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Role of Small GTPases in Phospholipase D Activation and Tumorigenesis

by

PAUL FRANKEL

A dissertation submitted to the Graduate Faculty in Biology in partial fulfillment of the requirements for the degree of Doctor of Philosophy
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ABSTRACT

Role of Small GTPases in Phospholipase D Activation and Tumorigenesis

by

Paul Frankel

Advisor: Professor David A. Foster

The activation of cellular phospholipase D (PLD) is commonly elevated in response to mitogenic signals. We reported previously that although the transformed phenotype induced by v-Src was dependent upon Raf-1, the PLD activity induced by v-Src was independent of Raf-1. This observation suggested to us that Raf would not likely be an activator of PLD. However, upon examination of PLD activity in v-Raf-transformed cells, surprisingly, we found that PLD activity is elevated to levels that were even higher than that observed in v-Src-transformed cells. To characterize the mechanism of v-Raf-induced PLD activity, we examined the dependence of v-Raf-induced PLD activity upon protein kinase C (PKC), the small GTPases RalA, and Rho, which have all been implicated in the activation of PLD. The v-Raf-induced PLD activity was inhibited by dominant negative mutants for both RalA and Rho. The dependence upon Ral was particularly surprising since RalA is a downstream target of Ras, which is an upstream activator of Raf.

Depleting cells of PKC by long term phorbol ester treatment actually increased PLD activity in v-Raf-transformed cells, indicating that v-Raf-induced PLD activity is not dependent on PKC. These data describe a novel mechanism for PLD activation by v-Raf that is independent of PKC, but dependent upon both RalA and Rho GTPases. The outcomes of these experiments questions the classical upstream/downstream paradigm used to describe signaling pathways. The requirement for Ral in v-Raf induced PLD activity may point to an emerging theme, that effectors may promote complex formation at the level of Ras, rather than simply forming a linear signaling pathway.

Given the result that RalA is involved in v-Src, v-Ras, and v-Raf induced PLD activity. We have investigated the role of RalA in tumorigenisis. Overproduction of urokinase-type plasminogen activator (uPA) and metalloproteases (MMPs) is strongly correlated with tumorigenicity and with invasive and metastatic phenotypes of human and experimental tumors. We demonstrated previously that overproduction of uPA in tumor cells is mediated by a phospholipase D (PLD)- and protein kinase C-dependent mechanism. The oncogenic stimulus of v-Src and v-Ras results in the activation of PLD, which is dependent upon RalA. We have therefore investigated whether RalA plays a role in uPA and MMP overproduction that is observed in response to oncogenic signals. We found that NIH 3T3 cells transformed by both v-Src and v-Ras, constitutively overproduce uPA and that expression of a dominant negative RalA mutant (S28N) blocks overproduction of uPA in both the v-Src-and v-Ras-transformed cells. v-Src and v-Ras also induced an upregulation of the activity of MMP-2 and MMP-9 as

detected by zymograms, however only the v-Src induction correlated with MMP protein levels detected by Western blot analysis. The dominant negative RalA mutant blocked increased MMP-2 and 9 overproduction induced by v-Src, but not the increased activity of MMP-2 and 9 induced by v-Ras. And, consistent with a role for the RalA/PLD pathway in mitogenesis and tumor development, the dominant negative RalA mutant completely blocked tumor formation by v-Src- and v-Ras-transformed NIH 3T3 cells injected subcutaneously in syngeneic mice. The data presented here implicate RalA and PLD as signaling mediators for tumor formation and protease production by transformed cells.

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I want to express my deepest gratitude to Dr. David Foster, my mentor, for his encouragement and guidance, as well as for the freedom to follow new ideas.

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To my brother Gary, and my sister Michelle. I wish to thank you both for your steadfast support and encouragement through some very trying times. I love you both very much.

Finally, I dedicate this dissertation and my doctorate to the memories of my beloved parents Marvin and Sydell. Without their love and support I never could have achieved all that I have.

In Memoriam

Marvin Frankel

Sydell Frankel

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LIST OF ABBREVIATIONS

DG	Diacylglycerol
DMEM	Dulbecco Eagle modified medium
DTT	Dithiothreitol
BFGF	Basic Fibroblast Growth Factor
GAP	GTPase Activating Protein
GDP	Guanosine-5'-diphosphate
GEF	Guanine nucleotide exchange factor
GTP	Guanosine-5'-triphosphate
LPA	Lyso-Phosphatidic Acid
MMP-2	Matrix Metalloproteinase-2
MMP-2	Matrix Metalloproteinase-9
PA	Phosphatidic Acid
Pbut	Phosphatidylbutanol
PC	Phosphatidylcholine
PDGF	Platelet Derived Growth Factor
PI3K	Phosphatydilinositol 3-Phosphate Kinase
PIP_2	Phosphatidyl inositol 4.5 diphosphate
PKC	Protein kinase C
PLC	Phospholipase C
PLD	Phospholipase D
PS	Phosphatidylserine
RalGDS	Ral GDP dissociation stimulator
TPA	12-O-Tetradecanoylphorbol 13-Acetate
uPA	Urokinase Plasminogen Activator
uPAR	Urokinase Plasminogen Activator Receptor

Chapter I

Introduction

Phospholipase D

Introduction

The class of enzymes known as phospholipases includes (Phospholipase D (PLD), PLA1, PLA2, and PLC) they function to hydrolyze membrane phospholipids to generate different biological molecules as shown in Figure 1. Phospholipase D (PLD) is present in bacteria, fungi, plants, and animals. It is widely distributed in mammalian cells, where it is regulated by a variety of hormones, growth factors, and other extracellular signals. Its major substrate is phosphatidylcholine (PC), which is hydrolyzed to phosphatidic acid (PA) and choline (Figure 1).

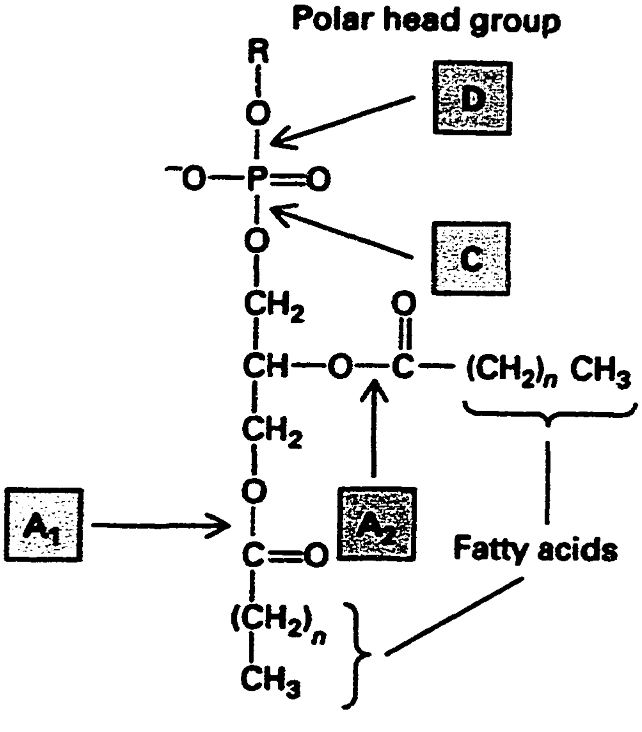


Figure 1. Phospholipase cleavage sites on phospholipids.

PLD catalyzes a phosphatidyl transfer reaction in which a primary alcohol, such as n-butanol, acts as nucleophilic acceptor in place of water. The resulting production of phosphatidyl alcohol represents a specific assay for PLD (Figure 2).

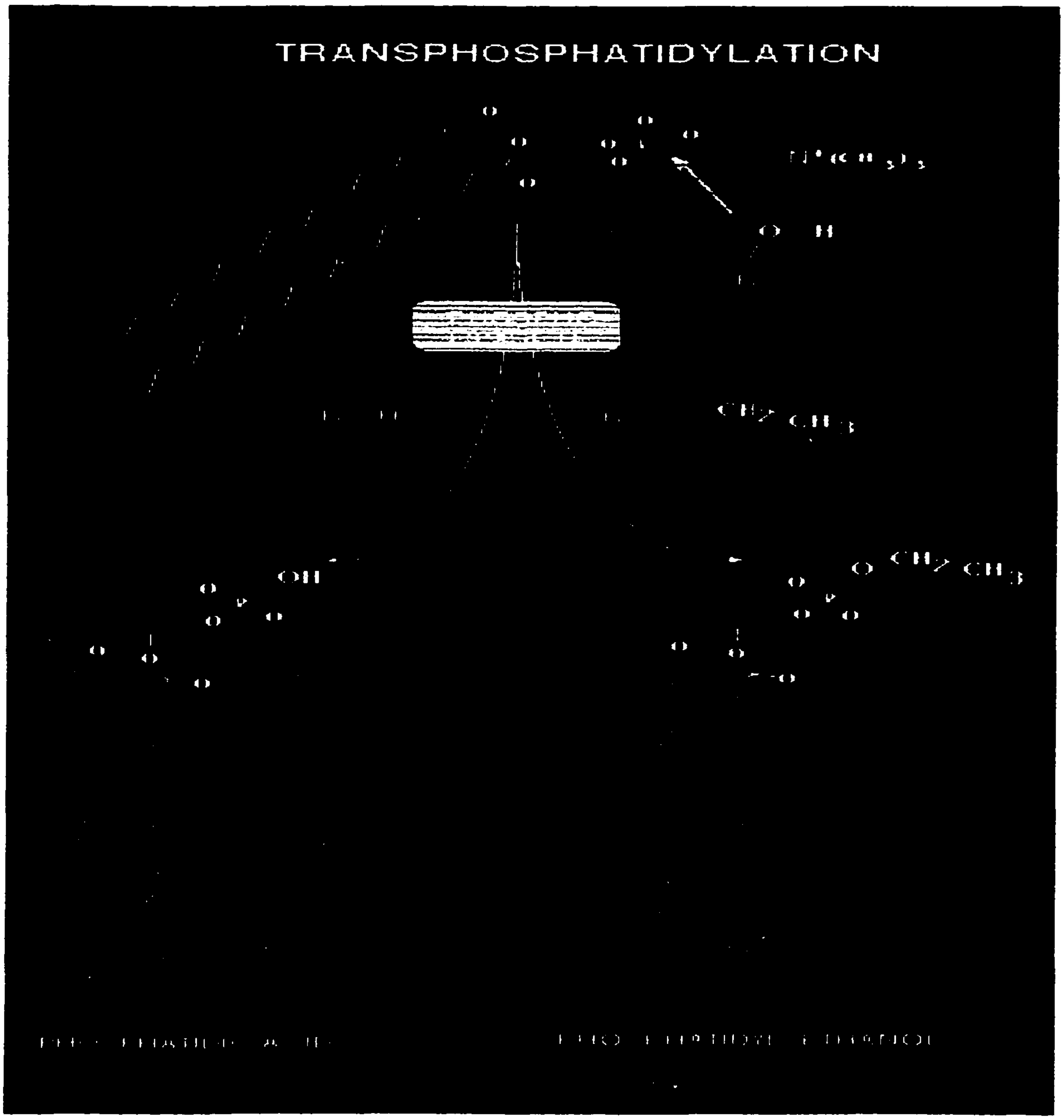


Figure 2. PLD specific transphosphatidylation reaction.

PLD Structure and Localization

PLD has recently been cloned from yeast, bacteria, plant, and mammalian sources (Morris *et al.*, 1996). Two separate mammalian PLD genes approximately 50% identical have been reported (hPLD1 and hPLD2;)(Colley *et al.*, 1997), with hPLD1 having two splice variants hPLD1a and hPLD1b. hPLD1a has 1072 amino acids and a molecular mass of 124 kDa (Hammond *et al.*, 1995). It is specific for PC and was obtained by using the yeast PLD gene (SPO14) (Rose *et al.*, 1995) to identify a human expressed sequence tag for screening a HeLa cDNA library. A shorter splice variant of hPLD1a with 1034 amino acids (hPLD1b) (Figure 3), which has similar regulatory properties, has been identified (Hammond *et al.*, 1997), and another PLD (PLD2) (Figure 3), which has 932 amino acids and 51% amino acid sequence identity to hPLD1a, has been cloned from a mouse embryonic library (Colley *et al.*, 1997). The amino acid sequence of PLD1 contains regions which are conservered with PLD2 as well as other nonmammalian species. In addition it contains a "loop region" that is unique to PLD1. Possible functions that have been proposed or demonstrated for these regions are shown in (Figure 4).

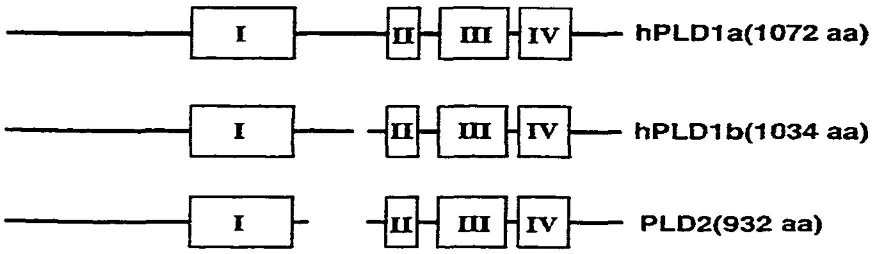


Figure 3. Alignment of conserved regions common to hPLD1a, hPLD1b, and PLD2. (Borrowed from Exotn. J.H. 1997)

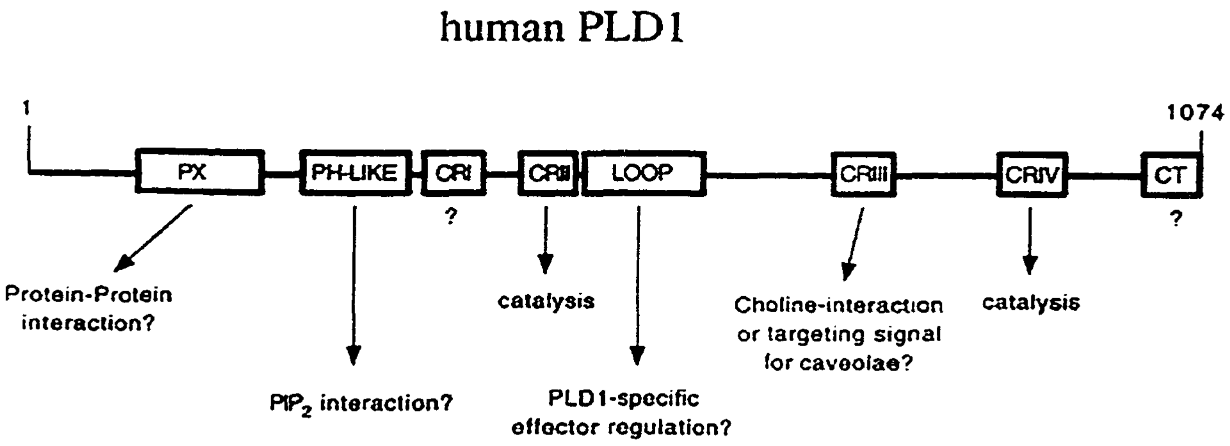


Figure 4. Conserved and unique features for human PLD1. The PLD1 amino acid sequence encodes regions of sequence that either are unique to PLD1 (loop region) or are conserved with mammalian PLD2 and some or all PLDs from nonmammalian species (other boxed regions). Possible functions that have been proposed or demonstrated for these regions are listed underneath each box. CT, carboxyl terminus; LOOP, loop region. (Borrowed from Sung et al., 1999)

The subcellular localization of PLD is still unclear at this time. Some groups have reported that in fibroblasts PLD2 localizes predominantly in the caveolin-rich membrane domains of the plasma membrane, whereas PLD1 is perinuclear, i.e. in endoplasmic reticulum, Golgi, and late endosomes (Colley *et al.*, 1997; Czarny *et al.*). While other groups, including our own, have found PLD1 and PLD2 to be localized in the cavolin enriched membrane fraction (Kim *et al.*, 1999; unpublished results) (Figure 5).

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

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

Regulation of PLD Activity

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

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

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

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

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

RhoA action on PLD is enhanced by other as yet unidentified cytosolic proteins (Shimooku *et al*., 1996; Kwak *et al*., 1995).

There is abundant evidence that PLD is regulated by PKC in most mammalian cells. This comes from studies of the effects of phorbol esters, PKC inhibitors, down-regulation of the enzyme, and overexpression and deletion of specific PKC isozymes (Lu *et al*., 1999; Hornia *et al*., 1999). Although a role for PKC in the actions of many agonists on PLD in many cells has been indicated, there are also instances where the enzyme does not seem to be involved, as in the case of v-Src and v-Ras (Exton JH. 1997; Song *et al*., 1993; See below).

Physiological Roles of PLD

The involvement of small GTPases like Ras (Jiang *et al*.,1995a), Rho (Ohguchi *et al*.,1995), and Arf (Hammond *et al*.,1995) in the activation of PLD suggest that PLD activation might play a role in cytoskeleton reorganization and intracellular protein trafficking. PLD has been implicated in the regulation of vesicle trafficking by ARF in Golgi. The formation of coated vesicles is also inhibited by ethanol, which reduces PA formation due to the production of phosphatidylethanol, and treatment of Golgi membranes with bacterial PLD promotes coated vesicle formation (Kistakis *et al*., 1996). Studies with ethanol and exogenous PLD in other cell types have also provided evidence that PLD is involved in vesicle transport (Cockcroft S. 1996). There are at least two potential mechanism for how PLD activation might influence vesicle formation (Figure 6).

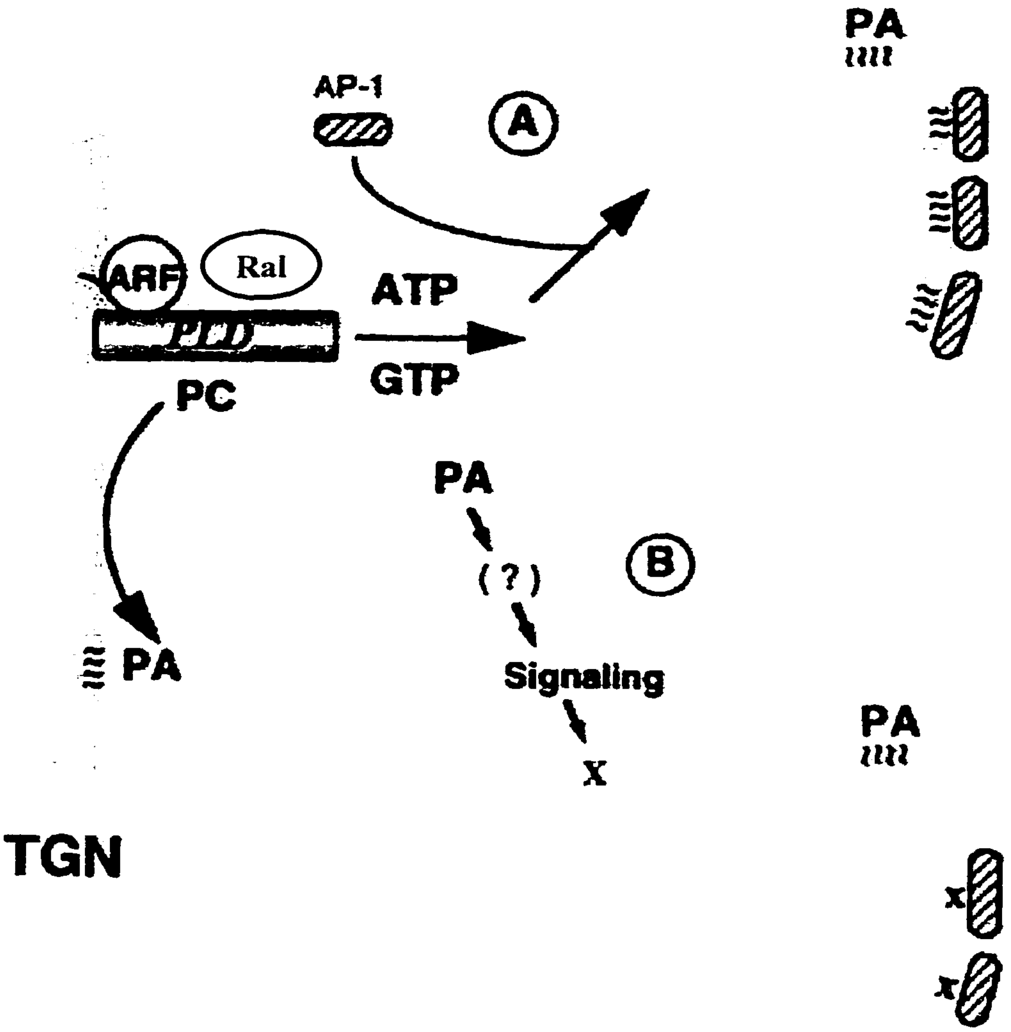


Figure 6. Possible effects of PLD activation in nascent vesicle formation. (A) PLD converts phosphatidylcholine to phosphatidic acid resulting in changes in both charge and pH at the membrane, which is hypothesized to alter membrane topology and facilitate vesicle formation. **(B)** PA is rapidly hydrolyzed to a number of possible intermediates which may be converted to other metabolites that could initiate a signaling cascade, the end product of which (X) mediates coat recruitment. (Modified from Chen et al., 1997)

One potential mechanism is changing the properties of cellular membranes by altering their lipid composition. Thus, by causing local changes in PC and PA the physical properties of the membranes could be substantially changed. A second mechanism is by generating PA. This lipid would probably remain in the membrane but could interact with proteins located in the membrane or cytosol (Figure 6). Many proteins have been shown to have their activities changed by PA *In vitro* (Exton JH. 1997), but evidence that they are targets of the lipid *In vivo* is largely lacking.

In addition to its role in vesicle formation PLD activation plays a major role in mitogenic signaling. As mentioned above. activation of PLD results in the production of PA which is rapidly converted to DG in most cells through the action of phosphatidate phosphohydrolase (Figure 7). Thus, while DG derived from PLC activation is responsible for early phase activation of PKC, the late phase of activation of PKC produced by agonists in many cells is mainly attributable to DG derived from PLD action (Jiang *et al.*, 1994; Jiang *et al.*, 1996; Ha *et al.*, 1993).

Lastly activation of PLD results in the generation of LPA through the action of a specific phospholipase A2 on PA (Figure 7). LPA has been to shown to be mitogenic via it's binding to the LPA receptor (Moolenaar & van Corven. 1990). This receptor is a classic serpitine receptor which couples to the heterotrimeric family of g-proteins (Moolenaar *et al.*, 1997). Interestingly, it has been recently reported that upon LPA stimulation, dynamin a small GTPase that facilitates the release of endocytic vesicles

(Vieira *et al.*, 1996) becomes tyrosine phophorylated and associated with the LPA receptor (Kranenburg *et al.*, 1999).

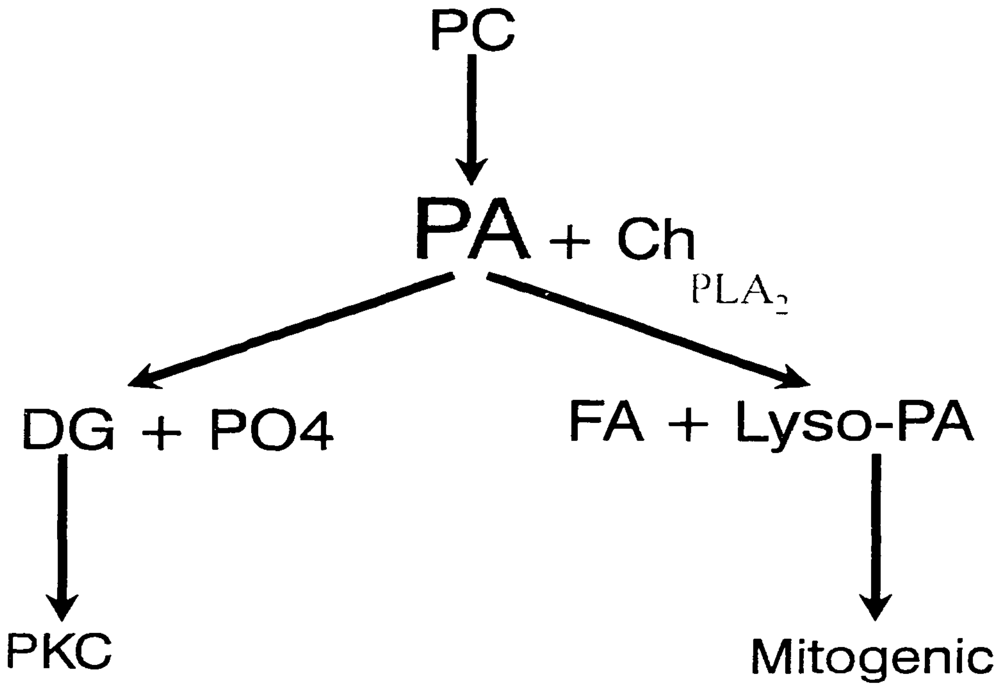


Figure 7. Effects of PLD activation on mitogenic signaling pathways. PA generated by PLD can be further metabolized to DG and free phosphate by the action of a PA-phosphohydrolase. DG can activate DG responsive PKC Isoforms. Alternatively PA can be converted to Lyso-PA and free fatty acid (FA). Lyso-PA can bind its classic serpintine receptor thereby leading to mitogenic signaling. See applicable references in text.

The potential function of PLD in the regulation of cell proliferation remains controversial. PA, LPA and bacterial PLD are mitogenic in several cell lines (Exton JH. 1997; van Dijk *et al.*, 1998), however there is a lack of correlation between PLD activity and mitogenesis in some cell lines (Paul *et al.*, 1994). Other signaling pathways are undoubtedly involved in growth control. The mechanisms by which PLD could control the cell cycle are unknown. However, Raf-1 kinase, which is involved in signal transduction from several receptors, has a binding site for PA and is translocated to membranes under conditions where PLD is activated (See Below) (Ghosh *et al.*, 1996).

Small GTPases

Introduction

At the surface of cells, many different kinds of receptors are expressed which allow the cell to respond to signals provided by its environment. Activation of these receptors leads to a large variety of biochemical events in which small GTPases play a crucial role (Figure 8). These proteins cycle between two conformations induced by the binding of either GDP or GTP. Guanine nucleotide exchange factors (GEFs) induce the dissociation of GDP to allow association of the more abundant GTP, which in its turn is hydrolysed to GDP by the intrinsic GTPase activity in combination with GTPase-activating proteins (GAP) (Figure 9).

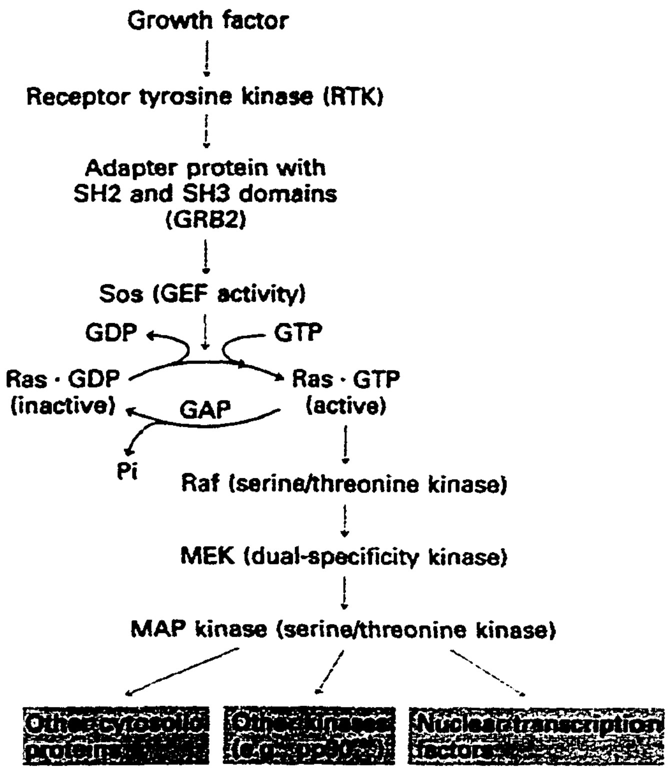


Figure 8. Growth Factor Receptor Tyrosine Kinase Signaling through the Small GTPase Ras.

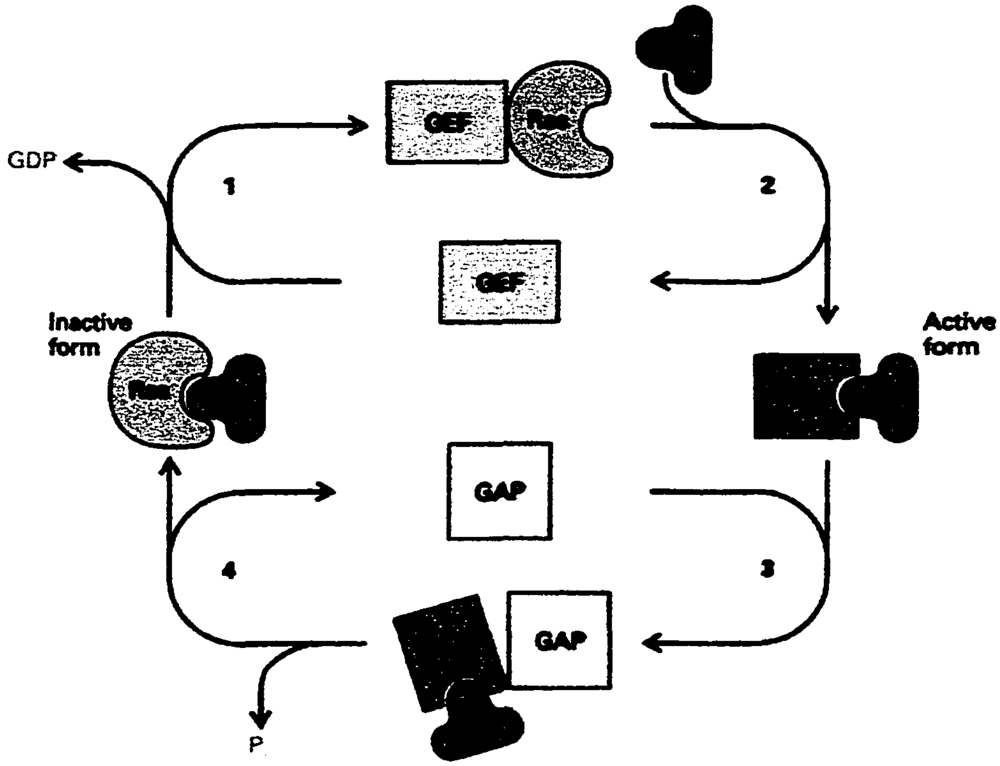


Figure 9. The Small GTPase Cycle. Cycling of the Ras protein between the inactive GDP bound form and the active GTP bound form. (1) Guanine nucleotide exchange factors (GEFs) facilitate the release of GDP. (2) GTP then binds spontaneously followed by release of the GEF yielding active Ras. (3,4) Hydrolysis of the bound GTP by the endogenous Ras GTPase activity is accelerated several orders of magnitude by association with a GTPase-activating protein (GAP). (Borrowed from Molecular Biology of the Cell., 1998).

The Ras superfamily of small GTPases includes the Ras family (Ras, Rap1,2, R-Ras, TC21, Ral, Rheb, and R-Ras3) (Bos JL.1997; Kimmelman *et al*., 1997; Matsumoto *et al*., 1997) and the Rho family (Rho A, B, and C isoforms), Rac (1 and 2 isoforms), Cdc42 (Cdc42Hs and G25K isoforms), RhoD, RhoG, RhoE, and TC10. These proteins are classified in this group due to similarities in the effector domain, a region that interacts with downstream targets. While Ras is clearly the best known and best-studied member of the superfamily, both Rho and Ral recently have attracted much attention: Ral because Ral specific GEFs are regulated by direct binding to Ras, and Rho because of its role in controlling the organization of the actin cytoskeleton in all eukaryotic cells.

The Ras signalling pathway

The Ras protein plays a critical role in cell proliferation, differentiation, and migration in response to extracellular signals. Ras proteins are ubiquitously expressed and located at the inner side of the plasma membrane. Binding of GTP to Ras is required for its interaction with downstream effector proteins including Raf-1, PI3-kinase and RalGDS (Lowy & Willumsen, 1993). Transforming mutations of Ras which decrease the rate of GTP hydrolysis result in its constitutive activation. Such oncogenic Ras mutations have been found in about 40% of human cancers and are thought to be a critical factor in the proliferation of these tumors (Boss JL. 1989). Four isoforms of Ras exist: Ha-Ras, N-Ras, Ki-Ras4A, and Ki-Ras4B (Lowy & Willumsen. 1993). They are products of three genes, with Ki-Ras4A (minor form) and Ki-Ras4B(major form) being splice variants of the same gene. Oncogenic mutations of the different isoforms predominate in different tumors (Boss JL. 1989). Ras protein sequences are 80% identical with major differences residing between residues 166 and 185 a region termed the hypervariable region. C-terminal to the HVR all Ras proteins terminate in a conserved CAAX motif (C, cysteine; A, aliphatic amino acid; X, methionine or serine) that directs post-translational prenylation by addition of a farnesyl group and is required for membrane localization. In addition to the conserved CAAX motif Ras Isoforms contain an additional membrane localizational signal within the HVR (Hancock *et al.*, 1989; 1991). In Ki-Ras 4B (major form) this second signal consists of a polylysine domain (lysine residues 175-180), whereas in Ha-Ras, N-Ras, and Ki-Ras 4A(minor form) the second signal comprises palmitoylation sites at cysteines 181 and/or 184 (Hancock *et al.*, 1990).

A large variety of signals induce the activation of Ras, most notably signals that induce receptor tyrosine kinases. Phosphotyrosines on these receptors serve as docking sites for the assembly of a complex containing the Ras-specific GEF SOS and the adaptor protein Grb2 (Figure 8 and 10). Currently, a large number of proteins have been identified which bind specifically to the active, GTP-bound conformation of Ras (Katz & McCormick, 1997). As mentioned above, for only a few of these proteins, additional biochemical and genetic evidence has been provided indicating that these proteins are genuine effector proteins of Ras. These include Raf proteins, members of the RalGDS family (RalGDS, Rlf and Rgl), the p85-p110 phosphatidylinositol 3 kinase (PI-3 kinase) PI3K, MEKK1, AF-6, PKC-z, and Ras GAP) (Figure 11).

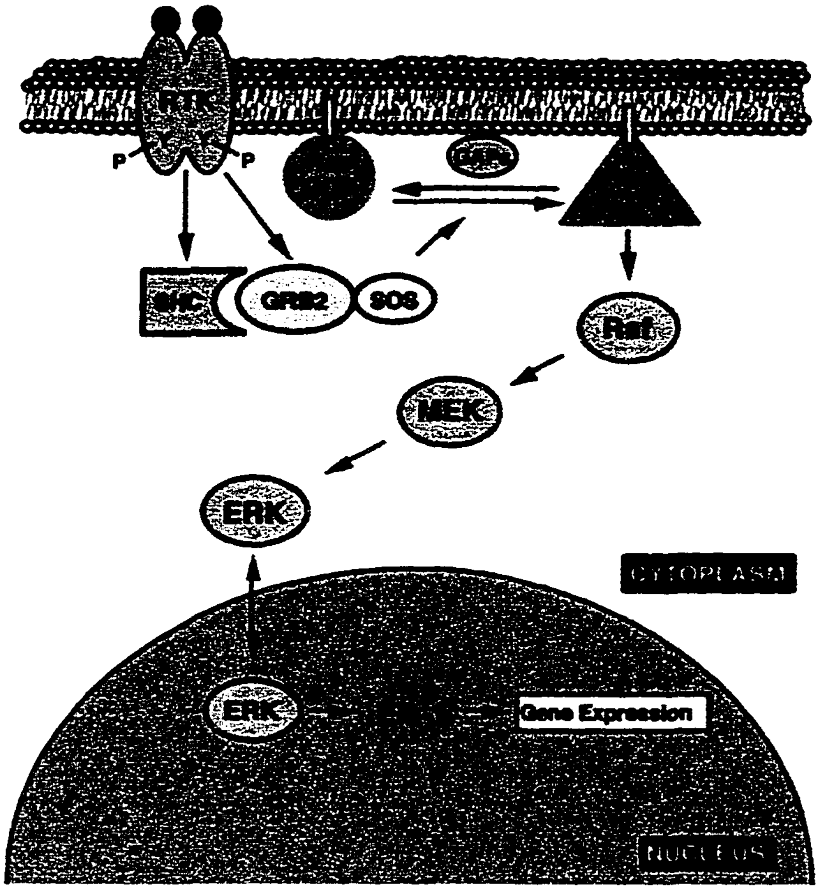


Figure 10. Activation of Ras by Receptor Tyrosine Kinases. Ras functions downstream of receptor tyrosine kinases (RTK) and upstream of a cascade of serine/threonine kinases (Raf > MEK > ERK) providing a link between the cell surface and the nucleus. Activated ERKs can translocate into the nucleus to phosphorylate and activate transcription factors, such as Elk-1. (Borrowed from Vojtek & Der, 1998)

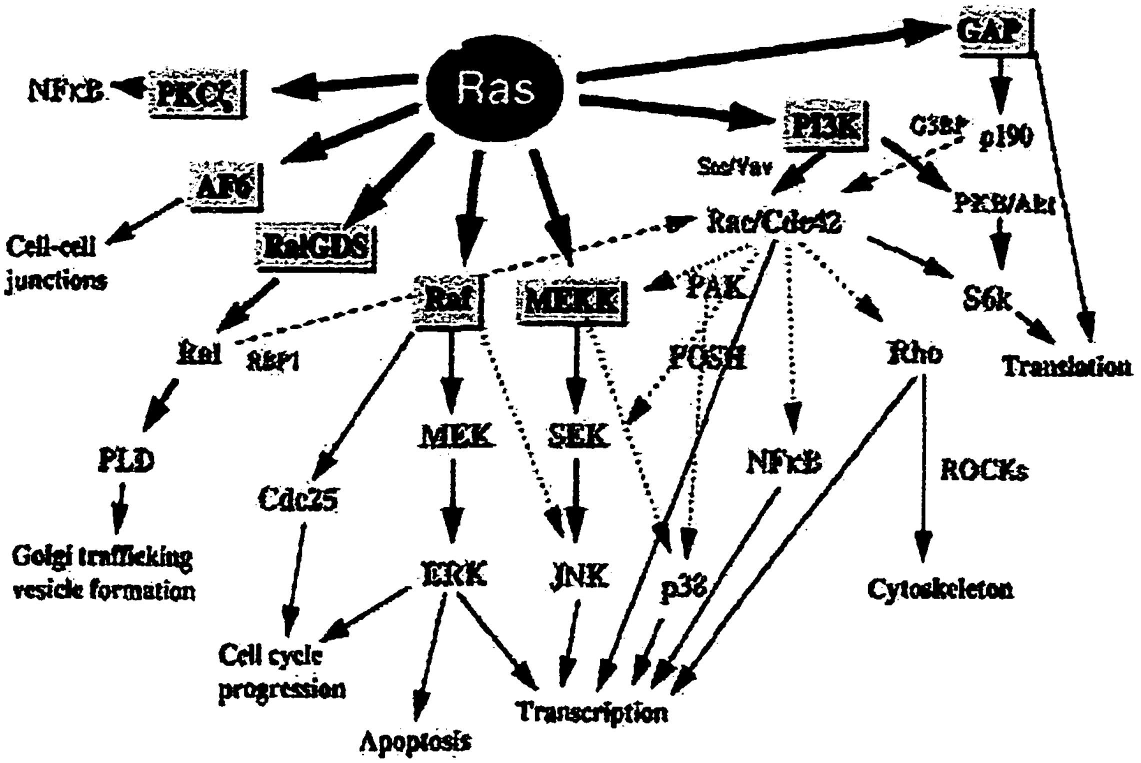


Figure 11. Ras effector and downstream pathways. Several different proteins have been demonstrated to bind Ras in a GTP-dependent manner: Raf proteins, members of the RalGDS family, PI3K, MEKK1, AF-6, PKC-z, and Ras GAP. These proteins are classified as Ras effectors based upon two criteria: (1) the effector protein binds preferentially to the activated GTP-bound form of Ras and (2) all of these proteins interact with some region in the so-called effector domain of Ras. The interaction between Ras and the effector molecule should elicit activation of the effector or production of a biochemical effect on downstream targets. Finally, a functional knockout of the putative effector (such as overexpression of a dominant negative mutant) should abolish part of the Ras-mediated signal. (Borrowed from Malumbres & Pellicer, 1998)

The ability of Raf to bind Ras in a GTP-dependent manner *In vitro* and *In vivo* is the principal biochemical evidence in support of a direct effector role for Raf (Moodie *et al.*, 1993; Vojitek *et al.*, 1993; Warne *et al.*, 1993; Zhang *et al.*, 1993). GTP-bound Ras binds cytoplasmic Raf-1 and translocates it to the plasma membrane where Rafl kinase becomes activated by a mechanism, which is still poorly understood but is Ras-independent (Stokoe *et al.*, 1994). Activated Raf phosphorylates MAP kinase-extracellular signal-regulated kinase (MEK) (Kyriakis *et al.*, 1992; Cowley *et al.*, 1994) which in turn activates the p42 and p44 MAPK/Erk kinases (Ahn *et al.*, 1991; Gomez *et al.*, 1991; Kosako *et al.*, 1992; Nakielny *et al.*, 1992). Erk phosphorylation promotes its homodimerization (Khokhlatchev *et al.*, 1998) and results in the translocation of the Erks into the nucleus leading to the activation by direct phosphorylation of transcription factors, such as p62TCF/Elk-1 and Ets-2 (Figure 10). These factors are involved in ternary complex formation at the serum response elements (SRE) (Gille *et al.*, 1992), which regulate the expression of immediate-early genes, such as the c-fos and HB-EGF genes and eventually to cell proliferation (Marshall CJ. 1994; Treisman R. 1996).

The activation of Raf-1 kinase activity requires its translocation from the cytoplasm to the plasma membrane where it is activated through a complex mechanism which includes the interaction with Ras, and possibly phosphorylation by PKC, and tyrosine kinases (Avruch *et al.*, 1994; Daum *et al.*, 1994; Morrison & Cutler, 1997) (Figure 12). Whereas, the precise nature of the events occurring at the plasma membrane remains unresolved, it is clear that the translocation of Raf-1 is crucial. Targeting Raf-1 to the plasma membrane by attaching the Ras membrane localization motif "caax box" to the C terminus of Raf-1 is sufficient for activation of the kinase (Leevers *et al.*, 1994), whereas trapping Raf-1 in the cytoplasm with cytosolic Ras prevents activation (Lerner *et al.*, 1995). However, translocation itself does not bring about the full activation of Raf-1. (Mineo *et al.*, 1997) used a mutant Ras protein that is deficient in binding to wild-type Raf-1-caax, but binds Raf-1(257L)-caax, to show that the interaction between Ras and Raf-1 stimulates Raf-1 kinase activity 3-fold better than targeting Raf-1 to the membrane alone. Thus, Raf-1 translocation and Raf-1 kinase activation are closely related but distinct phenomena.

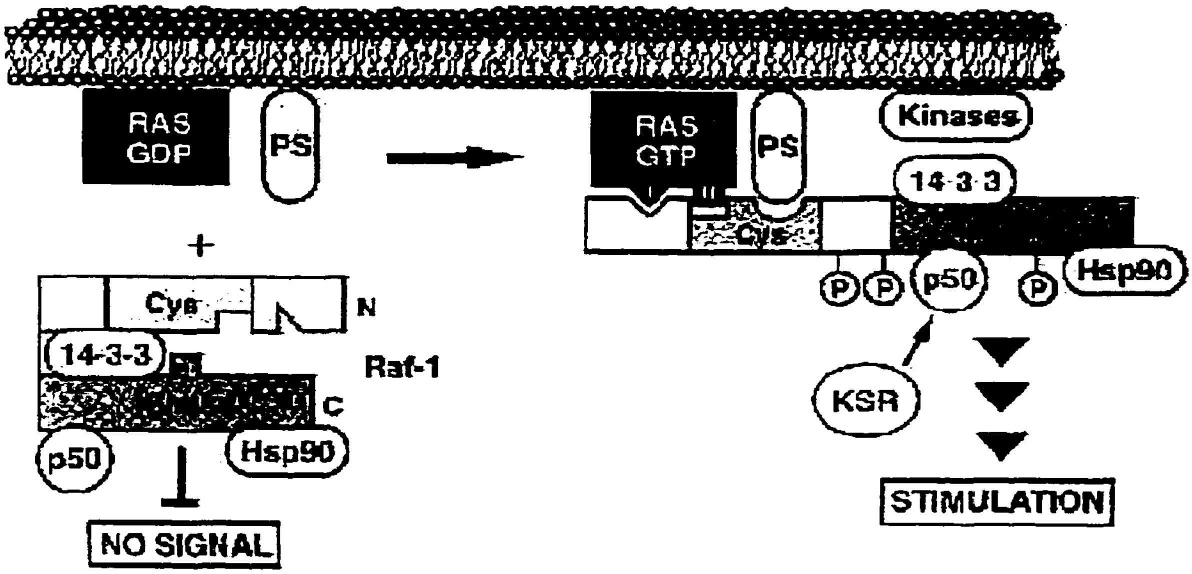


Figure 12. Proposed events involved in Ras mediated Raf-1 activation. Although Ras mediated Raf activation involves localizing Raf to the plasma membrane, it has been proposed that other events as well are required for full Raf kinase function. These include: 1) Ras interaction with a additional site the cytesine-rich domain (CRD); 2) Interaction with 14-3-3 proteins on multiple sites leading to negative regulation; 3) Additional interaction with several molecules such as kinase suppresor of Ras (KSR), p50, and heat shock protein 90 (Hsp90). (Borrowed from Campbell et al., 1998)

It has been assumed for some time that the interactions of Raf-1 with Ras are the primary mechanism driving the recruitment of Raf-1 to the cell membrane. Recently, other mechanisms that may play an important role in the recruitment of Raf-1 to the membrane have been investigated. For instance, (Ghosh *et al.*, 1996) have explored the interactions of Raf-1 with phosphatidylserine and PA *In vitro*. Phosphatidylserine appears to bind to the cysteine-rich domain (CRD) of Raf-1 (Figure 12). (Luo *et al.*, 1997) replaced the Raf-1 CRD with the analogous zinc finger domain found on PKC and found that DAG activated this chimera independently of Ras activation, demonstrating that interaction of an effector with the CRD is critical in the activation of Raf-1. Other effectors, such as ceramide (Huwiler *et al.*, 1996) and Rap1A (Hu *et al.*, 1997), interact with Raf-1 at this site and consequently have effects on its activation. The PA-binding site proposed by (Ghosh *et al.*, 1996) does not lie in this crucial lipid binding regulatory domain on Raf-1 but on a second lipid-binding site near the catalytic domain of Raf-1. The influence of effector binding at this site on Raf-1 kinase activity, if any, has not been fully characterized at the present time. (Ghosh *et al.*, 1996) also showed that inhibition of PLD-mediated generation of PA with ethanol inhibited phorbol ester-induced Raf-1 translocation to cell membranes. This suggests that the generation of PA may play an important role in the recruitment and/or activation of Raf-1 kinase. In agreement, (Rizzo *et al.*, 1999) recently showed that stimulation of the MAPK pathway by insulin is dependent on PLD activation, and this effect is mediated through the induction of Raf-1 translocation to the plasma membrane by PA. They found that PA was required for the complete activation of Raf-1 in response to insulin. However, PA alone was not sufficient to activate the Raf kinase *In vivo*, had no effect on Raf kinase activity *In vitro*, and could not activate the MAPK cascade. In addition, they showed that PA induced Raf-1 translocation to the plasma membrane and that the generation of PA was essential for the induction of Raf-1 translocation by insulin. Finally, in agreement with a role for PLD and PA in vesicle formation and mitogenic signaling, Raf-1 was found associated with intracellular vesicles containing the insulin receptor and clathrin after stimulation with insulin. Furthermore, Raf-1 association to endocytic vesicles was dependent on the generation of PA, suggesting a model in which Raf-1 migrates along with endocytic vesicles during receptor-mediated endocytosis via its interaction with PA.

The most recently established Ras effectors are the members of the RalGDS family, which connect Ras to the small GTPase Ral.

The Ral signalling pathway

Until recently, little was known about the regulation and function of Ral, but this changed when RalGDS, a RalGEF, was found to interact with the GTP-bound form of Ras (and several other Ras-like GTPases) in a yeast two-hybrid screen (Hofer *et al.*, 1994; Spaargaren & Bischoff, 1994). RalGDS exhibits exchange activity towards Ral *In vivo*, which is augmented by co-expression of active Ras (Urano *et al.*, 1996; Murai *et al.*, 1997; Wolthuis *et al.*, 1997). Insulin- and epidermal growth factor (EGF)-induced activation of Ral is inhibited by dominant-negative Ras, establishing that the RalGDS-Ral pathway is a downstream signalling pathway of Ras (Wolthuis *et al.*, 1998b, Lu *et al.*, 1999). However Ral can also be activated in a Ras independent manner (Hofer *et al.*, 1998).

Potential downstream targets for Ral proteins include a Cdc42/Rac GTPase-activating protein, RalBP1, and PLD1. RalBP1, a putative effector of Ral, binds specifically to the GTP-bound form of Ral but not to other GTPases. This protein has GTPase activating activity for Cdc42 and, to a lesser extent, Rac1 but not RhoA, linking the Ral pathway with the regulation of the Rho family proteins (Cantor *et al.*, 1995; Jullien-Floores *et al.*, 1995; Park & Weinberg, 1995). Whether the association of RalBP1 with active Ral results in an activation or an inactivation of these GTPases awaits further investigation. However, a recent report indicates nerve growth factor (NGF) treatment in PC12 cells leads to RalBP1 binding to Ral and activation of it's gap function, resulting in retraction of neurite outgrowth in PC 12 cells (Goi *et al.*, 1999). RalBP1 can also bind to Reps1, an Eps-homology domain protein which is tyrosine phosphorylated in response to EGF stimulation of cells and binds to the SH3 domains of the adaptor proteins Crk and Grb2 (Yamaguchi *et al.*, 1997). This chain of proteins may coordinate the cellular actions of activated EGF receptors, Ras, Ral and Rac/Cdc42/Rho proteins (Figure 11).

Although the functions of Ral have long remained elusive, it is known that Ral is required for Src- and Ras-dependent activation of phospholipase D1 (PLD1) (See above) (Jiang *et al.*, 1995b; Luo *et al.*, 1997; 1998). PLD1 associates directly with RalA, but RalA has no effect upon the activity of PLD1. However, PLD1 is activated when a functional complex of PLD1-RalA-Arf is formed, where activated Arf is able to activate

PLD. Inhibition of the Arf GDP-GTP exchange inhibits PLD activity in Src and Ras transformed cells, implicating Arf in the transduction of intracellular signals activated by Src and mediated by the Ras/RalA/PLD1 cascade. These results suggests a connection between the Ras/RalA/PLD1 complex and vesicle formation and trafficking in the Golgi, which is stimulated by both Arf and PLD1 (Chen,et al., 1997).

RalGEFs are also involved in the activation of gene expression by Ras, since Rlf is able to stimulate c-fos expression on its own (Wolthius *et al.*, 1997). However activation of c-fos is not limited to the RalGDS pathway since RalGDS and Raf act synergistically to stimulate c-fos expression (Okazaki *et al.*, 1997).

Although Ral seems to be one of the targets of Ras-RalGDS signaling, several observations indicate than RalGDS could signal to other molecules besides Ral. The effects of RalGDS on cellular responses are different from those of active Ral. RalGDS, for instance, can induce low serum and anchorage-independent growth, and tumorigenicity in NIH 3T3 cells, whereas active Ral has no effect (Bos JL. 1997; unpublished results). In addition, they show a differential ability to induce c-fos (Okazaki *et al.*, 1997) or to cooperate with Raf in cell transformation (Urano *et al.*, 1996; White *et al.*, 1996). Interestingly a dominant negative Ral mutant is able to inhibit Ras transformation, this mutant is also able to inhibit cell transformation by Raf, suggesting that this inhibition may be a more general phenomenon (Bos JL. 1997; Urano *et al.*, 1996; See Below).

Proteases

Introduction

Extracellular proteolytic enzymes have been implicated in the spread of cancer. Release of proteolytic enzymes from tumors is thought to facilitate cancer cell invasion into the surrounding normal tissue through the breakdown of basement membranes and extracellular matrix (ECM) and by tissue remodeling. Tissue remodeling requires the concerted action of a number of extracellular proteinases. Among these enzymes, urokinase plasminogen activator (uPA) and a variety of matrix metalloproteinases (MMPs) play important roles (Mignatti *et al.*, 1986; 1989; Kliener & Stetler-Stevenson, 1993; Mignatti & Rifkin, 1993).

Urokinase-type plasminogen activator (uPA)

uPA is an extracellular serine protease which converts plasminogen, a ubiquitous extracellular proenzyme, to plasmin, a serine protease with a wide spectrum of substrates (Sakseka & Rifkin, 1988). It is highly expressed in many transformed cells and cell lines (Dano *et al.*, 1985; Sappino *et al.*, 1987; Jankun *et al.*, 1991), but is also expressed in cells that exhibit dynamic changes in morphology such as those involved in embryogenesis (Valinsky & Le Douarin, 1985) and wound healing (Grondahl-Hansen *et al.*, 1988). uPA promotes the degradation of ECM proteins either acting alone or through activation of plasminogen to plasmin (Mignatti & Rifkin, 1993) (Figure 13). A role for uPA in invasion and metastasis has been demonstrated by experiments in which metastasis was inhibited by anti-uPA antibodies and by the expression of uPA sense or antisense cDNAs in transformed cells that either promoted or inhibited, respectively, tumor cell invasion and metastasis (Ossowski L. 1988; Axelrod *et al.*, 1989; Yu & Schultz, 1990).

Matrix Metalloproteinases (MMPs)

Matrix metalloproteinases (MMP's) are a family of Zn2+ dependent endopeptidases with a broad spectrum of proteolytic activity for several components of the extracellular matrix (Matrisian LM. 1990). Tumor cells secrete MMP's, which destroy basement membranes and local connective tissue, allowing tumor cells to gain access to the lymphatic or blood circulation (Liotta *et al.*, 1980). Once established at a secondary site, tumor cells continues to secrete MMP's that degrade connective tissue enhancing local growth (Fiedler & Balch, 1987). MMP's also appear to promote the growth of new blood vessels that nourish metastatic deposits.

Two MMPs, MMP-2 or gelatinase A, and MMP-9 or gelatinase B, degrade a variety of ECM proteins, including type IV collagen, fibronectin, laminin and interstitital (type I) collagen; in addition, they efficiently degrade denatured collagens (i.e. gelatins) of all types (Matrisian LM. 1990; Kleiner and Stetler-Stevenson. 1993; Aimes and Quigley, 1995). These proteinases, collectively referred to as type IV collagenases or gelatinases, have been implicated in a variety of invasive processes, including tumor invasion and metastasis both in experimental models and in human malignancies (Garbisa *et al.*, 1987; Bernhard *et al.*, 1990; Bernhard *et al.*, 1994; Levy *et al.*, 1991; Montgomery *et al.*, 1993; Crawford & Matrisian, 1995; Himelstein *et al.*, 1995).

Both uPA and MMPs are secreted as inactive zymogens (pro-uPA, proMMPs) and activated extracellularly by limited proteolysis (Peterson *et al.*, 1988). Plasmin through its activation by uPA degrades a variety of ECM components and activates several MMPs with different substrate specificities, including MMP-1 (interstitial collagenase), MMP-3 (stromelysin 1), and to a certain extent, MMP-9 (HE *et al.*, 1989; Wilhelm *et al.*, 1989; Okada *et al.*, 1992).

As described above RalA and PLD have been implicated in many different functions in the cell including, vesicle trafficking, mitogenic signaling, and the regulation of extracellular proteolytic enzymes. However the exact nature of this involvement is not fully understood.

Chapter II

Ral and Rho Dependent Activation of Phospholipase D in NIH 3T3 Fibroblasts

Introduction

There is a strong correlation between the activation of phospholipase D (PLD) and mitogenesis (Boarder, 1994). In response to most, if not all mitogenic signals, there is an increase in PLD activity. PLD activity is elevated in response to a variety of mitogens including platelet-derived growth factor (Plevin *et al.*, 1991) epidermal growth factor (Kaszkin *et al.*, 1992; Song *et al.*, 1994), and insulin (Karnam *et al.*, 1997; Shome *et al.*, 1997). PLD activity is also elevated in cells transformed by a several transforming oncogenes including v-Src (Song *et al.*, 1991; Wyke *et al.*, 1992), v-Ras (Teegarden *et al.*, 1990; Jiang *et al.*, 1995a; Carnero *et al.*, 1994; Martin *et al.*, 1997), and v-Fps (Jiang *et al.*, 1994). The activation of PLD activity results in the production of phosphatidic acid (PA), which has been implicated as a lipid second messenger that stimulates a variety of signaling molecules (Exton *et al.*, 1994; Exton *et al.*, 1994; Singer *et al.*, 1997) and in the formation of vesicles for membrane trafficking (Ktistakis *et al.*, 1996; Chen *et al.*, 1997; Roth *et al.*, 1997). A role for PLD and PA in the transduction of mitogenic signals is not clear; but the observation that PLD is elevated in response to most if not all mitogenic stimuli suggests that PLD may be an integral component of mitogenesis.

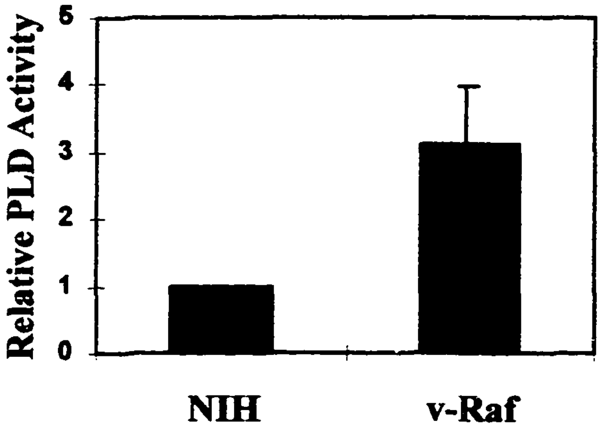
We demonstrated previously that although Raf is required for induction of the transformed phenotype by the oncogenic tyrosine kinase v-Src (Qureshi *et al.*, 1993), Raf was not required for the activation of PLD by v-Src (Jiang *et al.*, 1995a; Qureshi *et al.*, 1993). The activation of PLD by v-Src is mediated by Ras (Jiang *et al.*, 1995a) and its downstream target Ral guanine nucleotide exchange factor, which activates the Ras-family GTPase RalA (Jiang *et al.*, 1995b). Thus, the v-Src induction of PLD is mediated by a Ras signaling pathway that is independent of the well characterized Ras/Raf pathway (Avruch *et al.*, 1994). These data led us to the prejudice that v-Raf would not likely activate PLD activity. However, the recent report that v-Raf-induced transformation was inhibited by a dominant negative RalA mutant (Urano *et* *al*., 1996) led us to further investigate the affect of v-Raf on PLD activity. In this report, we present evidence indicating that PLD activity is activated in response to v-Raf via a novel mechanism involving both Ral and Rho GTPases.

RESULTS

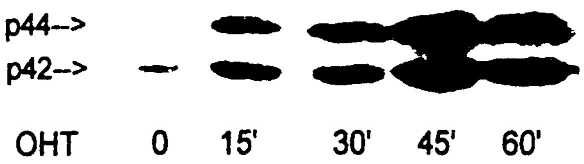
PLD activity is elevated in v-Raf-transformed NIH 3T3 cells---A unique property of mammalian PLD is its ability to catalyze a transphosphatidylation reaction. In this reaction short chain primary alcohols act as nucleophilic acceptors in place of H_2O. This results in the formation of a biologically stable phosphatidylalcohol instead of PA. Formation of phosphatidylalcohols has been used widely as an assay for PLD activity. To investigate the PLD activity in v-Raf-transformed cells, cells were prelabeled with [^{3}H]-myristate, which is incorporated exclusively into phosphatidylcholine (Song *et al.*, 1993), the substrate for PLD and then examined for the transphopshatidylation of phosphatidylcholine to PBt in the presence of exogenously-provided n-butanol. As shown in Figure 13a, the v-Raf-transformed cells displayed greater than 3 fold elevated PLD activity than the parental NIH 3T3 cells. To determine if the elevated levels of PLD activity in the v-Raf-transformed cells was an acute response of activated Raf or due to a secondary effect of transformation, we looked at conditional activation of Raf-1 in NIH 3T3 cells expressing an inducible form of an oncogenic activated c-Raf-1 protein (BxB-ER^{TM}) (Kerkhoff *et al.*, 1997). This Raf protein is a fusion of an activated Raf-1 with the estrogen receptor that confers responsiveness to the estrogen analogue 4-hydroxytamoxifen (Kerkhoff *et al.*, 1997). NIH 3T3 cells stably expressing the BxB-ER^{TM} Raf-1 were established as described in Experimental Procedures. We first investigated the ability to induce MAP kinase phosphorylation which is an early response to activation of Raf (Kyriakis *et al.*, 1992; Howe *et al.*, 1992). The cells expressing BxB-ER^{TM} c-Raf-1 were treated with 4-OH-T and the phosphorylation state of MAP kinase was examined at various time points after induction. As observed previously (Kerkhoff *et al.*, 1997) increased MAP kinase phosphorylation could be detected within 15 min after induction and reached maximal levels by 45 min (Figure. 13b). We next examined the ability of 4-hydroxytamoxifen to induce PLD activity in these cells; and, as shown in Figure 13c, increased PLD activity could be detected within two hr after treatment. It has been reported that oncogenic activation of c-Raf-1 kinase induces the expression of HB-EGF (McCarthy *et al.*, 1995). The action of the autocrine growth factor leads to a delayed activation of the Mekk/Sek/Jnk signal transduction cascade 16 to 20 hr following oncogenic activation of a c-Raf-1-ER fusion protien (McCarthy *et al.*, 1995; Minden *et al.*, 1994). The significance of this autocrine growth factor production for the mitogenic function of oncogenic c-Raf-1 was evaluated previously by analyzing the kinetics of HB-EGF expression and the induction of the Jnk1 kinase in response to 4-OH-T stimulation of quiescent N-BxB-ER^{TM} cells. Addition of 4-OH-T to quiescent N-BxB-ERTM cells leads to an induction of HB-EGF mRNA expression between 6 and 12 hr after stimulation and the activation of Jnk kinase could not be detected before 16 hr of 4-OH-T induction (Kerkhoff *et al.*, 1997). Thus, the rapid induction of both PLD activity and MAP kinase phosphorylation seen here indicate that the activation of PLD is a response to v-Raf kinase activity and not an indirect consequence of the transformed phenotype induced by v-Raf . These data indicate that the activation of PLD is a response to v-Raf kinase activity and not an indirect consequence of the transformed phenotype induced by v-Raf.

Figure 13. PLD activity is elevated in v-Raf-transformed NIH 3T3 cells. (a) NIH 3T3 (NIH) and v-Raf-transformed NIH 3T3 cells (v-Raf) were grown to confluence and placed in low serum for 24 hr to reduce background PLD activity. The cells were then prelabeled with [^{3}H]-myristate as described in Experimental Procedures. n-Butanol (0.8%) was then added and cellular lipids were extracted 25 minutes later. Transphosphatidylation products were separated by TLC and the PLD activity in these cells was determined by measuring the intensity of the corresponding PBt band. The data are presented as fold effect relative to the PLD activity in the NIH 3T3 cells. The error bar represents the standard error for duplicate experiments repeated three times. **(b)** The kinetics of MAP kinase phosphorylation in cells expressing BxB-ER^{TM} Raf-1 (BxB-ER^{TM}) after induction with 4-hydroxytamoxifen (4-OH-T) was determined by Western blot analysis of MAP kinase protein using an antibody raised against phosphorylated MAP kinase. **(c)** The kinetics of PLD activation in cells expressing BxB-ER^{TM} Raf-1 after induction with 4-OH-T was determined as in **(a)**. The data are presented as fold effect relative to the PLD activity in the uninduced cells.

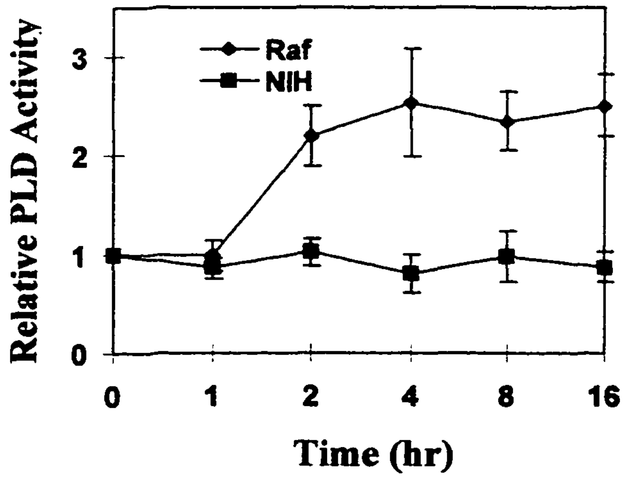
a)


Relative PLD Activity
0
1
2
3
4
5
NIH
v-Raf

b


p44-->
p42-->
OHT
0
15'
30'
45'
60'

c)


Relative PLD Activity
0
1
2
3
Raf
NIH
0
1
2
4
8
16
Time (hr)

v-Raf transformed cells have elevated level of Phosphatidic acid--- It is not yet clear what role PLD-generated PA might play in the transduction of intracellular signals. however PA can be metabolically converted to the PKC agonist DG by phosphatidate phosphohydrolase. We therefore examined the relative levels of PA and DG in both the v-Raf-transformed and parental NIH 3T3 cells. As shown in Figure 14a, there was about 3 fold more PA in the v-Raf-transformed cells relative to the parental NIH 3T3 cells; whereas there was no difference in DG levels between the two cell lines (Figure 14b). Thus. the activation of PLD by v-Raf results in increased levels of PA.

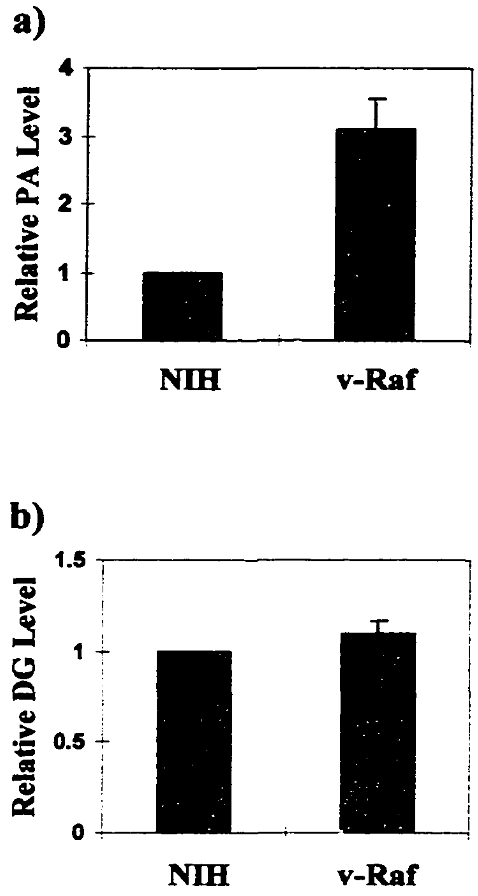


Figure 14. PA, but not DG, levels are elevated in v-Raf-transformed cells. v-Raf-transformed (v-Raf) and parental NIH 3T3 (NIH) cells were prelabeled with [^{3}H]-myristate as in Fig. 13. Cellular lipids were then extracted and subjected to TLC as described in the Experimental Procedures section. The PA **(a)** and DG **(b)** bands were determined with standards and quantified by densitometric analysis of autoradiographs. The data are presented as fold effect relative to NIH 3T3 cells and represents the mean (+/-) standard error of triplicate experiments repeated two times.

v-Raf induced PLD activity is insensitive to depleting cells of PKC---It has been reported that activation of PLD by either v-Src or v-Ras is independent of PKC (Song and Foster, 1993; del Paso *et al.*, 1996). However, several groups have shown that activation of PLD by other mitogenic stimuli including epidermal growth factor, fibroblast growth factor, platelet-derived growth factor, and lysophosphatidic acid is dependent on PKC (Plevin *et al.*, 1991; Yeo and Exton, 1995; Yeo *et al.*, 1994; van der Bend *et al.*, 1992). We therefore examined whether depleting cells of PKC affected the PLD activity in v-Raf-transformed cells. The v-Raf-transformed cells were treated with 12-O-tetradecanoylphorbol-13-acetate (TPA) for 24 hr, which leads to the ubiquitination and downregulation of DG-responsive PKC isoforms (Lu *et al.*, 1998). This treatment had no inhibitory effect on the levels of PLD activity in the v-Raf-transformed cells. In fact, we actually observed a small, but reproducible increase in PLD activity compared to untreated v-Raf-transformed cells (Fig. 15). In contrast, increases in PLD activity in NIH 3T3 cells stimulated with platelet-derived growth factor and basic fibroblast growth factor was substantially reduced when the cells were pretreated with TPA for 24hr (Fig. 15). These data suggest that the activation of PLD by v-Raf is independent of PKC.

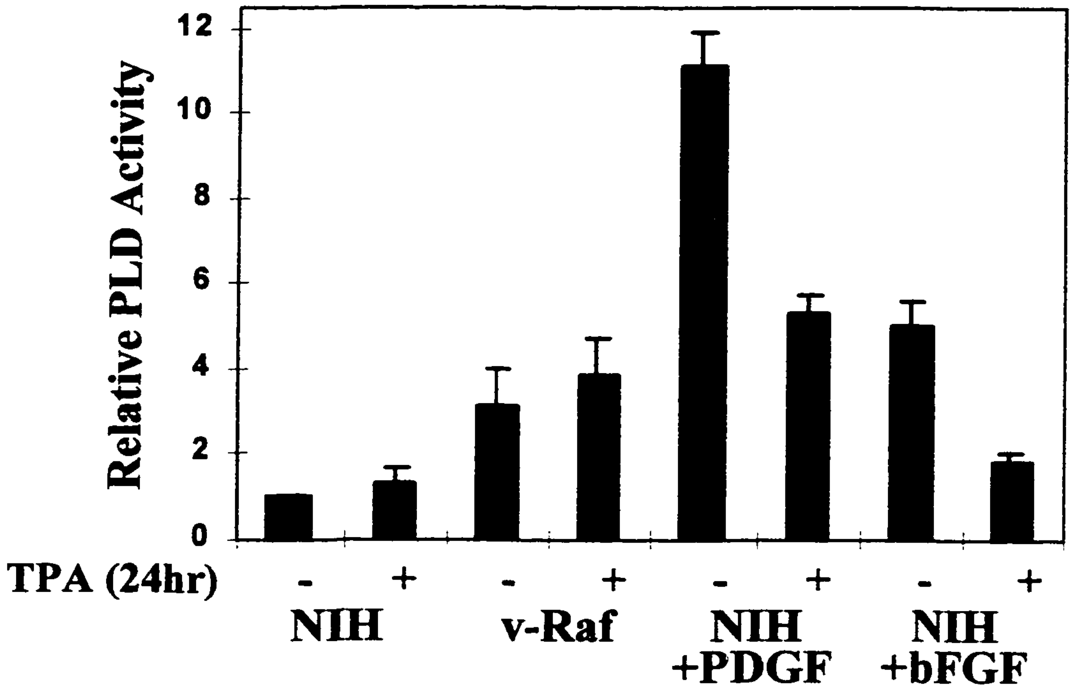
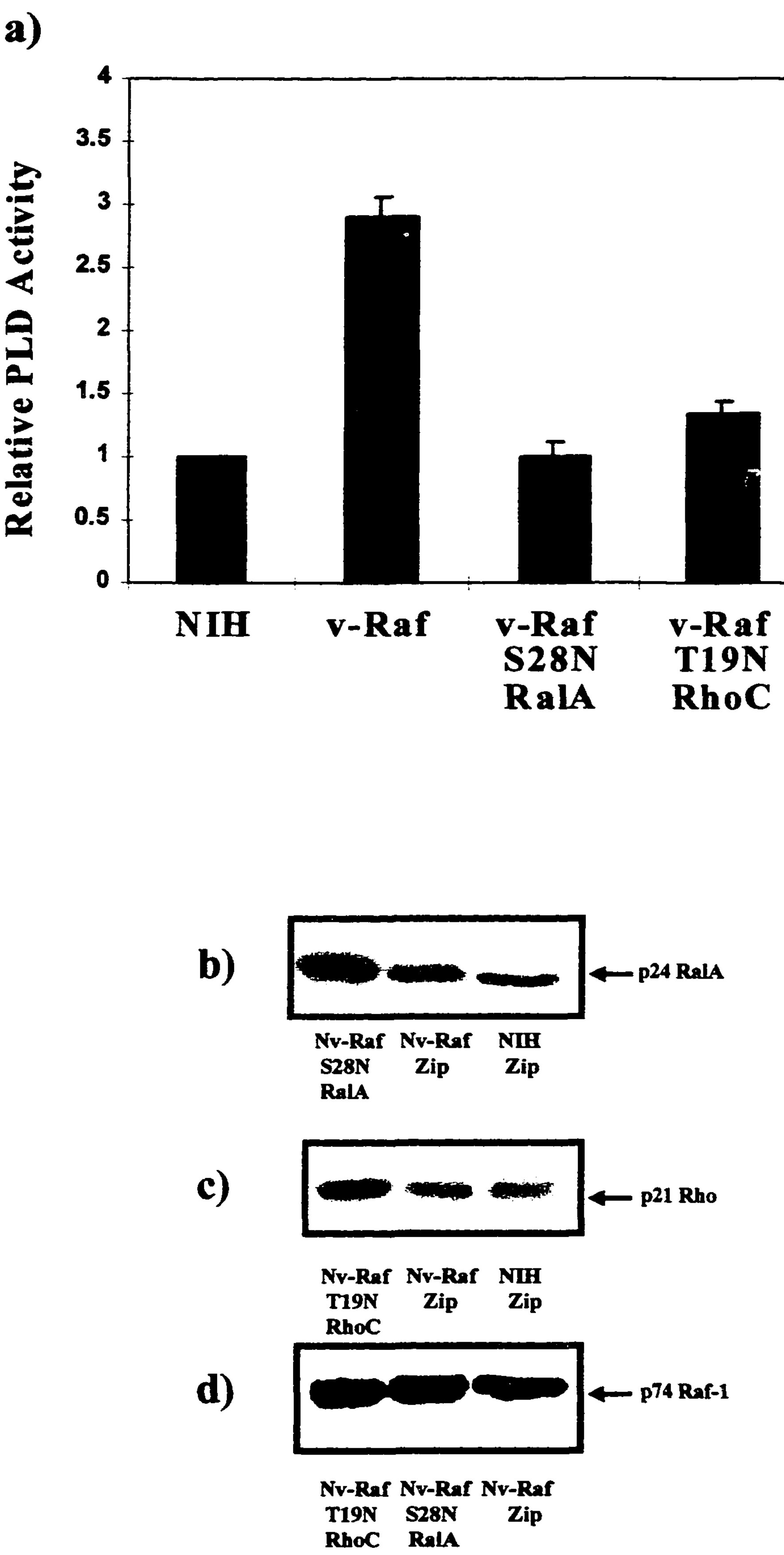


Figure 15. v-Raf induced PLD activity is insensitive to depleting cells of PKC. PLD activity was determined in v-Raf-transformed (v-Raf) and parental NIH 3T3 (NIH) cells, and in NIH 3T3 cells treated with platelet-derived growth factor (PDGF) and basic fibroblast growth factor (bFGF) (100 ng/ml, 15 min). Where indicated, cells were pretreated with TPA (350 ng/ml, 24 hr) to deplete cells of PKC. PLD activity was determined as in Figure 13. The data are presented as fold effect relative to the PLD activity observed in the untreated NIH 3T3 cells and represents the mean (+/-) standard error of duplicate experiments repeated three times.

Activation of PLD by v-Raf is mediated by Ral and Rho GTPases---It was reported previously that Raf-1-induced transformation of NIH 3T3 cells was dependent on RalA (Urano *et al.*, 1996). We therefore wished to determine whether RalA is required for the v-Raf activation of PLD. v-Raf-transformed cells that stably express a dominant negative mutant of RalA (S28N) (Jiang *et al.*, 1995b) was established along with empty vector controls as described in Experimental Procedures. We then examined the PLD activity in these cells relative to v-Raf-transformed and parental NIH 3T3 cells that had been transfected with the empty vector. As shown in Figure 16, the S28N-RalA mutant reduced the PLD activity in the v-Raf-transformed cells to that seen in the parental NIH 3T3 cells.

Although Rho family GTPases have never been implicated directly in Raf-1 signaling, RhoA has been implicated in the regulation of PLD (Bowman *et al.*, 1993; Malcolm *et al.*, 1994; Hammond *et al.*, 1997). We therefore investigated the effect of a dominant negative Rho mutant on the PLD activity in v-Raf-transformed cells. We introduced a RhoC mutant that contained the T19N mutation that confers a dominant negative phenotype to all of the Ras superfamily GTPases that have been examined. This mutant should act as a dominant negative mutant for both RhoA and RhoC since they interact with the same exchange factors (Feig, 1994). As shown in Figure 16, the T19N RhoC mutant also reduced the PLD activity in the v-Raf-transformed cells to that seen in the parental NIH 3T3 cells. Figure 16 b and c. shows levels of expression of the dominant negative RalA and Rho mutants. Expression of the dominant negative mutants had no effect upon Raf protein levels as shown in Figure 16d. Thus the reduced PLD activity observed in cells expressing the dominant negative RalA and Rho mutants observed in Figure 16a are not due to reduced levels of the transforming oncoprotein in response to the RalA and Rho mutants. These data indicate that that v-Raf-induced PLD activity is dependent upon both Ral and Rho family GTPases.

Figure 16. Activation of PLD by v-Raf is mediated by RalA and RhoA. **(a)** PLD activity was determined in NIH 3T3 . v-Raf-transformed NIH 3T3 cells and v-Raf-transformed cells expressing a dominant negative RalA mutant (S28N-RalA) and a dominant negative RhoC mutant (T19N-RhoC). The relative PLD activity in these cells was determined as in Figure 13. The data are presented as fold effect relative to the parental NIH 3T3 cells and error bars represents the standard error of duplicate experiments repeated three times. **(b)** Western blot analysis of levels of RalA protein in the NIH 3T3 Zip neo. v-Raf Zip neo. and v-Raf-S28N-RalA cells. **(c)** Western blot analysis of levels of RhoC protein in the NIH 3T3 Zip neo. v-Raf Zip neo. and v-Raf-T19N-RhoC cells. **(d)** Western blot analysis of levels of Raf protein in the v-Raf Zip neo. v-Raf-S28N-RalA and v-Raf-T19N-RhoC cells.


a)
Relative PLD Activity
0
0.5
1
1.5
2
2.5
3
3.5
4
NIH
v-Raf
v-Raf S28N RalA
v-Raf T19N RhoC
b)
p24 RalA
Nv-Raf S28N RalA
Nv-Raf Zip
NIH Zip
c)
p21 Rho
Nv-Raf T19N RhoC
Nv-Raf Zip
NIH Zip
d)
p74 Raf-1
Nv-Raf T19N RhoC
Nv-Raf S28N RalA
Nv-Raf Zip

DISCUSSION

In this report, we have shown that PLD activity is elevated in cells transformed by v-Raf. This result was surprising since the activation of PLD by v-Src was independent of Raf, yet v-Src activates Raf-1 and Raf-1 is required for v-Src-induced transformation (Qureshi *et al.*, 1993). The increased PLD activity was not an indirect effect of transformation since the activation of an inducible Raf protein resulted in increased PLD activity within two hours which is long before expression of the autocrine growth factor HB-EGF reported previously (Kerkhoff *et al.*, 1997). Thus, oncogenic activation of the c-Raf-1 kinase in quiescent N-BxB-ER^{TM} cells is apparently sufficient to activate PLD. The mechanism of PLD activation by v-Raf appears to be unique involving both Ral and Rho GTPases.

In v-Src- and v-Fps-transformed cells, the PA that is generated by PLD is rapidly converted to DG by phosphatidate phosphohydrolase (Song *et al.*, 1991; Jiang *et al.*, 1994). In contrast, growth factor stimulation of NIH 3T3 cells results in a biphasic induction of DG. The first phase due to direct accumulation of DG via activation of PLC, followed by a delayed increase in DG due to increased PLD activity generating PA which is then further converted to DG through the action of PAP. However, in the v-Raf-transformed cells, increased levels of PA were readily apparent, whereas DG levels remained constant. In this respect, it is interesting to note that Raf has a PA binding site which has been reported to be required for Raf-1 translocation to the membrane in response to mitogenic stimuli (Ghosh *et al.*, 1996). Thus, the activation of PLD and subsequent PA production may be important for maintaining Raf on the membrane. It was recently reported that in response to epidermal growth factor, the induction of interaction between Ras and Raf takes place on caveolar membranes (Mineo *et al.*, 1996). And although it has been reported that simply localization of Raf to the membrane is sufficient for activity (Stokoe *et al.*, 1994) specific targeting to Ras enhances the activation (Mineo *et al.*, 1997). Thus, activation of PLD may facilitate localization of Raf to a membrane microdomain where Ras and appropriate substrates are localized. It has been hypothesized that caveolae and caveolae-like membrane domains are regions where signaling complexes are organized (Okamoto *et al.*, 1998). And, interestingly, we have found that caveolar membranes are highly enriched for PLD activity and RalA (Xu et al., manuscript in preparation.).

The PLD activity induced by v-Raf was inhibited by a dominant negative mutant of RalA. We reported previously that the activation of PLD by v-Src and v-Ras is blocked by the dominant negative RalA mutant (Jiang *et al.*, 1995b). RalA, which interacts directly with PLD1 (Luo *et al.*, 1997) and apparently mediates interaction with the PLD1 activator protein Arf (Luo *et al.*, 1998), constitutes a distinct downstream signaling pathway from Ras through its exchange factor Ral-GDS (Ral-guanine nucleotide exchange factor), which, like Raf, is a direct downstream target of Ras that interacts with the Ras effector domain (Cantor *et al.*, 1995). Although v-Raf-induced transformation was dependent upon RalA, a link between Raf-1 and the RalA-PLD1 pathway is not readily apparent since Raf is generally considered a downstream target of Ras. Since it is believed that the dominant negative RalA mutant functions by interacting with and rendering inactive Ral-GDS, the sensitivity of v-Raf-induced PLD activity to this RalA mutant suggests a role for Ral-GDS. How Ral-GDS might be activated by Raf is unclear, but it suggests that simple upstream and downstream models based on genetic studies may not sufficiently explain what is actually happening. The requirement for Ral in v-Raf induced PLD activity may point to an emerging theme, that effectors may promote complex formation at the level of Ras, rather than simply forming a linear signaling pathway. The involvement of a Rho family GTPase was indicated by a sensitivity to a dominant negative mutant of RhoC. The Rho family GTPases are reported to be involved in the regulation of the actin cytoskeleton and specifically in the formation of focal adhesions and actin stress fibers (Ridley and Hall, 1992; Hotchin and Hall, 1996; Tapon and Hall, 1997). RhoA has also been shown to be involved in the regulation of PLD both in-vitro and in-vivo (Bowman et al., 1993; Malcolm et al., 1994; Hammond et al., 1997;Schmidt et al., 1996; Hess et al., 1997). Whether the involvement of Rho in the regulation of the actin cytoskeleton and the formation of focal adhesions and actin stress fibers has any thing to do with its apparent role in regulating PLD activity remains to be determined. However, actin stress fiber formation has been reported to be mediated by activation of PLD (Cross et al., 1996). Moreover, transformation results in dramatic changes in cytoskeletal structure, so it is possible that the Rho requirement for PLD activation by Raf involves regulation of cytoskeletal structure. Recently several groups have reported that high intensity Raf signaling causes cell cycle arrest in NIH 3T3 fibroblasts by inducing p21 Wafl/Cip1 expression (Kerkhoff and Rapp, 1998; Sewing *et al.*, 1997; Woods *et al.*, 1997). In addition, it was recently suggested that activated Rho acts synergistically in transformation by Ras and Raf by blocking p21 Wafl/Cip1 expression (Olson et al., 1998) indicating a previously unsuspected role for Rho in Ras and Raf signaling. Thus, it is possible that Rho-mediated inhibition of p21 Wafl/Cip1 expression is through PLD activation.

Chapter III

The RalA pathway is involved in protease production and tumor formation induced by oncogenic signals.

Introduction

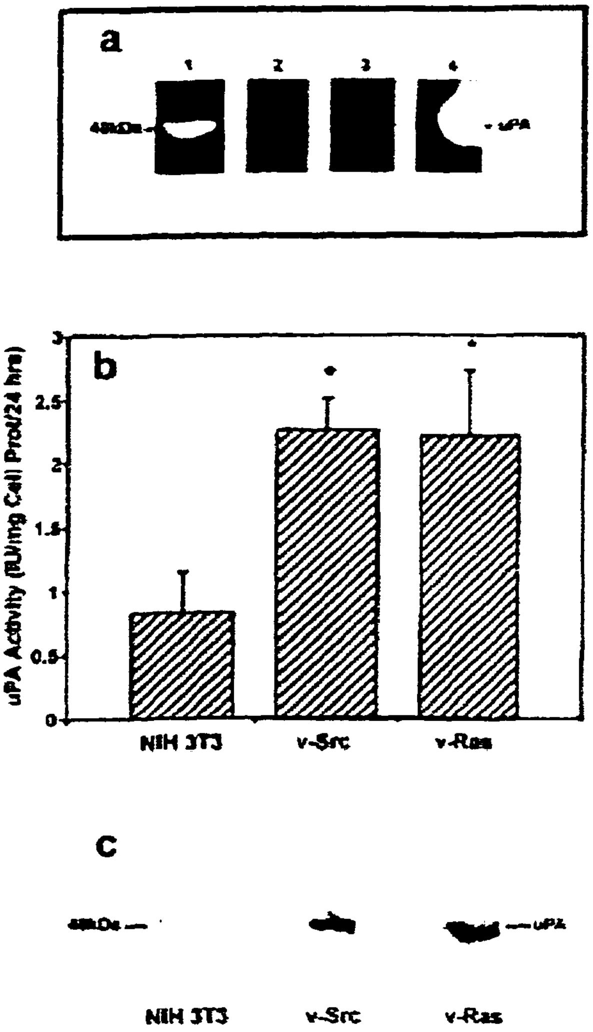
Metastasis is the last and most lethal stage of tumor progression. This process follows several steps including the exit of tumor cells from the primary tumor. intravasation after degradation of the interstitial and the blood vessels extracellular matrix, dissemination through the lymphatic or arterial system, and finally extravasation. settlement. and growth in a distant organ (Hart *et al.*, 1989). In several of these steps. as well as during primary tumor organization, proteases like urokinase-type plasminogen activator (uPA) and metalloproteases (MMPs) are highly involved (Liotta and Stetler-Stevenson 1991: Ossowski, 1992). Tumorigenicity has been described as being dependent on the expression of tumor- or host-derived proteases, since they enable the remodeling of the local tissue and angiogenesis, two critical steps for the successful development of a primary tumor (Liotta *et al.*, 1991; Werb *et al.*. 1996). Strong correlations between uPA and/or MMP production and the invasive and metastatic ability of tumor cells have been described (Ossowski 1992; Moller 1993; Boyd 1996). uPA is a serine-protease that converts plasminogen to the active plasmin (Moller 1993; Yu and Schultz, 1990). Enhanced pericellular proteolysis. primary tumor organization, and invasiveness are mediated via the uPA/uPA receptor/plasmin system. Upregulation of uPA and the uPA receptor enables tumor cells to keep their surface completely saturated with these enzymes (Ossowski and Reich 1983; Yu *et al.*, 1997). MMPs are a group of matrix proteases, such as the type IV collagenases (MMP-2/gelatinase B. MMP-9/gelatinase A), which are implicated in tissue remodeling, angiogenesis, inflammation, invasion, and metastasis (Matrisian, 1992).

Tumor proteases like uPA and MMPs have been reported to be upregulated upon transformation by v-Src and v-Ras (Sato *et al.*, 1993; Bell *et al.*, 1993; Silberman *et al.*, 1997; Gum *et al.*, 1996). Signaling pathways activated by v-Src and v-Ras (Burgering and Bos, 1995) include those involving phosphatidylinositol-3-kinase (Carpenter and Cantley, 1996), protein kinase C (Song *et al.*, 1991), Raf-1 (Blobe *et al.*, 1994) the extracellular regulated kinases (ERK-1 and 2) (Burgering and Bos, 1995) and phospholipase D (PLD) (Song *et al.*, 1991). It has been reported that Raf-1, MEK-1 and ERK1 mediate signal leading to uPA, the uPA receptor, and MMP-9 upregulation (Lengyel *et al.*, 1996; Lengyel *et al.*, 1997; Gum *et al.*, 1997). We recently reported that overexpression of uPA in murine mammary adenocarcinoma cells is controlled by a PLD and protein kinase C-dependent pathway (Aguirre Ghiso *et al.*, 1997). Additionally, MMP-2 expression induced by laminin has been reported to be coupled to PLD activation (Reich *et al.*, 1995). PLD converts phosphatidylcholine to phosphatidic acid, which has been implicated in vesicle budding and transport, as well as the transduction of intracellular signals (Exton, 1998; Roth and Sternweis, 1997). Phosphatidic acid may also be metabolized further to the biologically active diacylglycerol or lysophosphatidic acid (Foster, 1993; Exton 1994). PLD activity is increased in response to most, if not all, mitogenic signals (Foster, 1993; Exton, 1998). A PLD activity was found to be associated with the Ras-family GTPase RalA, which is required for the activation of PLD by both v-Src and v-Ras (Jiang *et al.*, 1995b) and the active complex includes PLD1, RalA and Arf (Luo *et al.*, 1997; 1998). In the present work, we have investigated the involvement of the RalA pathway in protease production and tumor formation induced by v-Src and v-Ras.

Results

uPA production is upregulated in v-Src- and v-Ras-transformed NIH 3T3 cells— Several reports have described increases in uPA production in response to transformation by v-Src and v-Ras (Bell *et al.*, 1993; Silberman *et al.*, 1997). To establish whether uPA production is elevated in NIH 3T3 cells in response to v-Src and v-Ras, we examined uPA expression levels in parental, and in v-Src-, and v-Ras-transformed NIH 3T3 cells. As shown in Figure 17a, NIH 3T3 cells secreted a 48 kDa plasminogen activator activity. This activity was is completely blocked by 1 mM amiloride or an anticatalytic anti-uPA antibody, confirming the identity of uPA. We then compared the uPA activity in conditioned medium (CM) from v-Src- and v-Ras-transformed cells relative to that in the parental NIH 3T3 cells. As shown in Figure 17b, both v-Src and v-Ras-transformed cells displayed increased levels of uPA activity relative to the parental NIH 3T3 cells (Figure 17b). Consistent with the increased uPA activity in v-Src and v-Ras-transformed cells, we also detected increased levels of uPA protein in these cells relative to the NIH 3T3 cells (Figure 17c).

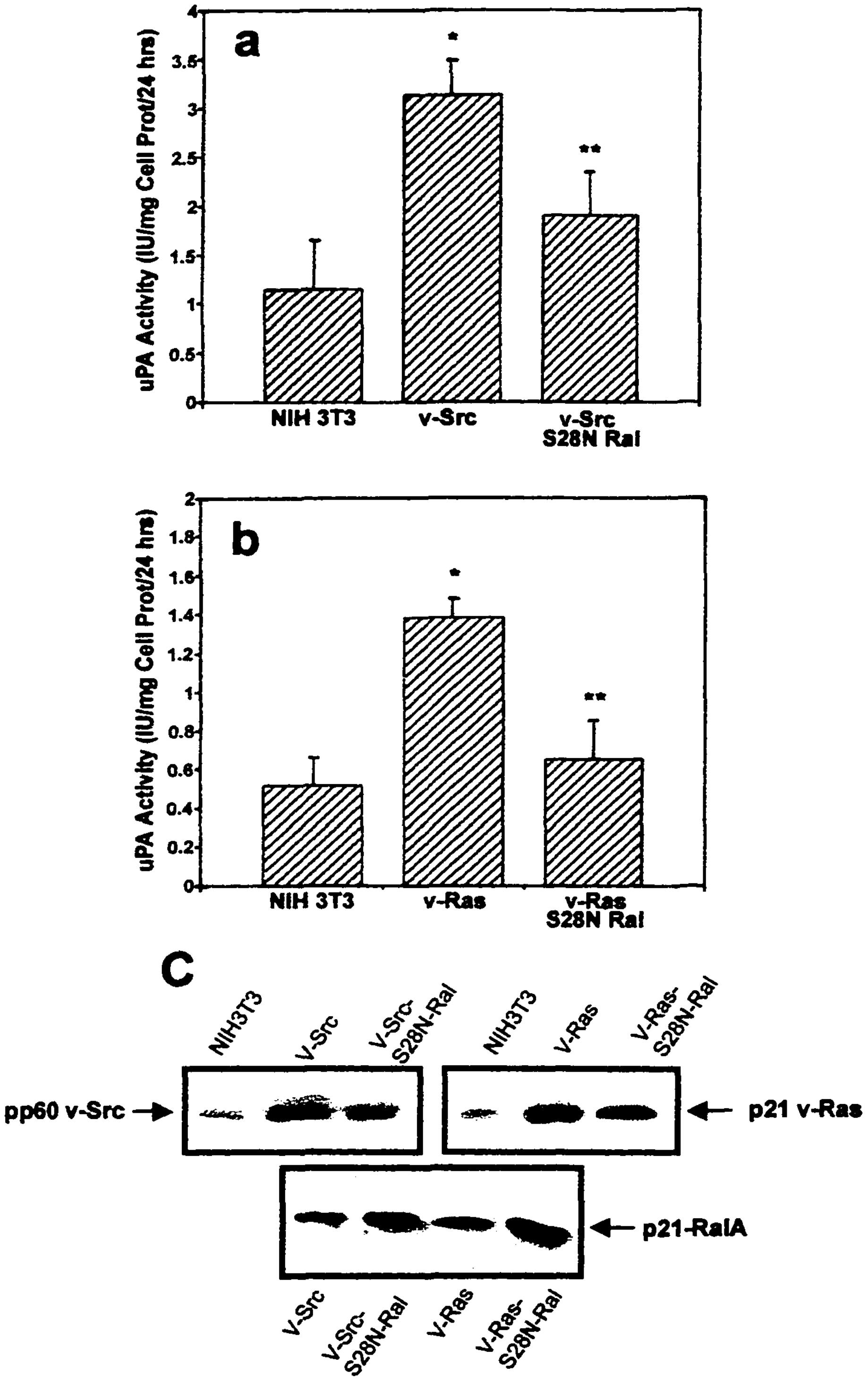
Figure 17. uPA production is upregulated in v-Src- and v-Ras-transformed NIH 3T3 cells. (a) Plasminogen activator activity was investigated by zymographic analysis on the CM from NIH 3T3 (pZip-neo transfectant) cells (lane 1). The same assay was performed in the presence of plasminogen-casein underlays prepared with anticatalytic anti-uPA antibody (10 ug/ml) (lane 2), or in the presence of the specific uPA inhibitor amiloride (1mM) (lane 3); LM3 cells, which overexpress uPA were used as a positive control (lane 4). **(b)** uPA activity in the CM from parental NIH 3T3-pZip-neo cells was compared with that present in the v-Src- and v-Ras-transformed NIH 3T3-pZip-neo cells. **(c)** uPA protein levels were determined by Western blot analysis of cell lysates from NIH 3T3-pZip-neo cells, v-Src-transformed NIH 3T3-pZip-neo cells and v-Ras-transformed NIH 3T3-pZip-neo cells.


a
1
2
3
4
48kDa
uPA
b
uPA Activity (IU/mg Cell Prot/24 hrs)
0
0.5
1
1.5
2
2.5
3
*
*
NIH 3T3
v-Src
v-Ras
c
48kDa
uPA
NIH 3T3
v-Src
v-Ras

Overproduction of uPA in v-Src- and v-Ras-transformed cells is mediated by RalA—

We demonstrated previously that uPA production is dependent on PLD (Aguirre Ghiso *et al.*, 1997), and that the activation of PLD by v-Src and v-Ras is dependent upon RalA (Jiang *et al.*, 1995b). We therefore wished to determine whether the elevated uPA levels observed in v-Src and v-Ras cells were dependent on RalA. To investigate the role of RalA on uPA overproduction induced by v-Src and v-Ras, we examined uPA activity in v-Src and v-Ras transformed cells stably expressing a mutant RalA protein (S28N), that functions as a dominant negative mutant for RalA (Jiang *et al.*, 1995b; Urano et al., 1996). This mutant blocked the increased PLD activity observed in response to both v-Src and v-Ras (Jiang *et al.*, 1995b). NIH 3T3 cells transformed by either v-Src (Figure 18a and 18c) or v-Ras (Figure 18b and 18c) that expressed the dominant negative S28N-RalA (Figure 18c) showed significant ($p < 0.01$) reductions in secreted uPA activity relative to the v-Src- and v-Ras-transformed cells. Zymographic analysis showed that the uPA activity reduced by the dominant negative mutant of RalA corresponded to a reduction of a 48 kDa band (Figure 18d). uPA protein levels as determined by Western blot analysis were also reduced in cells expressing the dominant negative RalA mutant (Figure 18e). As reported previously (Jiang *et al.*, 1995b), expression of the dominant negative RalA mutant had no effect upon either Src or Ras protein levels, or on the level of phosphotyrosine in the cells expressing v-Src (Figure 18c and data not shown). Thus the reduced uPA levels in cells expressing the dominant negative RalA observed in Figure 18 are not due to reduced levels of the transforming oncoprotein in response to the RalA mutant.

Figure 18. Overproduction of uPA in v-Src- and v-Ras-transformed cells is mediated by RalA. **(a)** Measurement of uPA activity present in the CM of NIH 3T3-pZip-neo cells, v-Src-transformed NIH 3T3-pZip-neo cells (v-Src) or v-Src cells expressing the S28N dominant negative RalA mutant (v-Src-S28N-Ral) was determined by radial caseinolysis as in Figure 17. **(b)** uPA activity in the CM from v-Ras-transformed NIH 3T3-pZip-neo cells (v-Ras) and v-Ras cells expressing the S28N dominant negative RalA mutant (v-Ras-S28N Ral) was determined as in **(a)**. The data in a and b represent the mean +/- the standard deviation for six samples from a representative experiment that was repeated three times. * indicates $p < 0.01$ for v-Src or v-Ras relative to the parental NIH 3T3-pZip-neo cells. ** indicates $p < 0.01$ for v-Ras-S28N-Ral or v-Src-S28N-Ral cells relative to the v-Ras or v-Src cells respectively as determined by the Students t test. **(c)** Western blot analysis of levels of Src protein in the NIH3T3, v-Src and v-Src-S28N-Ral cells (upper left panel), and levels of Ras protein in the NIH3T3, v-Ras and v-Ras-S28N-Ral cells (upper right panel), or levels of RalA protein in the v-Src, v-Src-S28N-Ral, and v-Ras and v-Ras-S28N-Ral cells. **(d)** Zymograms of the uPA activity present in NIH 3T3-pZip-neo cells and v-Src- and v-Ras-transformed NIH 3T3-pZip-neo cells and these cells expressing the dominant negative Ral S28N mutant are presented along with the densitometric analysis of the zymogram. **(e)** Western blot analysis of uPA protein levels was performed on cell lysates from the NIH 3T3, v-Src-transformed, v-Src-transformed cells expressing the S28N RalA mutant, as well as the v-Ras-transformed and v-Ras-transformed cells expressing the S28N RalA mutant.


a
uPA Activity (IU/mg Cell Prot/24 hrs)
0
0.5
1
1.5
2
2.5
3
3.5
4
*
**
NIH 3T3
v-Src
v-Src S28N Ral
b
uPA Activity (IU/mg Cell Prot/24 hrs)
0
0.2
0.4
0.6
0.8
1
1.2
1.4
1.6
1.8
2
*
**
NIH 3T3
v-Ras
v-Ras S28N Ral
C
NIH3T3
V-Src
V-Src-S28N-Ral
NIH3T3
V-Ras
V-Ras-S28N-Ral
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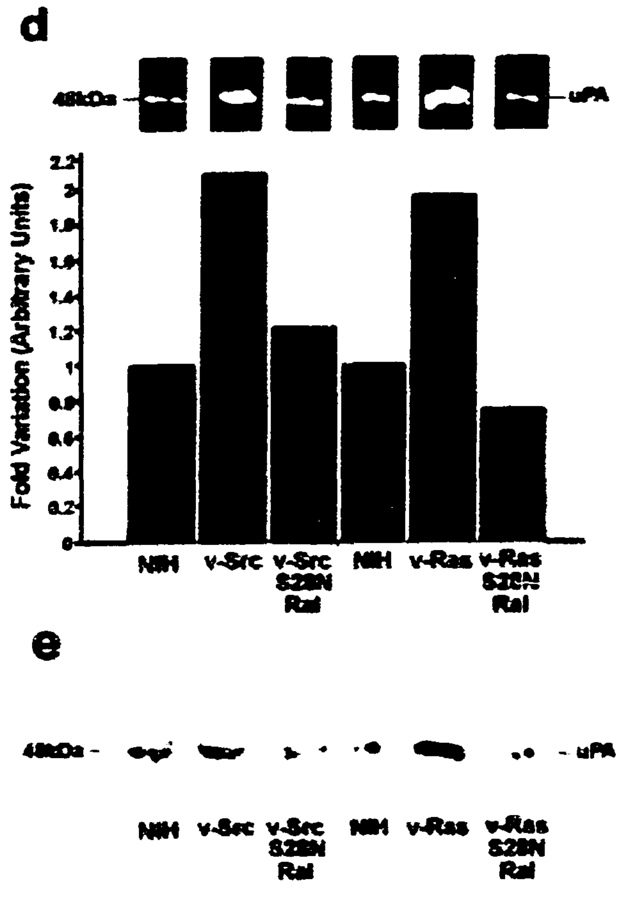
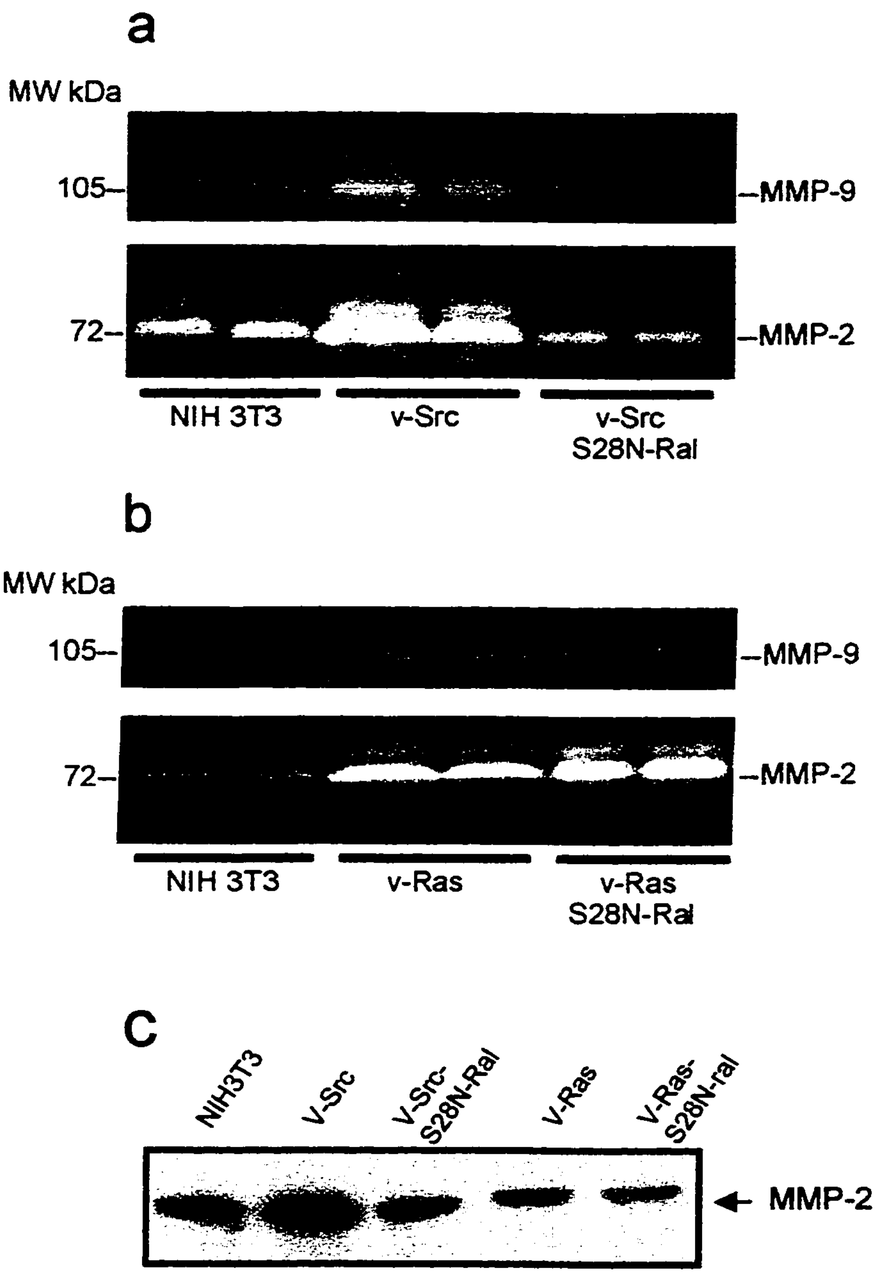
V-Ras
V-Ras-S28N-Ral


d
48kDa
uPA
Fold Variation (Arbitrary Units)
2.2
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
NIH
v-Src
v-Src S28N Ral
NIH
v-Ras
v-Ras S28N Ral
e
48kDa
uPA
NIH
v-Src
v-Src S28N Ral
NIH
v-Ras
v-Ras S28N Ral

Increased MMP-2 and MMP-9 production by v-Src, but not v-Ras is dependent on RalA—It has been suggested that MMPs play a role in tumorigenesis and metastasis formation (Liotta and Stetler-Stevenson, 1991; Werb *et al.*, 1996). Consistent with this hypothesis, increased MMP production has been reported in response to transformation by v-Src and v-Ras (Sato *et al.*, 1993; Gum *et al.*, 1996). MMP production in tumor cells has been reported to be regulated by Ras (Sato *et al.*, 1993) and dependent on PLD (Reich *et al.*, 1995). We therefore examined the role of RalA in the v-Src and v-Ras induction of MMPs. Zymograms of the CM from v-Src- and v-Ras-transformed NIH 3T3 cells showed an enhancement of proenzyme forms of a 72 kDa (Gelatinase B/MMP-2) and a 105 kDa (Gelatinase A/MMP-9) production compared to the control parental NIH 3T3 cells (Figure 19a and 19b). MMP-2 and MMP-9 activity was also elevated in cell lysates from v-Src- and v-Ras-transformed NIH 3T3 cells (data not shown). v-Src-transformed cells expressing the dominant negative S28N RalA had substantially reduced levels of both MMP-2 and MMP-9 in the CM (Figure 19a). Consistent with these results Western blots of cell lysates from NIH, v-Src or v-Src-S28N-Ral cells showed elevated MMP2 protein in the v-Src-transformed cells and a reduction in these cells expressing the dominant negative S28N RalA (Figure 19c). In contrast, the increased MMP-2 and MMP-9 activity induced by v-Ras was largely unaffected by the S28N RalA mutant (Figure 19b). Surprisingly, MMP-2 protein levels were not elevated in the v-Ras and v-Ras-S28N-Ral cells relative to the parental NIH 3T3 cells (Figure 19c). This may explain why the dominant negative RalA prevents v-Src-induced increases MMP2, but not v-Ras-induced increases in MMP2. These data suggest two distinct mechanisms for elevating MMP-2 activity, one involving increased expression that is dependent upon RalA and one that involves a change in specific activity that is independent of RalA. Also of interest here were the additional low molecular weight bands observed for MMP-2 in both v-Ras- and v-Src-transformed cells (Figure 19a and 19b). These low molecular weight species may represent the active forms of MMP-2. Interestingly, this form of MMP-2 was reduced in the v-Ras-transformed cells expressing the dominant negative RalA.

Figure 19. Dependence of increased MMP-2 and MMP-9 activities in v-Src- and v-Ras-transformed cells upon RalA. **(a)** Zymograms showing the MMP activities secreted into CM by NIH 3T3, v-Src-transformed or v-Src-transformed cells expressing the S28N dominant negative RalA mutant. **(b)** MMP activity from NIH 3T3, v-Ras-transformed, and v-Ras-transformed cells expressing the S28N dominant negative RalA mutant. Duplicate lanes represent the activities secreted in two independent samples. MMP activity was abolished in gels incubated in the presence of 40 mM EDTA and unaffected in the presence of aprotinin (not shown) indicating their specificity as metalloproteases. **(c)** Western blot analysis of MMP-2 protein in cell lysates of NIH 3T3, v-Src- and v-Ras-transformed NIH 3T3 cells, and these cells expressing the dominant negative Ral S28N mutant.


a
MW kDa
105
72
MMP-9
MMP-2
NIH 3T3
v-Src
v-Src S28N-Ral
b
MW kDa
105
72
MMP-9
MMP-2
NIH 3T3
v-Ras
v-Ras S28N-Ral
c
NIH3T3
V-Src
V-Src-S28N-Ral
V-Ras
V-Ras-S28N-ral
MMP-2

The dominant negative S28N RalA mutant prevents v-Src and v-Ras induced tumorigenicity---It was reported previously that the dominant negative S28N RalA inhibited v-Ras- and v-Raf-induced focus formation in cultured cells (Urano *et al.*, 1996). However, the inhibitory effect of the dominant negative RalA on focus formation was not as strong as that observed for dominant negative mutants of Ras and Raf on v-Src-induced transformation where colony formation in soft agar was inhibited almost completely (Qureshi *et al.*, 1993; Jiang *et al.*, 1995a; 1995b). We therefore investigated the effect of the dominant negative RalA on the ability of the v-Src- and v-Ras-transformed cells to form colonies in soft agar. As shown in Figure 20, the dominant negative RalA mutant reduced the colony forming efficiency of the v-Src- and v-Ras-transformed cells; however, as reported previously for the focus assay (Urano *et al.*, 1996), the dominant negative RalA only partially inhibited the colony formation. The S28N mutant reduced colony formation to about 75% that seen in the Ras-transformed cells and 54% of that seen in the Src-transformed cells. Another RalA mutant, D49N, which was slightly more effective at inhibiting colony formation where the efficiency was reduced to 44% and 51% for the Ras and Src transformed cells respectively.

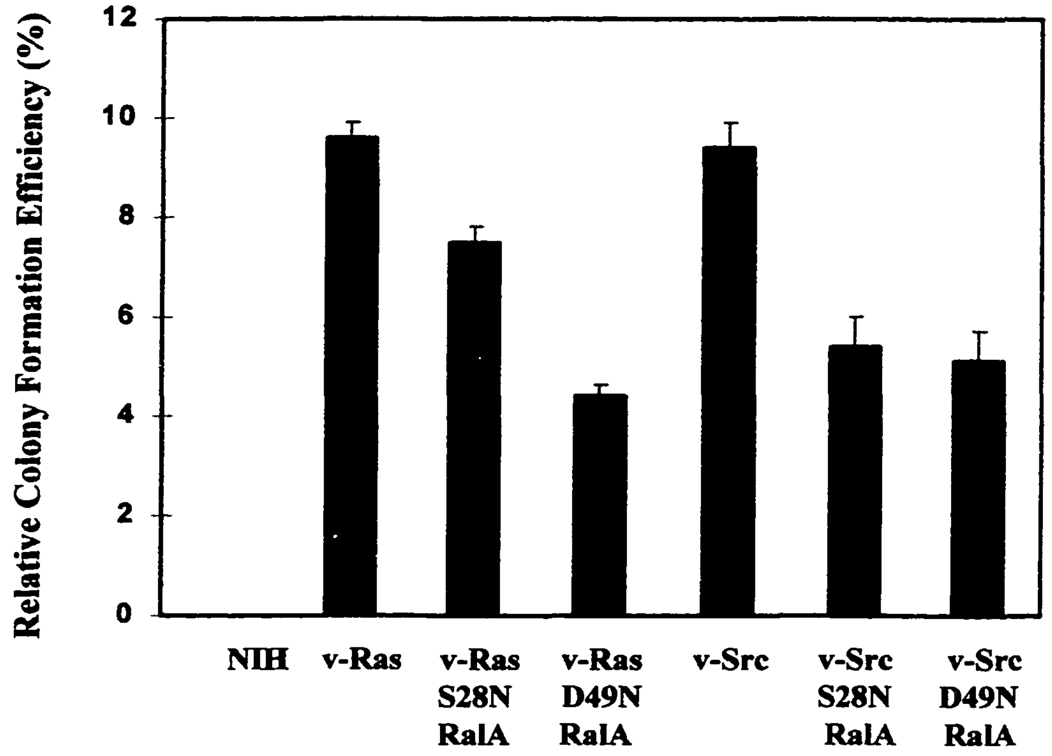


Figure 20. v-Src- and v-Ras-induced colony formation is partially dependent upon RalA. 1 x 10^3 NIH 3T3-pZip-neo cells, v-Src- and v-Ras-transformed cells, and v-Src and v-Ras-transformed cells expressing the S28N RalA mutant or the D49N RalA mutant were suspended in soft agar and the percentage of cells forming colonies was determined. Error bars represent the standard errors for triplicate samples from duplicate experiments.

We next examined the effect of the dominant negative RalA on the ability to form tumors in syngeneic mice. Cell suspensions from v-Src- and v-Ras-transformed NIH 3T3 cells and these cells expressing the S28N dominant negative RalA mutant were injected subcutaneously and tumor formation was monitored. As shown in Figure 21, both v-Src and v-Ras cells developed subcutaneous tumors that could be detected after a 5-7 day latency period, with an incidence of 50% and 55% respectively. In contrast, v-Src- and v-Ras-transformed cells expressing the dominant negative S28N RalA mutant did not develop subcutaneous tumors after injection when followed for 13 weeks. Tumor growth was evaluated up to 28 days when mice were sacrificed and tumors removed. The histopathology revealed sarcoma-like tumors for both v-Src and v-Ras cell lines (data not shown).

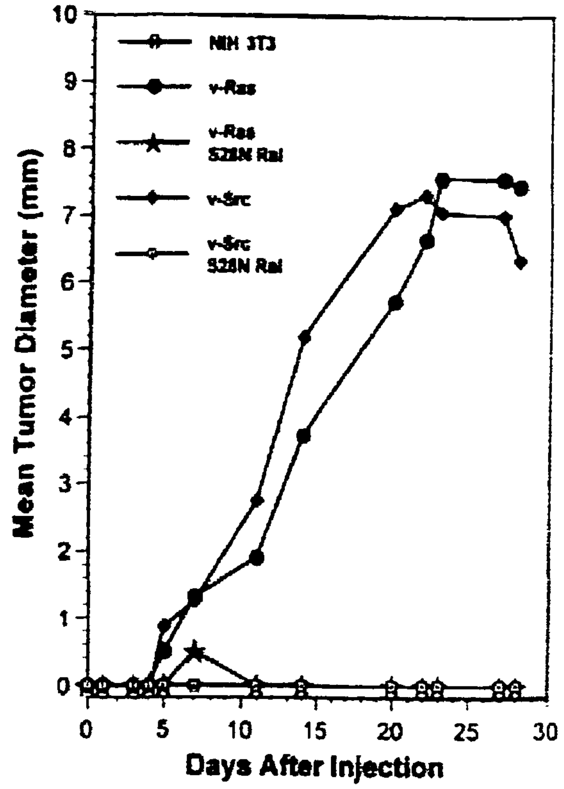


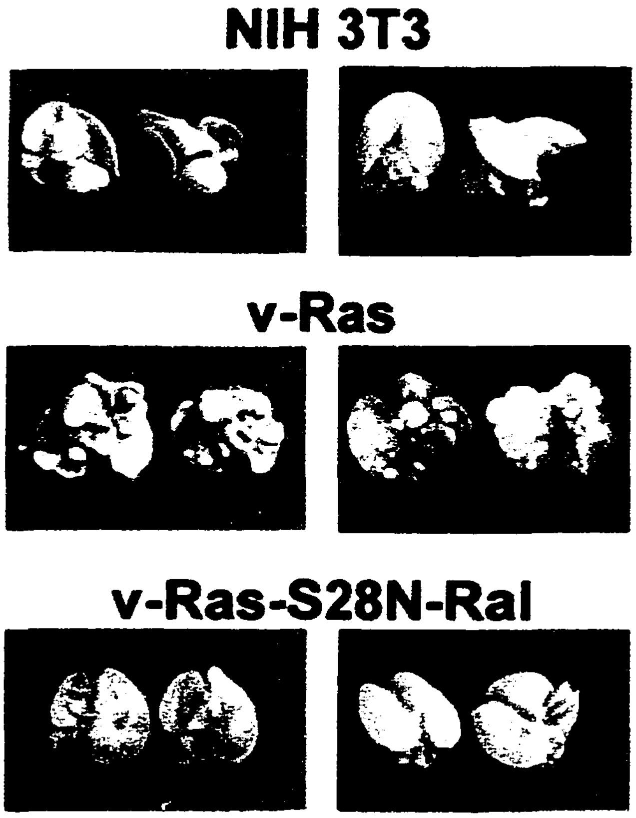
Figure 21. v-Src- and v-Ras-induced tumorigenicity is dependent upon RalA. NIH 3T3-pZip-neo cells, v-Src- and v-Ras-transformed cells, and v-Src and v-Ras-transformed cells expressing the S28N RalA mutant were injected in the subcutaneous flank of syngeneic normal BALB/c mice at 4 x 10^5 cells/mouse. Tumor size was monitored 3 times per week for 28 days at which time mice with tumors were sacrificed. Tumor size represents the average tumor size for of least 10 mice for each group. Mice without tumors were monitored for tumors for 13 weeks. The figure shows a representative experiment, which was repeated twice.

We also investigated whether the S28N RalA mutant interfered with the ability of v-Src- and v-Ras-transformed cells injected into the tail vein to colonize in the lung. Upon injection of either v-Src- or v-Ras-transformed cells into the tails of syngeneic mice, large colonies were detected in the lungs with high incidence (Table 1, Figure 22). The histopathological analysis showed the presence of not only subpleural but also parechymal nodules consisting of sarcomatoid cells and almost devoid of extracellular matrix (data not shown). The ability of v-Ras and v-Src cells to colonize the lung and form nodules was dramatically impaired in cells overexpressing the S28N-RalA mutant. As shown in Table 1, both the incidence and number of lung colonies was significantly reduced in mice inoculated with either v-Ras- or v-Src-transformed cells expressing the S28N RalA mutant. These data in Figures 20, 21, and 22 indicate that, while colony formation in soft agar is only partially dependent upon RalA, tumor formation is completely dependent upon RalA.

			Lung Tumor	Nodules
Cells	Mice with colonies Total Mice	Incidence (%)	Median	(Range)
NIH 3T3-pZip-neo	0/7	0	0	(0-0)
v-Ras	10/11	90	7	(0-31)
v-Ras + S28N RalA	1/7	14	0	(0-2)
v-Src	7/7	100	3	(1-15)
v-Src + S28N RalA	0/8	0	0	(0-0)

Table 1. The effect of S28N RalA on the lung colony formation ability of v-Ras- and v-Src-transformed cells. NIH 3T3 cells, v-Src and v-Ras-transformed cells, and the v-Src and v-Ras cells co-expressing S28N RalA were injected into the lateral tail veins of syngeneic mice and lungs from these mice were examined 21 days later. The incidence represents the percentage of mice with tumor lung colonies. Median represents the median number of nodules per lung and the range represents the maximum and minimum number of nodules in the lungs examined. The table shows a representative experiment repeated twice.

Figure 22. v-Ras-induced tumorigenicity as measured by colony formation in the lung is dependent upon RalA. NIH 3T3-pZip-neo cells, v-Ras-transformed NIH 3T3-pZip-neo cells, and the v-Ras-transformed cells expressing RalA-S28N were injected into the tail veins of syngeneic mice and the lungs were excised 21 days later. Representative lungs are shown here. Quantification of the data is presented in Table 1.


NIH 3T3
v-Ras
v-Ras-S28N-Ral

Discussion

In this report, we have investigated whether RalA plays a role in (i) the upregulation of proteases and (ii) tumor formation induced by the oncogenic v-Src and v-Ras. We first observed that uPA expression levels were elevated in the CM from NIH 3T3 cells transformed by v-Src and v-Ras. The upregulation of uPA could be reversed almost to the control levels by the expression of a dominant negative RalA mutant. We previously demonstrated that upregulation of uPA production is dependent upon PLD activity (Aguirre Ghiso *et al.*, 1997), and since PLD1 activation by v-Src and v-Ras is dependent upon RalA (Jiang *et al.*, 1995b; Luo *et al.*, 1997), the data presented here suggest that constitutive uPA overproduction in v-Src and v-Ras transformed cells is dependent upon a RalA-PLD1 signaling pathway.

MMP activity was elevated in v-Src-transformed cells as detected by zymography and MMP protein was elevated as determined by Western blot analysis of cell lysates. The increased MMP production induced by v-Src, like increased uPA production, was reduced in the cells expressing the dominant negative RalA. However, the increased MMP activity observed in v-Ras-transformed cells was insensitive to the dominant negative RalA. Interestingly, the increased MMP activity seen in zymograms was not observed at the MMP protein level in the v-Ras-transformed cells. This suggested that MMP activity may regulated differently in the v-Src and v-Ras-transformed cells and may explain why the dominant negative RalA prevents v-Src-induced increases MMP2, but not v-Ras-induced increases in MMP2. These data suggest that the increased expression of MMP in the v-Src-transformed cells is dependent upon RalA and the elevated specific activity observed in the v-Ras-transformed cells is independent of RalA. Exton and co-workers recently reported that MMP-9 secretion induced by phorbol esters was dependent on PLD and phosphatidic acid production (Williger et al., 1999). It is unlikely that the mechanism was the same as that reported here since phorbol esters activate PLD by activating protein kinase C, which can activate PLD directly (Exton, 1998). Thus, RalA is not likely to be involved in this case, however these data suggest the possibility that both RalA-dependent and independent mechanisms for MMP production exist.

More importantly, the studies presented here clearly indicate a role for RalA in v-Ras and v-Src induced tumorigenicity. Previous studies using cells in culture have suggested a role for RalA in mitogenic signaling and transformation (Urano et al., 1996). However, dominant negative RalA mutants were less efficient in blocking the transformed phenotype than dominant negative mutants of Ras and Raf (Qureshi et al., 1993; Jiang et al., 1995a; 1995b; Urano et al., 1996). The studies provided here demonstrate conclusively that RalA inhibits completely the ability to form tumors. Extracellular matrix remodeling by tumor cells has been proposed to be a necessary process for invasion and metastases establishment (Liotta *et al.*, 1991; Werb *et al.*, 1996). The inhibitory effect of the dominant negative RalA on the upregulation of uPA expression in v-Src and v-Ras-transformed cells may be one of the mechanisms by which RalA-dominant negative mutant modulates tumorigenicity. This result does not directly correlate the effect of S28N RalA on MMP expression in v-Ras-S28N-RalA cells with the tumorigenicity data. An explanation for this result may derive from recent data showing that MMPs produced in cell culture or *In vivo* are non-functional in the absence of the uPA/plasmin system (Mazzieri *et al.*; Carmeliet *et al.* 1997). In this regard although MMP expression levels are not reduced in the Ras S28N Ral cells, they are not active due to reduced levels of uPA. Additional low MW bands that may represent the active form of MMPs were observed for MMP-2 in v-Ras cells, but were absent in v-Ras-S28N-Ral cells. These data suggest that uPA downregulation in v-Ras-S28N-RalA cells may account for the lack of the MMP-2 low MW bands (active form) since the active form is generated in response to uPA.

Reduced protease production and/or activity in cells expressing the dominant negative RalA may contribute to the more stringent effect of the dominant negative RalA mutant on tumor formation relative to colony formation in soft agar and focus formation. We have recently found that fibronectin downregulation in NIH3T3 cells by v-Ras or v-Src is completely restored in v-Src or v-Ras cells expressing the S28N-RalA mutant (our unpublished data). This result suggests that reducing RalA signaling in v-Ras or v-Src transformed cells may restore the proper expression of not only uPA and MMPs in the case of v-Src, but of several other genes that influence the tumorigenic and metastatic phenotype explaining the dramatic effects observed on tumorigenicity.

This report provides (i) new insight on the role of RalA in the signals controlling protease expression and (ii) demonstrates a role for RalA in tumor formation in the NIH3T3 model. In addition, our data are consistent with mounting information (Silberman *et al.*, 1997, Gum *et al.*, 1996, Lengyel *et al.*, 1996, Lengyel *et al.*, 1997, Gum *et al.*, 1997) indicating that mitogenic signaling pathways stimulate the upregulation of uPA and MMPs. In this regard, an understanding of the mechanism(s) that tumor cells use to become tumorigenic and govern the production of proteases, which apparently includes the RalA-PLD1 pathway, may prove to be an important target for therapeutic intervention in tumor progression.

Chapter IV

Methods and Materials

Materials

G418 and Dulbecco's modified Eagle medium (DMEM) were obtained from GIBCO/BRL Laboratories. Trypsin and amiloride were purchased from Sigma. Anti-murine uPA antibody was kindly provided by Dr. G. Hoyer-Hansen (Copenhagen, Denmark). Anti MMP-2 antibody was from Neomarkers (Union City, CA). Anti-RalA antibody was from Trandsuction laboratories (Lexington, KY). Antibody for RalA was obtained from Transduction Laboratories. Antibodies for Raf-1, RhoC and Phospho-Erk were from Santa Cruz Biotechnology. Biotinylated anti-rabbit IgG polyclonal antibody and streptavidin-alkaline-phosphatase were purchased from GIBCO/BRL. Purified human urokinase was provided by Serono Laboratories (Buenos Aires). Plasminogen was purchased from Chromogenics (Sweden). Nitrocellulose membranes were purchased from BioRad (USA). [3H]-Myristate (NET-830). was obtained from New England Nuclear. Phosphatidylbutanol (PBt) , PA, and diacylglycerol (DG) standards were obtained from Avanti Polar Lipids. Precoated silica 60A thin layer chromatography (TLC) plates were from Scientific Products.

Cells and cell culture conditions.

All cell lines were maintained in Dulbecco's modified Eagle medium (DMEM, Life Technologies) supplemented with 10% calf serum except for cells expressing the BxB-ERTM Raf-1 which were maintained with phenol red-free DMEM. v-Raf-transformed NIH 3T3 cells were generated by amplification of a transformed focus induced by transfection of the p3611-MSV-v-Raf expression plasmid (Rapp *et al.*, 1983) into NIH 3T3 cells. The generation of cells expressing the BxB-ERTM Raf-1 was described previously (Kerkhoff & Rapp, 1997). v-Raf-transformed cells overexpressing the S28N Ral A mutant were generated by transfection of the p-Zip-RalA (S28N) expression vector followed by selection of G418 resistant colonies which were then pooled. Construction of the S28N RalA mutant was described previously (Urano *et al.*, 1996). The dominant negative RhoC T19N mutant was constructed using overlap PCR as described previously (Farnsworth *et al.*, 1995). v-Raf-transformed cells overexpressing the T19N RhoC mutant was generated by transfection of the pZip-RhoC (T19N) expression vector followed by selection of G418 resistant colonies. The v-Src and v-Ras-transformed NIH 3T3 cells and the v-Src- and v-Ras-transformed cells overexpressing the S28N RalA mutant were pooled clones generated as described previously (Jiang *et al.*, 1995b). Empty vector control cell lines were also established for v-Src, v-Ras and v-Raf-transformed and parental NIH 3T3 cells and used as controls in all experiments. Transfection was performed using Lipofectamine (Life Technologies) according to the vendors instructions. RalA, RhoC, Raf-1, and Phospho-ERK protein levels were verified by western blot analysis using commercially-obtained antibodies as described previously (Jiang et al., 1995a, 1995b). Cell cultures were made quiescent by growing to confluence and then replacing with fresh media containing 0.5% newborn calf serum overnight (~16hr). For induction of BxB-ERTM Raf-1 with 4-hydroxytamoxifen (4-OH-T), a solution of 1 mg/ml in ethanol was added to the medium to a final concentration of 200 nM (5,000 fold dilution).). For growth of cells in soft agar, 1 x 10^3 cells were suspended in top agar (DMEM, 20% calf serum, 0.38% agar) and overlaid onto hardened bottom agar (DMEM, 20% calf serum, 0.7% agar) as described previously (Qureshi *et al.*, 1993).

Prelabeling of phospholipids

Unless otherwise indicated, cells in 60 mm culture dishes were prelabeled for 4 to 6 h with 3 μCi of [3H]-Myristate in 2 ml of Dulbecco's modified Eagle media containing 0.5% newborn calf serum.

Extraction of lipids

Extraction of lipids was performed according to procedures described by Song et al. (Song *et al.*, 1991,1993) with minor modifications. Cells were washed with isotonic tris-saline buffer and rapidly treated with 0.5 ml of MeOH:6N HCl (50:2). Lipids were extracted by the addition of 0.5 ml of CHCl3. Phase separation was obtained by adding 155 μl of 1M NaCl. The organic phase was reextracted with 0.12 ml of 1M NaCl, 0.35 ml of H_2O and 155 μl of MeOH and recovered. An aliquot (10 μl) of the organic phase was removed and total cpm incorporated into cellular lipid/sample was determined. Samples were normalized for total cpm, dried under N_2 and redissolved in $CHCl_3$:MeOH (9:1).

Characterization of phospholipid metabolites by TLC

Extracts of phospholipid metabolites were characterized by TLC as described previously (Song *et al.*, 1993). The following solvent systems were used: For DG, hexane:diethylether:MeOH: glacial acetic acid (90:20:3:2); for PBt, the organic phase of ethylacetate:trimethylpentane:acetic acid:H20 (100:50:20:100). Lipid standards were visualized by treating TLC plates with iodine vapor. TLC plates were sprayed with EN^3HANCE (Dupont) and exposed to Kodak XAR-5 film at -70^0C for 2-3 days. Relative levels of PLD activity was then determined by measuring the intensity of the corresponding PBt band in the autoradiogram using a Molecular Dynamics scanning densotometer and ImageQuant software.

Preparation of conditioned media (CM) and cell lysates

uPA activity was investigated in CM and cell lysates. Briefly, semiconfluent cell monolayers were washed in phosphate buffer saline to eliminate serum. Serum-free DMEM/0.5% bovine serum albumin/25 mM HEPES was added and incubation was continued for 24 hours. CM was harvested, centrifuged (600 x g), concentrated with a Centricon filter (MW cut off 30,000), aliquoted, stored at -40°C, and used only once after thawing. The remaining monolayers were washed, scrapped and sonicated at 4°C; protein content determinations were performed and the cell lysate samples were then stored at -40°C and used only once after thawing.

Zymography and radial caseinolysis for detection of uPA activity

SDS-polyacrylamide gel electrophoresis was performed using 9% separating and 4% stacking gels. Gels were washed with 2.5% Triton X-100, and incubated on the surface of a plasminogen-rich casein-agarose underlay, as described previously (Aguirre Ghiso *et al.*, 1997, Saksela 1981). To confirm uPA activity in the zymograms, plasminogen-casein-agarose underlays were prepared with an anticatalytic uPA antibody, amiloride (1 mM) with or without plasminogen. uPA activity was quantified by a radial caseinolysis assay (Saksela, 1981), using plasminogen-rich (2 ug/ml) casein-agarose plates. uPA activity was referenced to a standard urokinase curve (0.1-100 IU/ml) and normalized to total cell protein.

Detection of MMP production

Collagenolytic activity present in CM or cell lysates was determined by electrophoresis in SDS-polyacrylamide gels co-polymerized with 0.1% gelatin as described previously (Pittman, 1985). After electrophoresis, gels were washed in 2% Triton x-100, washed with distilled water and incubated for 48-72 hours in 0.25 M Tris-HCl:1 M NaCl:25 mM CaCl2 (pH 7.4) for specific activity detection, or in the same solution containing 40 mM EDTA to detect non-specific activity. Gels were fixed and stained with Coomassie Blue. Activity bands were visualized by negative staining. The activity bands for MMP or uPA activity were quantitated with a densitometer as described below.

Immunodetection

v-Src, v-Ras, Raf-1, RalA, RhoC, MMP-2 and Phospho-ERK protein levels were verified by western blot analysis using commercially-obtained antibodies as described above and previously (Jiang et al., 1995a, 1995b). Blots were developed with the Pierce Super Signal system and exposed to Kodak XAR-5 film at room temperature. Intensity of bands were visualized using a Molecular Dynamics scanning densotometer.

uPA protein levels were verified by western blot analysis using a Rabbit IgG anti-murine uPA antibody and a biotinylated-IgG anti-rabbit antibody as described previously (Aguirre Ghiso *et al.*, 1997). The streptavidin-alkaline-phosphatase system was used to develop the signal (Alonso *et al.*, 1993). Protein bands were quantitated with a Molecular AnalystTM /PC Densitometer Model GS-700 (Bio-Rad USA) and analyzed with the Image Analysis Software for Model GS-700 (Bio-Rad USA).

Mice

Inbred BALB/c mice 2-4 months old. were obtained from the Animal Production Division of the Research Area of the Institute of Oncology "Angel H. Roffo". Mice were bred with a 12 hour light-12 hour darkness cycle and a constant temperature of 20°C. Water and food were administered ad libitum.

Tumorigenicity assays

To evaluate local tumor growth, NIH 3T3 cells, v-Src- and v-Ras-transformed cells, and v-Src- and v-Ras-transformed cells expressing the S28N dominant negative RalA mutant were trypsinized, washed three times with DMEM, and allowed to recover for 1 hr. Cells were assayed for viability with the Trypan Blue exclusion test prior to injection. Tumorigenicity was then examined in two ways. Method 1: Cells were injected in the subcutaneous flank of syngeneic BALB/c mice (4×10^5 cells in 0.2 ml of DMEM). Growth kinetics of tumors were established by measuring the average tumor diameter with a caliper three times per week. Method 2: cells prepared as above were injected into the lateral tail vein of normal mice ($3\text{x}10^5$ cells in 0.3 ml of DMEM). Growth of these cells was then investigated by histopathological analysis of the lungs and other organs 21 days after injection. Lung subpleural tumor nodules were counted under a stereoscopic magnifier after fixation for 5 h with Bouin's solution.

Chapter V

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