro is shown. A representative experiment that was repeated four times is shown.

The transphosphatidylation reaction is considered to be a very reliable way to measure PLD activity. However the long exposure times required in order to achieve sufficient band intensity on the X-Ray films is a distinct disadvantage of this technique. . In order to expedite our future research, we developed an *in vitro* assay for measurement of phospholipase D activity based on the release of radiolabeled choline from phosphatidylcholine of cellular membranes. Ras-transformed and parental NIH 3T3 cells were labeled to equilibrium with [^{3}H]-choline chloride. Cellular membranes from these cells were isolated and assayed for membrane-bound phospholipase activity. Incubation of these membranes at 37 ^{O}C results in the release of water-soluble radioactivity from the lipid fraction. This release can be mediated by the action of any one of the three phospholipases. However, it is possible to find out the relative contribution of each class of the phospholipases by analyzing the aqueous products of these reactions. Release of choline, phosphocholine and glycero-phosphocholine will be indicative of the action of phospholipases D, C and A respectively (see Figure 2). TLC analysis of aqueous metabolites released from membranes in an *in vitro* reaction demonstrates that choline was the predominant species (Figure 5, compare lines 1 and 2; and lines 4 and 5), indicating that phospholipase D is the major phosphatidylcholine hydrolyzing activity in isolated membranes. Similar results have been reported by other groups (Cabot et al., 1988; Cook and Wakelam, 1992; Carnero et al., 1994).

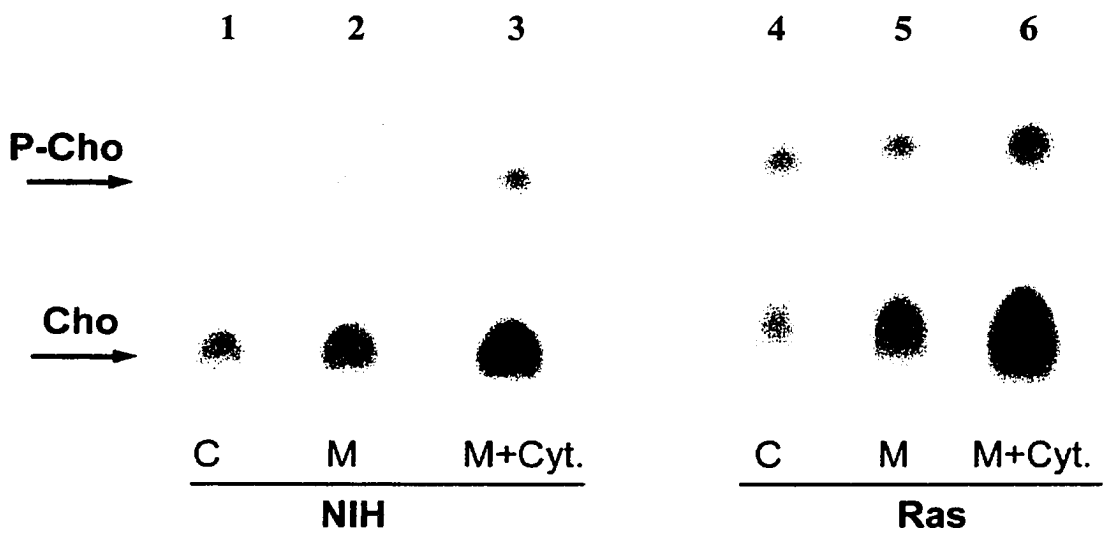

Figure 5. Soluble cytosolic factors elevate PLD activity in isolated membranes. Membranes were isolated from [^{3}H]-choline-labeled NIH 3T3 and Ras-transformed cells and incubated for 1 hour at 37 ^{o}C. Membranes were either incubated alone (M) or in the presence of 50 μg of corresponding cytosolic fraction (M + Cyt). Control tubes (C), were maintained on ice throughout. Aqueous metabolites released from membranes were analyzed as described in Materials and Methods. Positions of standards for Choline (Cho) and phosphorylcholine (P-Cho) are shown. A representative experiment that was repeated three times is shown.

Therefore, we employed the choline release assay for measurement of PLD activity *in vitro* in our subsequent experiments.

As mentioned above, data presented in Figure 4 strongly implicates existence of a cytosolic factor that is responsible for PLD activation in Ras-transformed cells. Therefore we performed *in vitro* reconstitution experiments with cytosolic fractions isolated from normal and Ras-transformed NIH 3T3 cells. Addition of these fractions to [^{3}H]-choline-labeled membranes increased the amount of choline released from these membranes (Figure 5, compare lines 2 and 3; and lines 4 and 5). Cytosol from Ras-transformed cells caused a significantly higher increase in released choline than cytosol from NIH 3T3 cells. In addition, cytosolic fractions from both cells caused a small increase in the amounts of phosphocholine (Figure 5). However, these species contributed to less than 5% of the total released radioactivity. This increase in the amount of phosphocholine is most likely due to the presence of soluble choline kinase activity, rather then phospholipase C (Ratnam and Kent, 1995).

2. PLD activity is present in cytosolic fractions

The higher levels of PLD activity observed upon addition of cytosolic fractions in *in vitro* experiments could be explained by the presence of soluble cytosolic PLD. The existence of such activity have been reported previously (Wang et al., 1991; Siddiqi et al., 1995). In order to investigate this possibility we performed *in vitro* reactions with cytosolic fractions and substrate supplied in the form of mixed

phospholipid liposomes. As shown in the Figure 6, cytosolic fractions from both normal and Ras-transformed fibroblasts contained PLD activity. All of this cytosolic PLD activity from both the parental and Ras-transformed cells was completely inactivated by heat treatment for 10 min at 100 ^{0}C (Figure 6).

3. Cytosol from Ras-transformed cells contains a heat-resistant activator of PLD

We then examined the ability of heat-inactivated cytosolic fractions to stimulate membrane-bound PLD. Interestingly, the heat-inactivated cytosol from the Ras-transformed cells was able to stimulate PLD activity in isolated membranes (Figure 7), whereas heat-inactivated cytosol from normal cells had no effect on membrane-bound PLD activity.

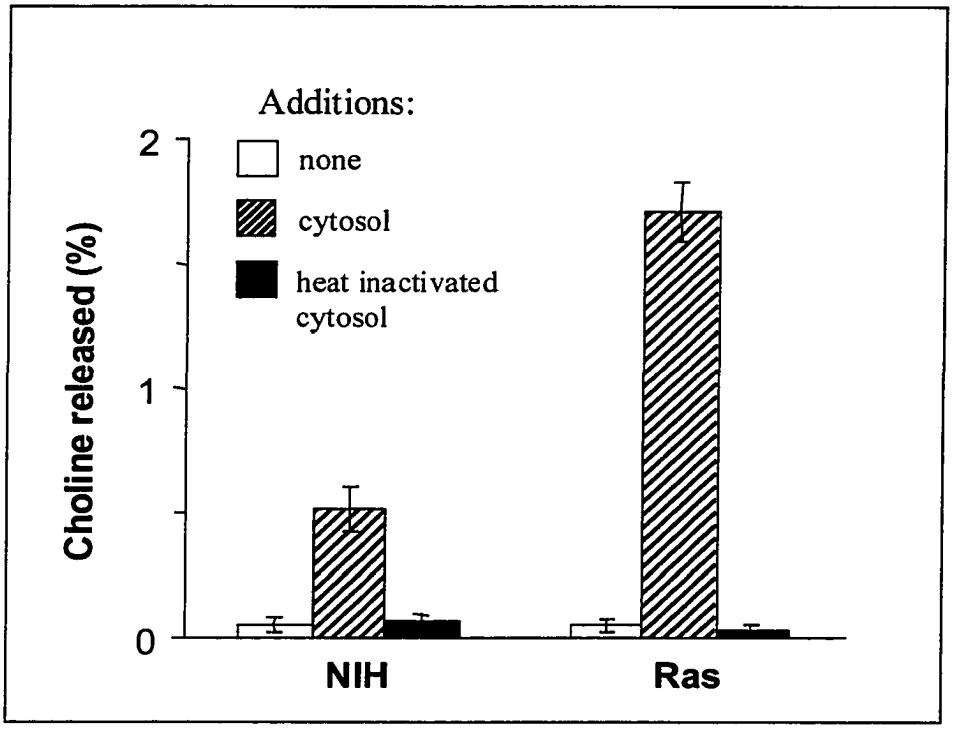

Figure 6. Cytosolic fractions from NIH 3T3 and Ras-transformed cells contain PLD activity. Cytosolic fractions were isolated from NIH 3T3 and Ras-transformed cells. For heat inactivation cytosolic fractions were incubated for 10 min at 100 ^{0}C cooled on ice and precipitated proteins were removed by centrifugation for 5 min at 12,000 X g. The PLD activity in the cytosol before and after heat treatment was determined by *in vitro* choline release assay as described in Materials and Methods except that substrate was [^{3}H]-choline-PC supplied as liposomes (White et al., 1993). 50 μg of cytosolic protein or 5 μg of heat-inactivated cytosolic protein was used in the assays. The values are expressed as a percent of total lipid label released from liposomes in 1 hour. Each data point represents the mean +/- standard error of three independent experiments performed in duplicate.

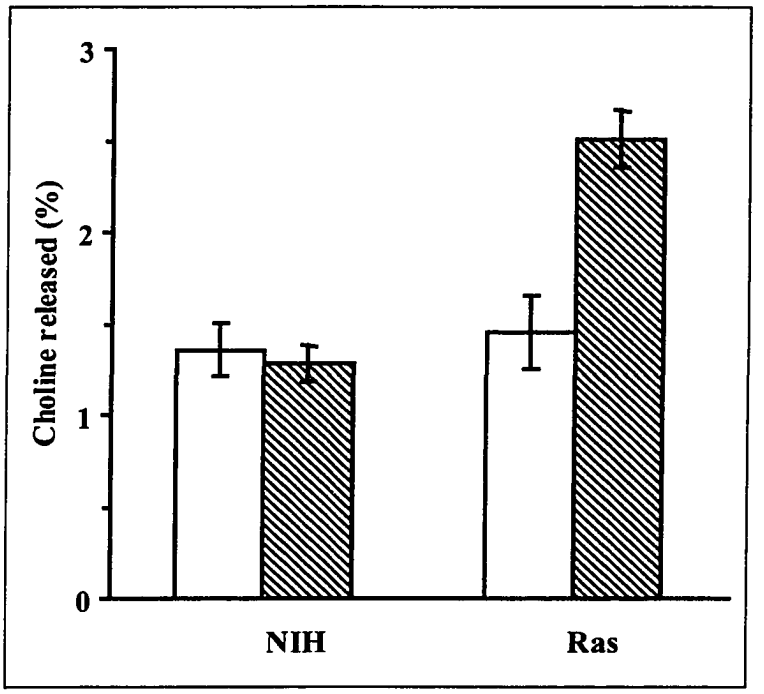

Figure 7. A heat-resistant activator of PLD is present in the cytosol of Ras-transformed cells.

Cytosolic fractions and [^{3}H]-choline-labeled membranes were isolated from parental and Ras-transformed NIH 3T3 cells as described in Materials and Methods. Cytosolic fractions were heat-treated as described in Figure 6 and 5 μg of protein was used for each reaction. PLD activity was determined by in vitro choline release assay as described in Materials and Methods. Isolated membranes were either incubated alone (open bars) or in the presence of corresponding heat-inactivated cytosol (hatched bars). The values are expressed as a percent of total lipid label released from membranes in 1 hour. Each data point represents the mean +/- standard error of eight independent experiments performed in duplicate.

Discussion

PLD activity is increased in cells transformed by a number of oncogenes including v-Ras. The molecular mechanisms responsible for this increase are not yet well understood. The Ral protein has been shown to potentiate activation of PLD by v-Src and v-Ras (Jiang et al., 1995b). An activated Ral mutant, however, had no effect on PLD activity when expressed in normal cells (Luo et al., 1998). An increased amount of PLD enzyme could account for the higher level of PLD activity observed in transformed cells. In the experiments described above we have shown that membranes isolated from normal NIH 3T3 and Ras-transformed cell contain similar level of PLD activity suggesting that there was no difference in the amount of PLD enzymes in these cells. This data is in agreement with our observation that soluble cytosolic fractions contain factors responsible for increased PLD activity in Ras-transformed cells (Figure 4). Further analysis of these factors was complicated by the presence of soluble PLD activity in cytosol from both NIH 3T3 and Ras-transformed cells. However, we have demonstrated that under the conditions where all of the cytosolic PLD activity was inactivated, cytosol from Ras-transformed cells was able to stimulate membrane-bound PLD, while cytosolic fractions from normal NIH 3T3 cells did not. These data indicate the existence of a heat-resistant PLD-stimulating factor (designated PLD-SF) that is elevated in the cytosol of Ras-transformed cells and which is responsible, at least in part, for increased PLD activity observed in these cells.

Chapter IV

Heat-Resistant Activator of PLD is a Small Peptide and It is Elevated in the Dividing Cells

Introduction

Several cytosolic proteins were identified in recent years as regulators of PLD activity. It is unlikely that any of these proteins is the PLD-SF we identified in the previous chapter. It is expected that the conditions we used for inactivation of cytosolic PLD would irreversibly denature these proteins. Therefore, the nature of the heat-resistant PLD-stimulating factor is unknown. In this chapter we describe experiments intended to elucidate the properties of PLD-SF. We also examined several cell lines and animal tissues for the presence of similar PLD-stimulating activity.

Results

1. Partial purification of PLD-stimulating factor

We first separated the heat-inactivated cytosol from the Ras-transformed cells into low and high molecular weight fractions using a 1 kDa molecular weight cut-off filter and found, that the PLD-stimulating activity was in the filtrate. The PLD-stimulating activity could be precipitated from the 1 kDa filtrate with either ethanol or

acetone (the latter being more effective). These data suggest that PLD-SF is a small molecule. We have tested several published methodologies for purification of small molecules and found one to be the most satisfactory (Kai et al., 1981). Based on this method we have designed a purification scheme that allowed us to obtain partially purified PLD-SF from Ras-transformed cells (see Materials and Methods). We were able to achieve purification of PLD-SF about 250 times (Table I). When added to isolated membranes this preparation caused a linear increase in membrane-bound PLD activity in response to increasing concentrations of the partially purified factor (Figure 8*a*). There was a similar increase in transphosphatidylation of PC to PPro in response to the partially purified factor (Figure 8*b*), confirming that the heat-resistant factor is a specific activator of membrane-bound PLD.

2. PLD-stimulating factor is a small peptide

We next examined the physical properties of partially purified PLD-SF in order to determine the molecular nature of this activity. PLD-SF was sensitive to both HCl and NaOH treatment and to baking at 350 ^{0}C (Table II), suggesting an organic molecule rather than a metal ion. The factor was completely resistant to DNAse and RNAse treatment (Table II). Although the PLD-SF was somewhat resistant to protease treatment, pronase, subtilisin and proteinase K all reduced the PLD-stimulating ability of the factor (Table II). We also examined the effect of several small molecules known to function as intracellular mediators for their effect upon

Table I. Partial purification of PLD-stimulating factor.

	Total protein (mg)	Specific activity*	Fold purification
1. Acid extract (0.1 N HCl)	96	0.05	1
2. Deproteinized (0.2 N $HClO_4$)	17.8	0.2	5
3. 1 kDa filtrate	1.54	1.25	40
4. Cyclohexyl	0.15	7.2	245

PLD-stimulating factor was purified from Ras-transformed NIH 3T3 fibroblasts as described in the Materials and Methods. At each stage of purification an aliquot was taken and prcipitated with 5 volumes of ice cold acetone. Samples were dried in SpeedVac, resuspended in assay buffer and added to [^{3}H]-choline-labeled membranes isolated from Ras-transformed NIH 3T3 cells. PLD activity was determined by *in vitro* choline release assay as described in Materials and Methods. Data from a representative isolation is shown.

* Specific activity determined as a percent of total choline released from membranes by 1 milligram of total protein in 1 hour. The amount of choline released from membranes incubated alone was subtracted from all experimental points.

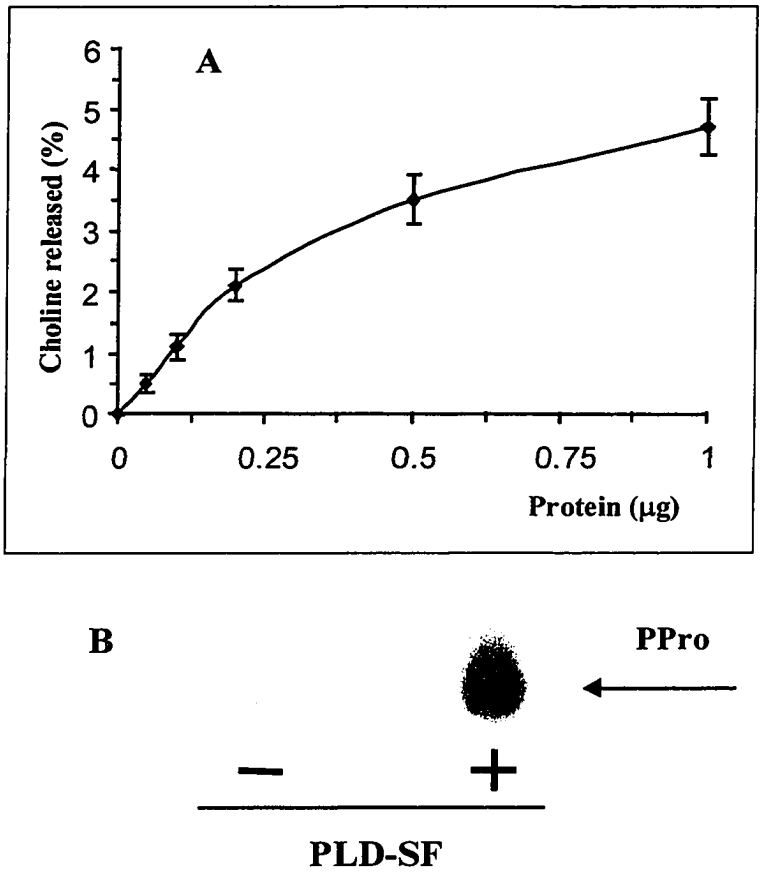

Figure 8. Activation of membrane-bound PLD activity by PLD-SF.

Partially purified PLD-SF was prepared from Ras-transformed cells as described in Materials and Methods. (*a*) Increasing concentrations of the PLD-SF preparation were added to [^{3}H]-choline-labeled Ras membranes and *in vitro* PLD activity was determined by the release of [^{3}H]-choline. The amount of choline released from membranes alone was subtracted from all experimental points. The values are expressed as a percent of total lipid label released from membranes in 1 hour. Each data point represents the mean +/- standard error of four independent experiments performed in duplicate. (*b*) *In vitro* transphosphatidylation reactions were performed with [^{3}H]-myristate-labeled Ras membranes and 1-propanol (1%). Where indicated, partially purified PLD-SF (1 μg of protein) was added to the reaction mixture. A representative experiment repeated three times is shown.

Table II. Characterization of PLD-SF.

Treatment	Stimulated PLD activity (%)[d]
None	100
NaOH[a]	40 +/- 6
HCl[a]	62 +/- 9
Baking, 350 ^{0}C[b]	3 +/- 2
DNAsed[c]	105 +/- 12
RNAse[c]	98 +/- 7
Trypsin[c]	99 +/- 5
Chymotrypsin[c]	96 +/- 9
Proteinase K[c]	59 +/- 11
Subtilisin[c]	14 +/- 3
Pronase[c]	5 +/- 2
Alkaline phosphatase[c]	2 +/- 7

PLD-SF was partially purified from Ras-transformed cells as described above and 1 μg of total protein from these preparations was used for each assay.

[a] PLD-SF preparations were resuspended in 1 N HCl or 1 N NaOH and incubated for 2 hours at 100^{0}C. Samples were cooled to room temperature and neutralized with either 1 N of NaOH or 1 N HCl. As a control for this treatment PLD-SF was incubated in 0.5 M NaCl.

[b] Dry preparation of PLD-SF was baked at 350 ^{0}C for 30 minutes, cooled to room temperature and extracted with H_2O.

[c] PLD-SF preparations were resuspended in 100 μl of 25 mM Tris-HCl (pH = 8.0) containing 50 μg of indicated enzyme and incubated for 24 hours at 37 ^{0}C. For protease digestion 0.5% SDS was included in the incubation mixture to denature proteins. After incubation PLD-SF was separated from the enzymes by centrifugation through 10 kDa molecular weight cut-off filter (Millipore). As a control for these treatments PLD-SF and enzymes were incubated in separate tubes and combined just prior to filtration.

[d] After these treatments, PLD-SF was recovered by precipitation with 5 volumes of cold acetone, dried in SpeedVac and its PLD-stimulating activity was examined as described in Figure 8*a*. The values were normalized to the PLD-stimulating activity of untreated PLD-SF. Each data point presented is the average of at least three independent experiments performed in duplicate +/- standard error.

PLD activity in isolated membranes. These included cAMP, cGMP, inositol-1,4,5-tris phosphate, Ca^{2+}, ATP, GTP and glutathione. None of these low molecular weight compounds (at physiological concentrations) elevated PLD activity in isolated membranes (Table III). These data suggest that PLD-SF is most likely a small peptide and since it is able to pass through a 1 kDa molecular weight filter, it is likely no more than 10 amino acids.

3. The presence of PLD-SF correlates with the cells proliferative state

We next investigated whether PLD-SF is unique to cells transformed by Ras oncogene or if it is elevated in other transformed cells. As shown in Figure 9, the cytosol from both the v-Src and v-Raf-transformed cells also contained elevated levels of PLD-SF, demonstrating that this activity is not unique to the Ras oncogene. Significant activity could also be detected in the cytosol from quiescent NIH 3T3 cells that had been stimulated with serum for 16-18 hours (Figure 9), suggesting that appearance of the factor correlates with the proliferation state of the cells rather than transformation. In agreement with this observation we also detected PLD-SF in several rat tissues and found that testes contained the highest levels of the activity (Figure 9). Interestingly, this tissue contains the most proliferating cells among the tissues examined. We also examined whether a similar PLD-SF could be detected in yeast. Both stationary and log phase *Saccharomyces cerevisiae* were analyzed for the presence of a low molecular weight cytosolic PLD-SF, and as shown in Figure 9, a

Table III. Small molecules that do not affect membrane-bound PLD activity.

Molecule	Range of concentrations tested
cAMP, cGMP	10-100 μM
ATP, GTP	0.1-1.0 mM
Ca^{2+}	0.01-1.0 mM
IP3	10-100 μM
Glutathione	0.1-5.0 mg/ml

[^{3}H]-choline-labeled membranes were isolated from Ras-transformed NIH 3T3 cells and PLD activity was determined by in vitro choline release assay as described in Materials and Methods. Membranes were incubated alone and in the presence of indicated molecules. At least four different concentrations were used for each molecule within given range. PLD activity in all samples did not differ more than 10% from PLD activity measured in membranes incubated alone.

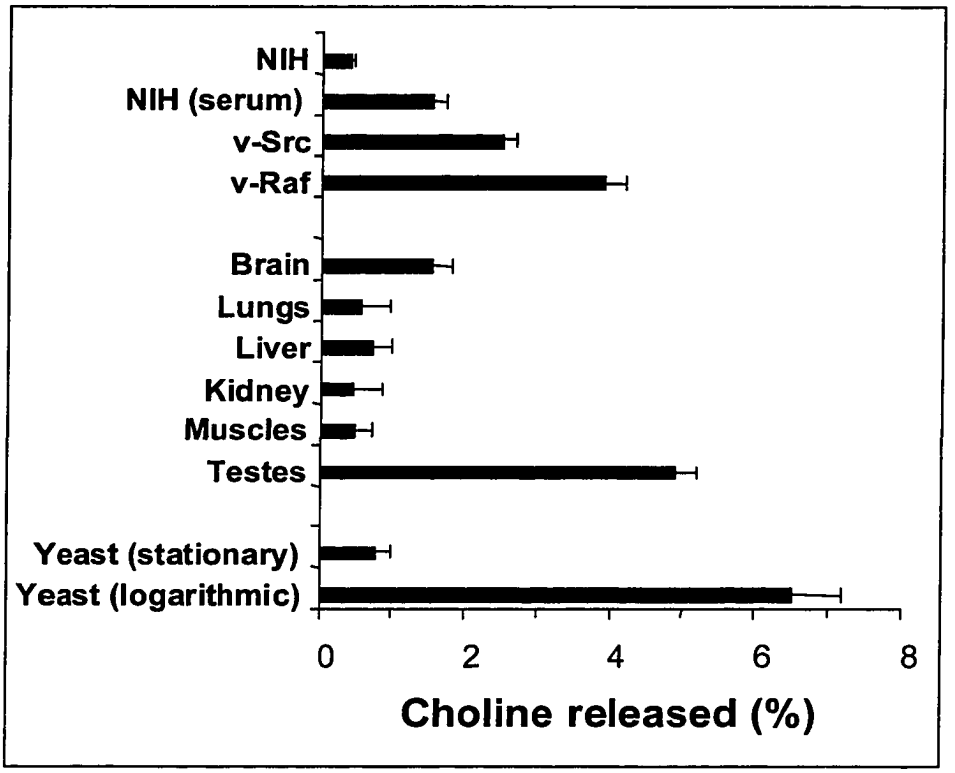

Figure 9. PLD-SF levels correlate with the proliferative state of the cells.

PLD-SF was purified from cultured cells, rat tissues (10 grams each) and yeast cultures (5 ml of packed cell volume) as outlined in Materials and Methods. 1 μg of protein from these preparations was then tested for the ability to stimulate PLD activity in [^{3}H]-choline-labeled membranes from Ras-transformed cells. The amount of choline released from membranes alone was subtracted from all experimental points. The values are expressed as a percent of total lipid label released from membranes in 1 hour. Each data point represents the mean +/- standard error of at least three independent experiments performed in duplicate. NIH – serum-starved NIH 3T3 cells; NIH (serum) - NIH 3T3 cells stimulated with serum for 18 hours; v-Raf, v-Src - NIH 3T3 cells transformed by v-Raf and v-Src respectively; Yeast (stationary) – yeast cells collected at $OD_{600} > 1$ AU; Yeast (logarithmic) – yeast cells collected at $OD_{600} < 0.3$ AU.

PLD-SF was detected that was 10-fold higher in the dividing cells than in the stationary cells. Thus, PLD-SF may be ubiquitously distributed and plays a role in cell division.

Discussion

In this chapter we have presented the evidence that PLD-stimulating factor from Ras-transformed cells is a small phosphorylated peptide of 10 or less amino acids. In this respect it is unlike any other known activators of PLD. There is a singe report in the literature which describes a similar activity in the cytosol from *Arabidopsis* plants (Pappan et al., 1997). The partially characterized cytosolic PLD activating factor from these plants, like PLD-SF, had a low molecular weight (< 6 kDa) and was also resistant to boiling. The plant factor was resistant to treatment with proteinase K, trypsin and thermolysin, whereas PLD-SF was partially sensitive to proteinase K and more highly sensitive to subtilisin and pronase. The difference in protease sensitivity could be explained by the different proteases used and the known resistance of small peptides to proteases. Thus it is possible that this plant PLD stimulating activity may be the same as or similar to PLD-SF.

The level of PLD-SF is substantially elevated in the cells that have higher PLD activity. It was found in the cytosol from cells transformed by v-Ras, v-Src, v-Raf and also in the cytosol from NIH 3T3 cells stimulated with serum. Similar PLD-stimulating activity was also present in several rat tissues but was highest in rat testes,

which contain the highest percentage of dividing cells of the tissues examined. Altogether these data demonstrates that PLD-SF is higher in dividing cells. PLD activity is elevated in response to a large variety of extracellular signals, especially those involved in cell division. The first PLD gene cloned was yeast SPO14 gene which is essential for meiosis (Rose et al., 1995). The fact that PLD-SF activity correlates with the proliferative state of cells strongly implicates PLD-SF as a factor that contributes to increased PLD activity in mitogenicly stimulated cells.

At this moment we can not say if PLD-SF is a product of degradation of a particular protein or if it is encoded by a separate gene. It is possible that hydrolysis of one of the known PLD-activating proteins yields a peptide which is still capable of binding PLD and increasing its catalytic activity. A 34 kDa proteolytic fragment of PKCα which contains a lipid binding domain has been shown to activate PLD (Singer et al., 1995). It is currently accepted that regulation of PLD is complex. However, it is still puzzling that so many unrelated proteins have been found to regulate PLD activity. Protein kinase C, small GTPases Arf, Rho, RalA and calmodulin have very little in common but a single feature. They all have a lipid interacting domain and under appropriate conditions can translocate from cytosol to membranes. Therefore, we would like to speculate that PLD-SF is small peptide, possibly derived from a larger protein, which has affinity for lipids. It can bind to membranes and its presence on the surface of a lipid bilayer is sufficient to activate membrane-associated PLD. It would be premature to assume that we know all possible mechanisms of PLD activity

regulation. It has been reported, for example, that yeast PLD1 and PLD2 are not activated by yeast Arf proteins (Rudge et al., 1998). We have found the presence of a small molecular weight PLD-stimulating factor in proliferating yeast cell. Therefore it is possible that PLD-SF represents a novel mechanism of PLD activity regulation which is present in yeast, plant and mammalian cells.

Chapter V

PLD-SF Activates PLD Associated with Caveolae-Related Membranes

Introduction

To date PLD has been found in virtually every subcellular fraction. It is present in cytosol (Wang et al., 1991; Siddiqi et al., 1995), in Golgi membranes (Ktistakis et al., 1995; Provost et al., 1996), in nuclei membranes (Balboa et al., 1995), in endoplasmic reticulum (Decker et al., 1996), in plasma membranes (Kim et al., 1999b) and caveolae (Czarny et al., 1998; Kim et al., 1999b). It is possible that specific mechanisms of PLD activation are distinct in different membranes. PLD activated by oncogenes has preference for the subclass of PC containing saturated fatty acids (Song and Foster, 1993). There was no increase in PLD activity in response to v-Src if the cells were prelabeled with polyunsaturated fatty acid arachidonate, which was incorporated efficiently into PC (Song and Foster, 1993). This suggests that v-Src and v-Ras activate PLD specific for PC present in membrane microdomains enriched with phospholipids containing saturated fatty acids (Simons and Ikonen, 1997). As we described in the previous chapters, PLD-SF is implicated as a mediator of PLD activation by v-Ras. Therefore it is possible that PLD activated by

PLD-SF may be restricted to a subset of cellular membranes. In this chapter we describe experiments performed to test this hypothesis.

Results

1. PLD-SF elevates PLD activity in a subset of membranes

Upon examination of the time course of PLD activation, we observed that PLD-SF not only elevated the PLD activity in isolated membranes, but ultimately resulted in a higher total level of PC hydrolysis (Figure 10). The amount of choline released by untreated membranes leveled off between 4 and 6 hours when approximately 5% of the labeled choline had been released. In contrast, in the presence of PLD-SF, the activity leveled off between 2 and 4 hours and the amount of choline released was close to 10% or roughly twice that seen in the untreated membranes. These data suggested the possibility that PLD-SF was acting on a PLD that was specific for a subpopulation of PC which is not utilized by the basal PLD activity present in the isolated membranes. Therefore PLD, which is activated by PLD-SF, must be associated with the distinct membrane fraction.

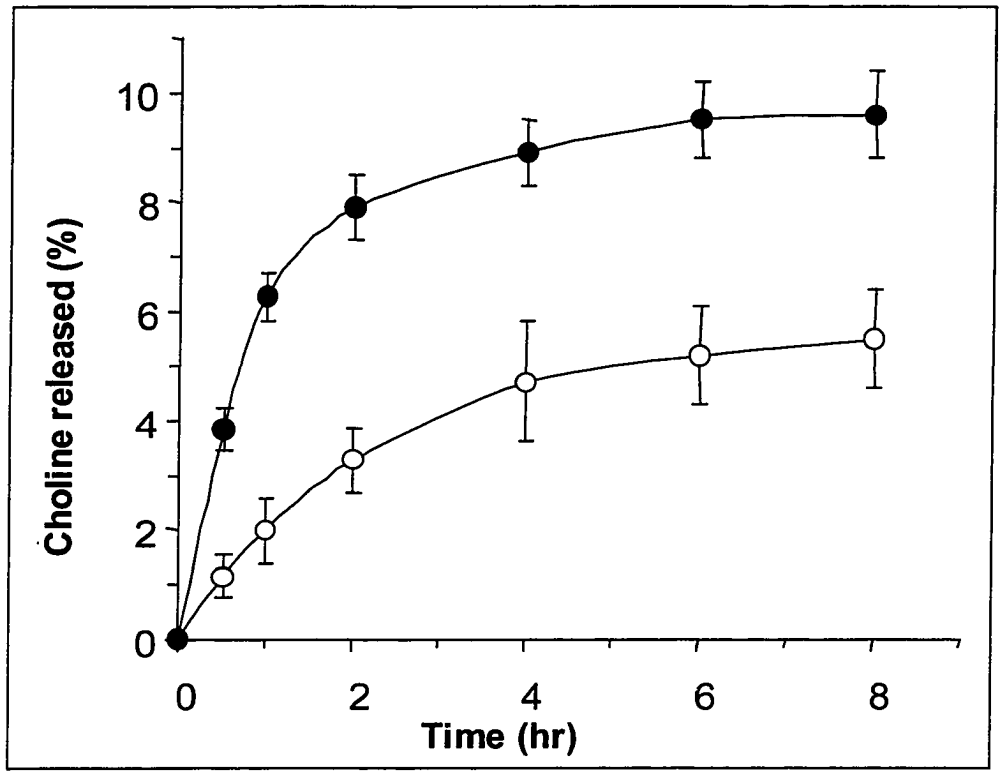

Figure 10. PLD-SF elevates PLD activity in a subset of membranes.

Membranes from Ras-transformed cells were labeled with [^{3}H]-choline and then isolated. Partially purified PLD-SF was prepared from Ras-transformed cells as described in Materials and Methods. The kinetics of choline release was then determined in the presence (solid circles) and absence (open circles) of the PLD-SF (1 μg of protein). The values are expressed as a percent of total lipid label released from membranes. Each data point represents the mean +/- standard error of three independent experiments performed in duplicate.

2. PLD stimulated by PLD-SF is localized in caveolae-enriched membranes

We next examined the effect of PLD-SF on PLD activity in different membrane fractions separated on the basis of density. Labeled membranes from Ras-transformed cells were isolated and disrupted by sonication as described in Materials and Methods. Membranes of different density were then separated by centrifugation through a discontinuous sucrose gradient (0.25 to 1.25 M sucrose). Membranes that layered on the 0.50, 0.75, 1.00 and 1.25 M sucrose cushions and the pellet were recovered. No membranes could be detected on top of the 0.25 M sucrose cushion. PLD activity in the recovered membrane fractions was then analyzed in the presence and absence of PLD-SF. As shown in Figure 11, similar levels of PLD activity were found in membranes from all fractions, however only the PLD activity from the lightest membrane fraction was stimulated by PLD-SF. This light membrane fraction generally corresponds to the cholesterol- and glycosphingolipid-rich membrane microdomains found in caveolae (Song et al., 1996). To verify this, we examined the presence of caveolin 2 protein in all membrane fractions, and as shown in Figure 12, caveolin 2 was observed almost exclusively in the light membrane fraction where PLD-SF elevated PLD activity. We also examined distribution of Ha-Ras protein and FGF receptor in our membrane fractions. In agreement with data published previously (Song et al., 1996), we found that Ha-Ras was also localized predominantly in the light membrane fraction

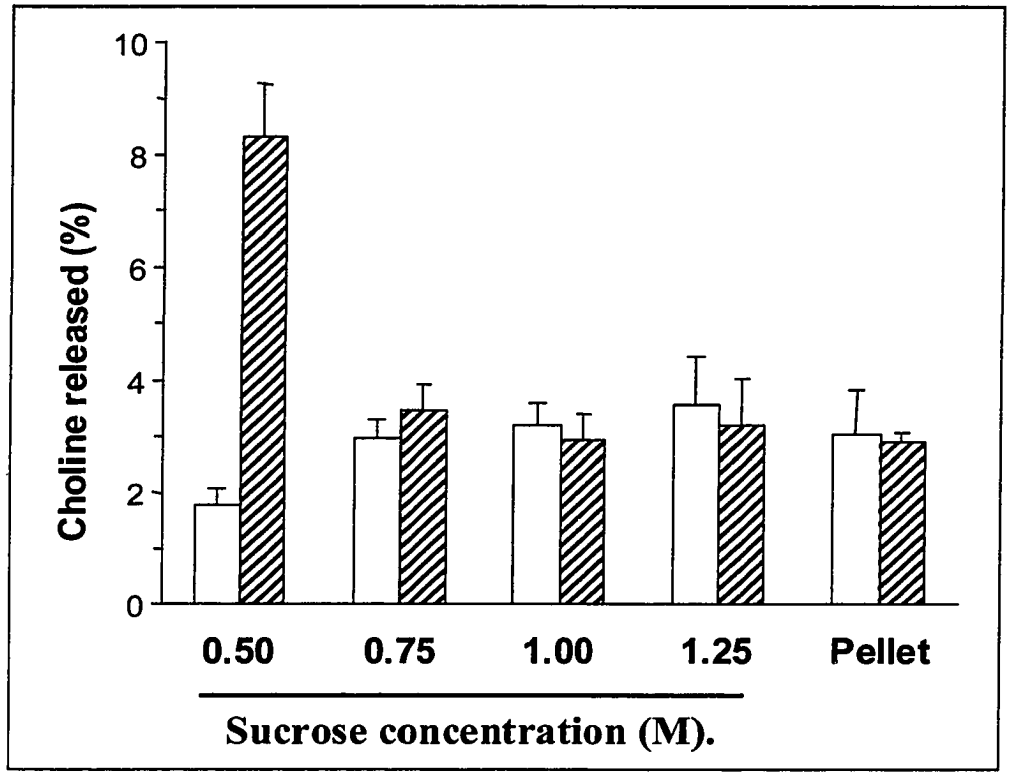

Figure 11. PLD-SF is specific for PLD in caveolae-related light membrane fractions.

Ras-transformed cells were labeled with [^{3}H]-choline and membrane fractions of different density were isolated by centrifugation through sucrose gradient as described in Materials and Methods. *In vitro* PLD activity was measured in the different fractions by the release of [^{3}H]-choline. Membranes were either incubated alone (open bars) or in the presence of partially purified PLD-SF (1 μg of protein) isolated from Ras-transformed cells (hatched bars). The values are expressed as a percent of total lipid label released from membranes in 1 hour. Each data point represents the mean +/- standard error of three independent experiments performed in duplicate.

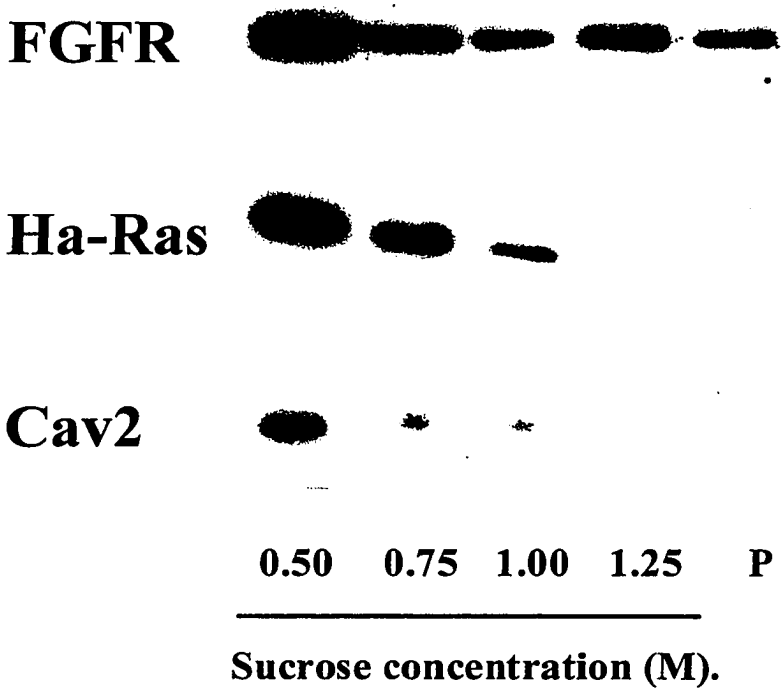

Figure 12. Distribution of caveolin 2, Ha-Ras and EGFR in the membrane fractions of different density.

Membrane fractions of different density were isolated from Ras-transformed cells as described in the legend in Figure 11. 20 μg of protein from each fraction were separated by SDS-PAGE and probed with specific antibodies as described in the Materials and Methods. The antibodies were raised against caveolin 2 (Cav2), Ha-Ras and the fibroblast growth factor receptor-1 (FGFR). A representative experiment repeated twice is shown.

(Figure 12). The fibroblast growth factor receptor was found in all membrane fractions, but was enriched approximately 2-3 times in the light membranes (Figure 12). These light membranes contained only a fraction of the total membrane protein (approximately 7 –8 %). The other fractions were as follow, 0.75 M – 10%, 1.0 M – 10 %, 1.25 M – 25% and membranes in the pellet contained about 40% of total protein.

Discussion

In this chapter we presented evidence that PLD-SF activates only a subset of PLD enzymes present in the cellular membranes. This subset is distinguished from other cellular PLDs by its localization to the specific subpopulation of membranes. The membranes have very low buoyant density, are enriched in phospholipids with saturated fatty acids and contain almost all of the cellular caveolin 2 protein. These data indicates that membranes are enriched in caveolae. Therefore, PLD that is activated by PLD-SF is exclusively localized in caveolae or caveolae-related membrane microdomains. It is interesting that the same fraction is highly enriched in Ras protein. It would make a lot of biological sense for two functionally linked membrane proteins to be localized in the close proximity on the cellular membranes.

Although, all of the membrane fractions contained detectable levels of PLD activity, only PLD in the light membranes was responsive to PLD-SF. First of all, this observation demonstrates that activation of PLD in caveolae-related membranes is

specific and not a result of a nonspecific action of PLD-SF on a membrane bilayer. Second, it suggests that PLD-SF exerts its effect on PLD in conjunction with another PLD activator such as RalA, Arf or Rho family GTPases. It is unlikely that the factor is working with PKC, since PLD-SF is elevated in v-Src- and v-Ras-transformed cells where the activation of PLD is independent of PKC (Song and Foster, 1993; del Paso et al., 1996). A more probable scenario is that activation of PLD by the factor requires RalA protein, which is involved in PLD activation in v-Src and v-Ras transformed cells (Jiang et al., 1995b; Luo et al., 1998). In support of this hypothesis we can add that preliminary experiments underway in our laboratory show that RalA protein is almost exclusively localized in the light membrane fraction in cells transformed by v-Src and v-Ras oncogenes. At the same time, Arf proteins were found to be uniformly distributed in membranes of different density (Lizhong Xu, personal communication).

Chapter VI

Differential Effects of PLD-SF on Normal and Transformed Cells *In vivo*

Introduction

In previous chapters we described identification of a small cytosolic molecule, presumably a peptide, which activates membrane-bound PLD in caveolae-related membranes. The level of this PLD-stimulating activity shows a high degree of correlation with cell's proliferative state, with higher activity observed in actively dividing cells and tissues which contain large number of dividing cells. Therefore we presume that PLD-stimulating factor, is responsible at least in part for increase in PLD activity observed in transformed and dividing cells.

PLD activity is clearly involved in mechanisms of cellular proliferation as it has been found to be activated by every growth factor tested (Exton, 1998). Hydrolysis of phosphatidylcholine by PLD also generates phosphatidic acid, a recognized mitogen and a precursor for other lipid signaling molecules. However, direct confirmation of PLD contribution to the mitogenic signaling is complicated since activation of PLD is only one of the many reactions induced by growth factors. For this reason it would be advantageous to have a specific inhibitor or a specific activator of PLD which could work on intact cells.

PLD-SF is a small molecule, less then 1000 Da, which likely able to interact with a lipid bilayer. It is possible that due to its small size PLD-SF might be able to penetrate cellular membranes. If so, the factor may activate PLD *in vivo*, most probably the PLD involved in mitogenic signaling. This could provide us with additional information regarding the role that PLD activity plays in mitogenic signaling. In this chapter we describe the experiments intended to investigate this hypothesis.

Results

1. *PLD-SF activates PLD in vivo*

We have tested the ability of PLD-SF to activate PLD in intact NIH 3T3 and Ras transformed NIH 3T3 cells. Partially purified PLD-SF was added directly to cell culture medium and PLD activity was measured by transphosphatidylation in the presence of 1-propanol. There were no changes in PLD activity in cultures treated with the factor for 1 and 2 hours (Figure 13). However, after 4 hours incubation with PLD-SF both NIH 3T3 and Ras-transformed cells show a significant increase in PLD activity (Figure 13). This data demonstrates that PLD-SF is able to penetrate cellular membranes and activate PLD in intact cells. The activation is not rapid and requires at least 4 hours of incubation of the cells in the presence of the factor, apparently needed for PLD-SF to reach sufficient intracellular concentration.

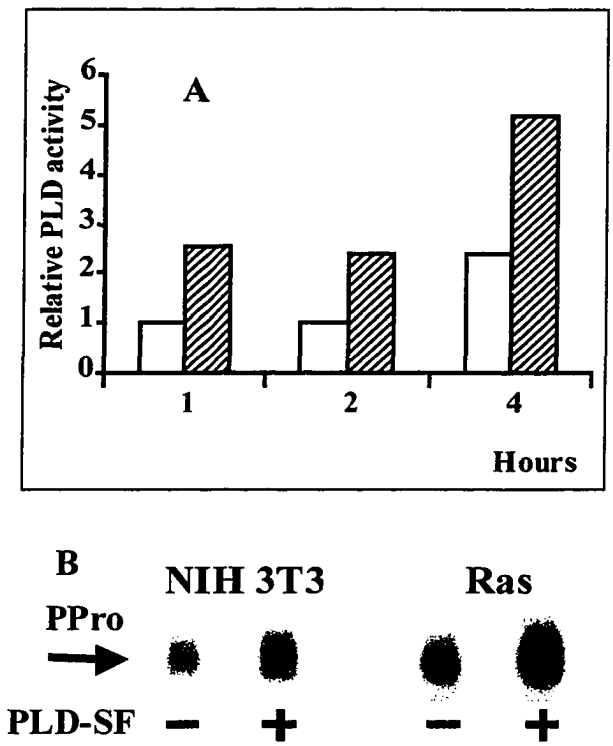

Figure 13. PLD-SF activates PLD in vivo.

NIH 3T3 and Ras-transformed cells were grown to 50 % confluence and made quiescent by changing the medium to DMEM with 0.5 % bovine calf serum and growing overnight. 2 μCi/ml of [^{3}H]-myristate was added to the medium. The following day, partially purified PLD-SF from rat testes was added to cell culture (0.5 mg/ml). 1-propanol was added to the medium (1 % final concentration) for 1 hour and products of transphosphatidylation reaction were analyzed as described in Materials and Methods. (A) NIH 3T3 (open bars) and Ras-transformed cells (hatched bars) were incubated with PLD-SF for different amount of time. (B) Cells were incubated with PLD-SF for four hours. A representative experiment, which was repeated three times, is shown.

2. PLD-SF causes apoptosis in normal but not Ras-transformed NIH 3T3 cells

We next examined the possibility that PLD-SF might have a growth promoting effect on cells in culture. Partially purified PLD-SF was added to cell culture medium and cells were examined microscopically at different times. NIH 3T3 cells begin to show distinct morphological changes after incubation with the factor for 6 to 8 hours. Their plasma membranes start shrinking and some cells detach from the surface of the plates. No changes could be seen in Ras-transformed cells at this time. Incubation with the factor overnight (16-18 hours) caused cell death in the NIH 3T3 cells, while Ras-transformed cells only display slightly altered morphology (Figure 14). We also tested different concentrations of PLD-SF for the ability to induce cell death in normal and Ras-transformed NIH 3T3 cells. As shown in the Figure 15, Ras-transformed cells were resistant to the cytotoxic effect of the factor, while the number of surviving NIH 3T3 cells shows a progressive decline with increased concentrations of PLD-SF. The concentration of the factor at which more than 90 %, of the normal cells were killed had no effect on the Ras-transformed cells (Figure 15).

The appearance of the dead NIH 3T3 cells resembled that of apoptotic bodies. Namely, they were significantly smaller in size than living cells and chromatin in their nuclei was condensed and fragmented (Figure 14). In order to verify that NIH 3T3 cells indeed died of apoptosis we extracted DNA from cells treated with PLD-SF and analyzed it by agarose electrophoresis. As shown in the Figure 16, DNA from

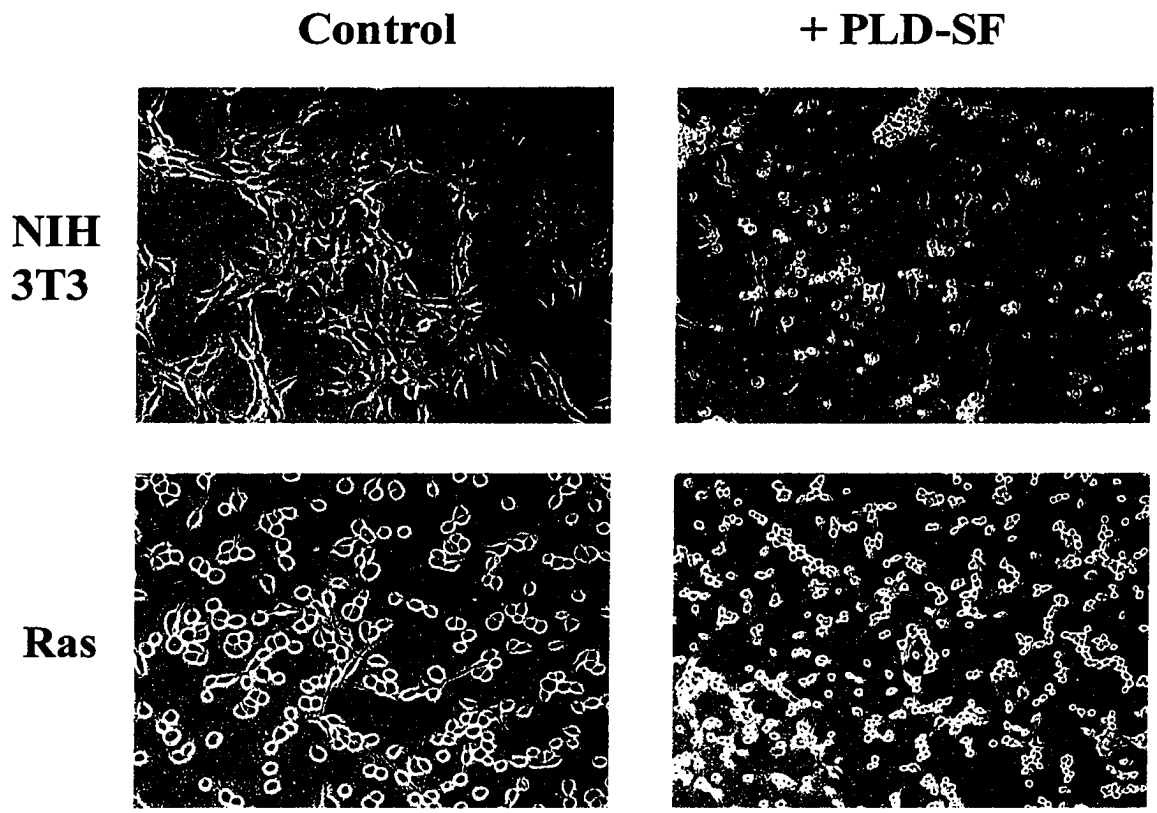

Figure 14. PLD-SF induces cell death in normal but not Ras-transformed cells.

NIH 3T3 and Ras-transformed cells were grown and made quiescent as described in the Figure 13. Partially purified PLD-SF from rat testes was then added to cell culture medium (0.5 mg/ml) and cells were incubated for additional 16-18 hours. Cells were photographed using Nikon Optiphot 2 microscope equipped with digital photo camera. Representative fields from an experiment that was repeated two times are shown.

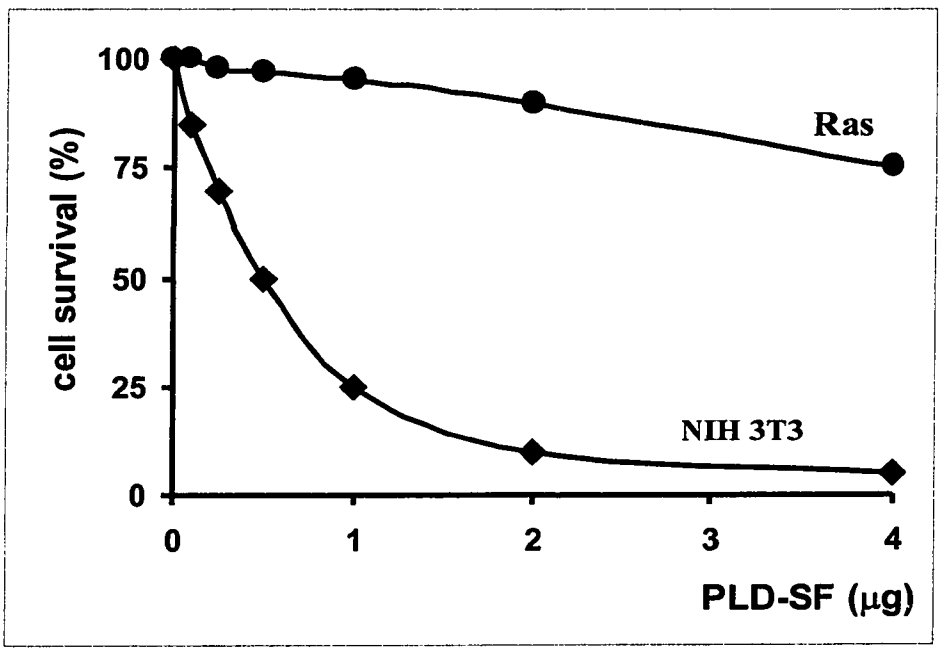

Figure 15. Differential sensitivity of NIH 3T3 and Ras-transformed cells to PLD-SF *in vivo*

NIH 3T3 and Ras-transformed cells were grown and made quiescent as described in the Figure 13. Different concentrations of partially purified PLD-SF from rat testes was then added to cell culture medium and cells were grown for additional 16-18 hours. Plates were washed and amount of surviving cells was determined as described in Materials and Methods. The number of cells in the cultures that were grown without PLD-SF was assigned a value of 100 %. Each data point represents the mean of two independent experiments performed in thriplicate.

NIH 3T3 cells treated with PLD-SF for 8 hours shows a distinct "laddering" pattern, a characteristic feature of internucleosomal DNA cleavage in apoptotic cells. This result confirms that PLD-SF treatment of normal fibroblasts induces them to undergo programmed cell death. There was no DNA fragmentation in similarly treated Ras-transformed cells (Figure 16).

The PLD –SF used in the previous experiments was only partially purified and we reasoned that it might contain a number of biologically active molecules. Therefore treatment of cells with the factor could have effected multiple cellular targets. In order to verify that it was the activation of PLD that caused apoptosis in NIH 3T3 cells, we examined the effect of primary and secondary alcohols on the PLD-SF-induced programmed cell death. As was mentioned above (see Chapter I, Introduction) this approach is often used to demonstrate involvement of PLD in processes that take place in complex biological systems. One of the unique properties of mammalian PLD is the ability to catalyze a transphosphatidylation reaction. Only primary alcohols are substrates for this reaction. The formation of a specific phosphatidylalcohol will interfere with production of the physiologically relevant product of PLD – phosphatidic acid. Secondary alcohols are usually used as a negative control for nonspecific alcohol action, since a high concentration of alcohol are required to achieve noticeable inhibition of PA production. In our experiments we examined the effect of different concentrations of 1- and 2-propanol on PLD-SF-induced apoptosis in NIH 3T3 cells. These two alcohols had a dramatically different

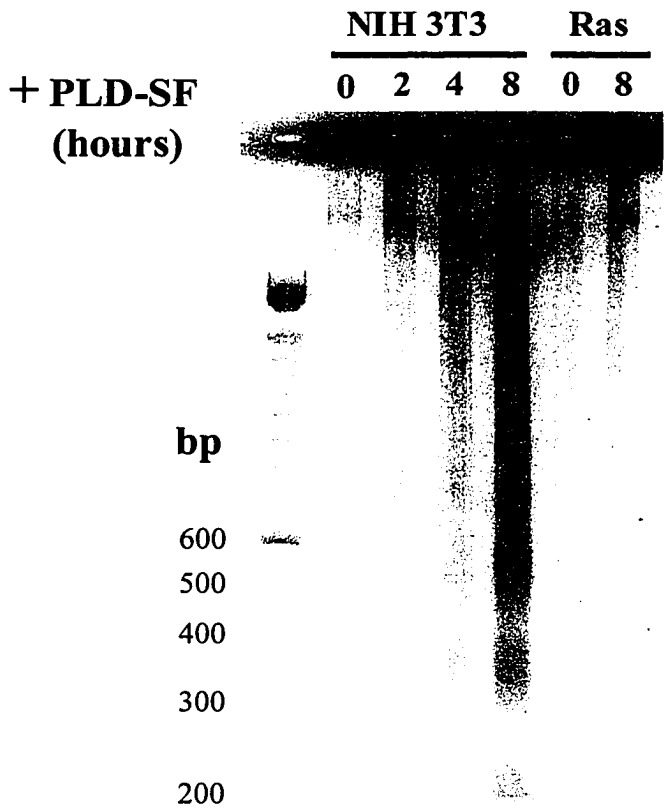

Figure 16. PLD-SF treatment causes characteristic apoptotic DNA fragmentation in NIH 3T3 cells. NIH 3T3 and Ras-transformed cell were grown and made quiescent as described in the Figure 13. Cells were treated with 0.5 mg/ml of partially purified PLD-SF from rat testes. At times indicated, DNA was extracted and analyzed by agarose electrophoresis as described in the Materials and Methods. Position of molecular weight DNA markers are shown. A representative experiment, which was repeated three times, is shown.

effect on survival of NIH 3T3 cells treated with PLD-SF. Primary propanol, in concentrations of up to 1.0 %, produced a concentration-dependent increase in the survival rate of NIH 3T3 cells when added to the cell culture medium (Figure 17). Higher concentrations show a reverse trend, probably due to general alcohol toxicity. At the same time, secondary propanol did not have any significant effect on survival of NIH 3T3 cells treated with PLD-SF at any concentration we have tested (Figure 17). This data strongly implicates PLD activity as a causative agent in the induction of apoptosis in NIH 3T3 cells treated with PLD-SF.

3. PLD-SF induces transformed phenotype in partially transformed cell

As was shown above, PLD-SF treatment led to apoptosis in NIH 3T3 and did not have a significant effect on proliferation of Ras-transformed cells (Figure 15). The lack of effect of PLD-SF on Ras-transformed cell could be explained by the fact that these cells are already highly transformed. The presence of an activated Ras oncogene in these cells might be sufficient to activate cell cycle progression at the maximum allowed speed and therefore, further mitogenic stimulation of these cells is not feasible. However, it is possible that PLD-SF might show its mitogenic potential on cells that are resistant to its apoptosis-inducing effect but not yet fully mitogenicaly stimulated. Therefore, we investigated the effect of PLD-SF treatment on a number of partially transformed cells. As shown in the Figure 18, 3Y1 rat fibroblast cells overexpressing c-Src protein or EGF receptors have normal

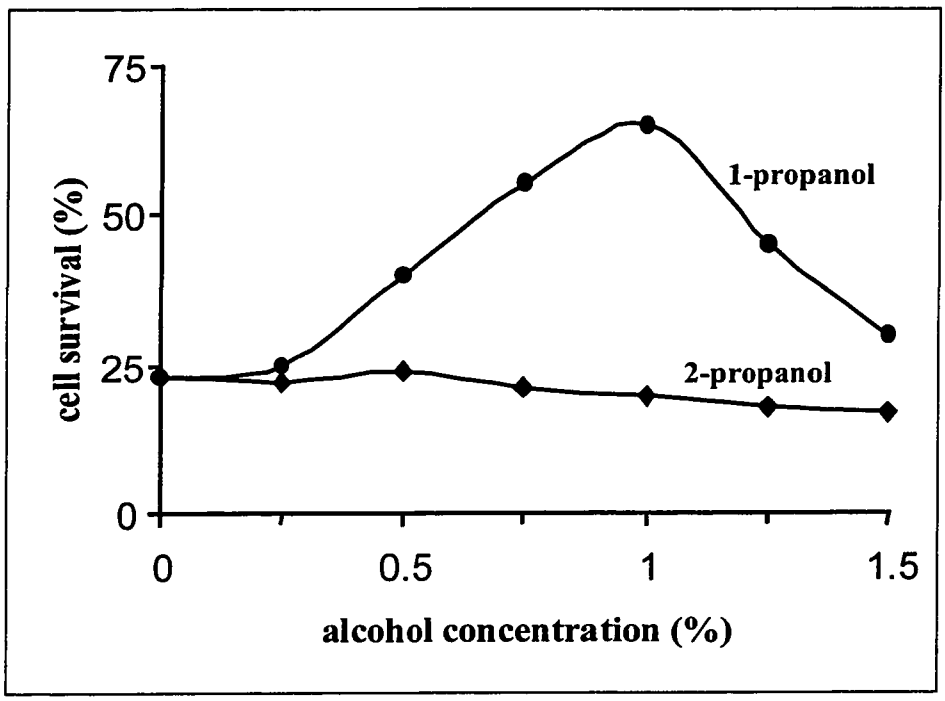

Figure 17. Differential effects of primary and secondary alcohols on survival of NIH 3T3 cell treated with PLD-SF.

NIH 3T3 were grown and made quiescent as described in the Figure 13. Cells were treated overnight with 0.5 mg/ml of partially purified PLD-SF from rat testes and different concentrations of 1- and 2-propanol. Plates were washed and amount of surviving cells was determined as described in Materials and Methods. The number of cells in the cultures that were grown without PLD-SF was assigned a value of 100 %. Each data point represents the mean of two independent experiments performed in thriplicate.

fibroblastic morphology. Treatment of these cells with PLD-SF led them to acquire a transformed phenotype. They become more refractile and formed small colonies on cell culture plates (Figure 18). This data suggests that PLD activity is an important part of mitogenic signaling, yet additional mitogenic signaling pathways are required for cells to initiate cell cycle progression.

Discussion

In this chapter we described the effects of PLD-SF treatment on intact cells. First we have shown that PLD-SF is able to activate PLD *in vivo* in normal NIH 3T3 cells and in Ras-transformed cells. This activation was not rapid, as several hours are required before increased PLD activity could be detected in treated cells. This time is apparently necessary for PLD-SF to reach sufficient intracellular concentration. This data suggests that PLD-SF is able to penetrate cellular membranes and therefore could be utilized to study the functions of PLD activity in intact cells.

PLD-SF treatment had a dramatically different effect on normal and transformed cells. Concentration of the factor that caused cell death in more then 90 % of NIH 3T3 cells had no effect on the survival rate of Ras-transformed cells (Figure 15). It is possible that Ras-transformed cells tolerate higher levels of PLD activity because they have an increased rate of PC biosynthesis (Ratnam and Kent, 1995). Cell death induced by PLD-SF in NIH 3T3 cells is apparently due to the

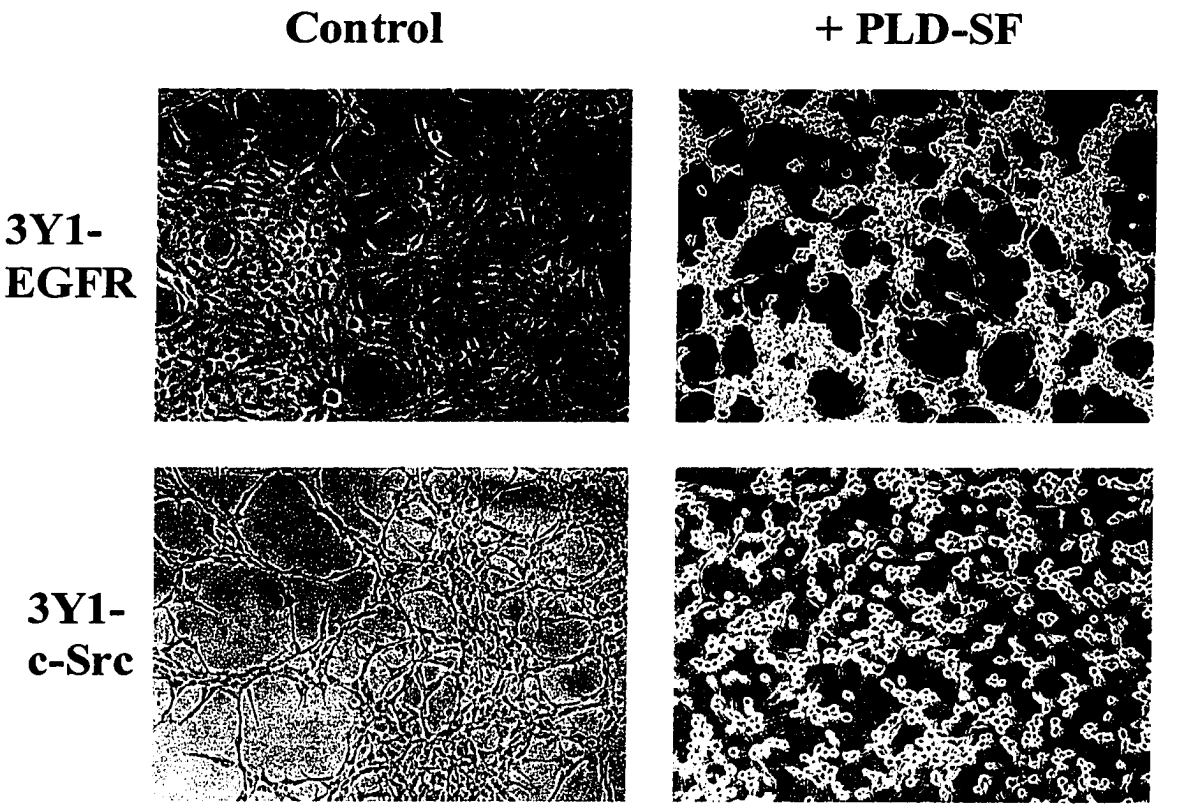

Figure 18. PLD-SF treatment induces transformed phenotype in partially transformed cells.

3Y1 rat fibroblast cells overexpressing c-Src or EGF receptor were grown and made quiescent as described in the Figure 13. Where indicated, cells were treated with 1 mg/ml of partially purified PLD-SF from rat testes for 48 hours. Cells were photographed using Nikon Optiphot 2 microscope equipped with digital photo camera. Representative fields from an experiment that was repeated four times are shown.

activation of their apoptotic program (Figure 16). We also demonstrated that increase in PLD activity, but not some other reaction, was in fact the cause of apoptosis in these cells after treatment with PLD-SF (Figure 17). These results are in agreement with the data obtained in our laboratory previously. It was shown that increased expression of PLD1 in normal 3Y1 rat fibroblasts is lethal to these cells. At the same time, cell that are partially transformed due to the presence of a large number of EGF receptors on their surface were able to tolerate higher levels of PLD1 expression (Lu et al., 1999).

Ras-transformed NIH 3T3 cells did not undergo apoptosis when treated with PLD-SF. Contrary to our expectations, however, we did not observe a significant effect of increased PLD activity on proliferation of these cells. It is possible that Ras-transformed cells have already fully activated their mitogenic signaling pathways. Therefore, further mitogenic stimulation of this cell might not produce any noticeable effect. We were able to detect a mitogenic effect of PLD-SF stimulation on cells that were partially transformed. 3Y1 rat fibroblast cells overexpressing c-Src or EGF receptors become morphologically transformed after treatment with the factor (Figure 18). This observation is in agreement with the results recently obtained in our laboratory. We have demonstrated that overexpression of PLD1 in 3Y1 cells that overexpress the EGF receptor leads to transformation of these cells in the absence of EGF (Lu et al., 1999). These data demonstrate that PLD activity does contribute to mitogenic signaling, although by itself it is not sufficient to stimulate cell cycle

progression in quiescent cells. Similar observations have been previously published. It has been shown, for example, that complete transformation of cells by Ras oncogene requires simultaneous activation of several distinct mitogenic signaling pathways (White et al., 1995).

Chapter VII

Summary

Lipids play an important role as precursors for a number of mitogenic signaling molecules. Phosphatidic acid, diacylglycerol and lysophosphatidic acid are just a few of the examples of such molecules. Phosphatidylcholine, the major phospholipid of cellular membranes, is an important source for these signaling lipids. It is currently accepted that the major reaction of phosphatidylcholine breakdown is catalyzed by PC-specific phospholipase D. The importance of this reaction for mitogenic signaling is underlined by the fact that many mitogens and growth factors cause rapid and robust increase in PLD activity (Exton, 1998). Elevated levels of PLD activity have also been found in a number of transformed cells (Song et al., 1991; Wyke et al., 1992; Carnero et al., 1994; Jiang et al., 1995a; Martin et al., 1997; Frankel et al., 1999), leading to the notion that sustained high PLD activity must be important for maintaining a transformed phenotype. Therefore there is a great interest to understand the molecular mechanisms leading to PLD activation in transformed cells, as such understanding might provide new molecular targets for diagnostics and possibly treatment of malignant diseases.

Work conducted in our laboratory has illuminated several important steps leading to PLD activation in cells transformed by v-Src and v-Ras oncogenes. Most

importantly, RalA, a small GTPase of the Ras family which is involved in intracellular signal transduction, has been shown to be required for PLD activation in transformed cells (Jiang et al., 1995b). RalA protein directly associates with PLD1 (Luo et al., 1997). The RalA/PLD1 complex next interacts in a GTP-dependent manner with Arf protein which results in the activation of PLD (Luo et al., 1998). These studies also implicated existence of additional factors present in transformed cells that contribute to PLD activation.

In this report we have described the discovery of a low molecular weight cytosolic factor which stimulates PLD activity in isolated membranes. The level of PLD-SF was substantially elevated in the cytosol of cells transformed by v-Ras. We also detected increased PLD-SF in the cytosol of cells transformed by v-Src and v-Raf and in NIH 3T3 cells stimulated with serum, suggesting that PLD-SF is increased in dividing cells. PLD-SF was present in several rat tissues but was highest in rat testes, which contains the highest percentage of dividing cells of the tissues examined. Thus, PLD-SF may contribute to increased PLD activity in response to mitogenic stimuli.

PLD-SF is apparently a peptide consisting of no more than 10 aminoacids. The fact that PLD-SF is sensitive to alkaline phosphatase treatment suggests that its activity may be regulated by phosphorylation. We do not know if this peptide is a product of degradation of a particular protein or if it is encoded by a separate gene since we were not able to purify PLD-SF to homogeneity. It is possible that

hydrolysis of one of the known PLD-activating proteins yields a peptide which is still capable of interacting with PLD and increasing its catalytic activity.

PLD-SF is evidently a ubiquitous molecule. We detected the presence of a small molecular weight PLD-stimulating factor not only in mammalian cells and tissues but also in proliferating yeast cells. A similar PLD-stimulating activity was reported in the cytosol from *Arabidopsis* plants (Pappan et al., 1997).

We demonstrated previously that the PLD activated in response to v-Src had an apparent substrate preference for PC that had been prelabeled with saturated fatty acids (Song et al., 1991; Song and Foster, 1993). The differential utilization of the PC prelabeled with saturated and unsaturated fatty acids in v-Src-transformed cells was lost when examined *in vitro* (our unpublished results), suggesting that there was no inherent substrate specificity for the v-Src-induced PLD. The apparent *in vivo* substrate specificity could be explained by a PLD that acted on PC localized to regions of the membrane that were enriched for saturated fatty acids. In recent years much interest has been generated in lipid rafts, membrane microdomains which are highly enriched in phospholipids containing saturated fatty acids (Harder and Simons, 1997; Simons and Ikonen, 1997). It has been proposed that Van der Waals interactions between fully extended saturated fatty acid tails and cholesterol is the thermodynamic driving force for the assembly of these membrane microdomains. These rafts, which can form caveolae in the presence of caveolin, have been suggested to be scaffolding sites for the assembly of signaling complexes since many

proteins implicated in the transduction of mitogenic signals including Ras, Raf, and Src are highly enriched in caveolae (Okamoto et al., 1998; Anderson, 1998). Thus, it is possible that the PLD activated in response to mitogenic signals is restricted to the caveolae-related light membrane fractions that are enriched in phospholipids containing saturated fatty acids. In our experiments we discovered that PLD-SF is apparently specific for PLD localized in caveolae-related light membrane fractions. Taken together with the fact that PLD-SF is elevated in dividing and mitogenically stimulated cells, these observations strongly implicate PLD-SF as a specific regulator of PLD activity involved in the transduction of mitogenic signals.

Although PLD activity is an important part of mitogenic signaling, activation of PLD alone apparently is not sufficient to induce proliferation in quiescent cells. In our hands, activation of PLD in normal NIH 3T3 cells by PLD-SF did not promote cell cycle progression but instead led to apoptosis. However in our preliminary experiments we observed that PLD-SF was able to induce a transformed phenotype in cells that have a low level of constitutive mitogenic stimulation. It is therefore most likely that PLD activity contributes to cell proliferation and transformation by cooperating with other mitogenic signaling pathways. Future research will undoubtedly identify specific signaling pathways involved and the exact mechanisms of this cooperation.

Chapter VIII

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